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Mosaic CDKL5 expression in heterozygous female mice is associated with age- and region-dependent remodeling of inhibitory circuits

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Keywords: CDKL5 Deficiency Disorder; Mosaic X-Chromosome Inactivation; Inhibitory Synapses; GABAergic Transmission; Hippocampus; Cortex.

ABSTRACT

CDKL5 Deficiency Disorder (CDD) is a severe X-linked encephalopathy that predominantly affects heterozygous females, in whom random X-chromosome inactivation generates mosaic CDKL5 expression. Although early-onset epilepsy is a hallmark of the disorder, the mechanisms by which mosaic CDKL5 loss destabilizes neural circuits over time remain poorly understood. Here, we demonstrate a dynamic, age- and region-dependent remodeling of inhibitory synaptic transmission in heterozygous *Cdkl5* (+/–) female mice. At 3 months, inhibitory function is selectively enhanced in the hippocampus, whereas cortical transmission remains unchanged. By 12 months, this profile diverges: hippocampal inhibitory input is reduced, while cortical inhibition is increased. These changes are accompanied by distinct structural alterations. In the hippocampus, presynaptic terminal density follows a biphasic trajectory, together with a persistent reduction of inhibitory postsynaptic sites. In the cortex, a reduction in GABAergic presynaptic markers is observed in association with impaired synaptic organization despite enhanced inhibitory neurotransmission, indicating a dissociation between synaptic structure and function. These alterations occur in the absence of major changes in interneuron density, supporting a model of synaptic and circuit-level remodeling. At the cellular and molecular levels, this phenotype is associated with early microglial activation, age-dependent reduction in GFAP-positive astroglial cells, and altered hippocampal transcriptional trajectories. The analysis of postmortem human cortical tissue suggests age-related changes in inhibitory presynaptic markers consistent with the mouse model. Together, these findings indicate that mosaic CDKL5 deficiency drives region-specific and temporally divergent remodeling of GABAergic circuits rather than a uniform loss of inhibition, identifying inhibitory circuit remodeling as a potential mechanism underlying disease progression in CDD.

INTRODUCTION

CDKL5 (Cyclin-Dependent Kinase-Like 5) is a serine/threonine kinase that plays a crucial role in brain development and synaptic function, including neuronal maturation, dendritic arborization, and synaptic plasticity [2, 15, 24, 55, 82]. Mutations in the *CDKL5* gene cause CDKL5 Deficiency Disorder (CDD) [20, 62], a rare and severe neurodevelopmental encephalopathy with an estimated incidence of approximately 1 in 40,000 to 60,000 live births [52]. CDD is characterized by early-onset epileptic seizures, severe intellectual disability, and motor impairment [36, 41, 52]. Seizure phenotypes are highly heterogeneous, including tonic, tonic-clonic, and myoclonic events, as well as atypical episodes lacking a consistent electroclinical signature. Although initial responses to broad-spectrum antiepileptic treatments may occur [51], seizure control is often transient [5], and seizures frequently re-emerge over time, becoming a persistent and lifelong clinical challenge [3].

Due to its X-linked inheritance, CDD predominantly affects females, in whom random X-chromosome inactivation (XCI) generates mosaic CDKL5 expression [37, 43]. This cellular mosaicism results in the coexistence of CDKL5-expressing and non-expressing neurons within the same circuits, contributing to phenotypic variability and potentially shaping network-level dysfunction. To date, hundreds of pathogenic CDKL5 mutations have been identified [31], the majority of which arise de novo and lead to loss of kinase function [48]. Together, these features make CDD a paradigmatic disorder in which cellular mosaicism may critically influence neural circuit organization and disease progression.

Despite advances in defining the genetic and molecular basis of CDKL5 deficiency, the synaptic and circuit-level mechanisms underlying the neurological manifestations of the disorder, including treatment-resistant epilepsy, remain incompletely understood. In particular, it is unclear whether CDKL5 dysfunction leads to a uniform disruption of excitatory/inhibitory (E/I) balance or, alternatively, to a dynamic and region-specific remodeling of inhibitory circuits across development

and aging. Elucidating these mechanisms is essential for understanding disease pathophysiology and identifying targeted therapeutic strategies.

Animal models of CDKL5 deficiency have provided important insights into synaptic dysfunction. Both constitutive knockout (KO) and conditional knockout (cKO) mouse models display impaired dendritic arborization, altered spine morphology, and deficits in synaptic plasticity [2, 15, 23, 46, 50, 53, 63]. In addition to these structural alterations, *Cdkl5*-deficient mice consistently exhibit disruption of E/I balance, affecting both excitatory and inhibitory synaptic compartments. In the hippocampus, *Cdkl5* KO and cKO mice show increased spontaneous excitatory postsynaptic activity, as well as alterations in inhibitory synaptic transmission and circuit excitability, particularly in CA1 pyramidal neurons and dentate gyrus interneurons [75, 80]. Region-specific changes have also been reported in the cortex, where altered excitatory terminal density and postsynaptic marker expression are accompanied by changes in inhibitory network maturation [53]. In the cerebellum, selective reductions in GABAergic terminals and structural abnormalities in inhibitory synapses have been described [58].

Notably, despite these widespread synaptic alterations, spontaneous seizures are not observed in full *Cdkl5* KO mice, suggesting that synaptic imbalance alone may not be sufficient to trigger epileptogenesis, and that additional mechanisms are required. In contrast, heterozygous female *Cdkl5* mutant mice develop spontaneous epileptic spasms later in life (median onset ~28-34 weeks) [47, 64], whereas hemizygous males and homozygous mutant females do not exhibit overt seizures within the same timeframe. These observations indicate that seizure susceptibility is specifically associated with the mosaic condition, arising from XCI in females, rather than complete CDKL5 loss. This distinction is particularly relevant given that most CDD patients are heterozygous females, and that a subset of affected males (32%) also exhibits somatic mosaicism [61]. Together, these findings suggest that circuit instability in CDD may emerge from the coexistence and interaction of CDKL5-

expressing and non-expressing neurons within the same network, leading to non-cell-autonomous effects and altered circuit organization.

Proper brain function depends on the precise balance between excitatory glutamatergic and inhibitory GABAergic transmission. Disruption of this balance can lead to network hyperexcitability and seizure generation. While early studies of CDKL5 deficiency primarily focused on excitatory synapses [55], increasing evidence highlights a critical contribution of inhibitory dysfunction. CDKL5 deficiency has been shown to impair inhibitory synaptic transmission, reduce clustering and surface expression of GABA_A receptors, and disrupt key scaffolding proteins such as gephyrin and collybistin [14, 53]. These findings indicate that inhibitory synapses are particularly vulnerable in CDKL5 deficiency. However, whether this impairment reflects a progressive loss of inhibition or a more complex and dynamic remodeling of inhibitory circuits remains unclear.

In particular, the interplay between mosaicism and aging in shaping inhibitory circuit function has not been fully elucidated. It is unknown whether inhibitory dysfunction follows a uniform degenerative trajectory or instead reflects region-specific adaptations and maladaptive remodeling processes that evolve over time.

In this study, we aimed to define the functional, structural, and molecular trajectory of inhibitory synapses in heterozygous female *Cdkl5* mutant mice across age. To address this, we compared young adult and middle-aged animals, combining whole-cell patch-clamp recordings of inhibitory synaptic transmission, quantitative analysis of synaptic markers, characterization of interneuron and glial populations, and hippocampal transcriptomic profiling. In addition, to assess the translational relevance of our findings, we performed an exploratory analysis of inhibitory presynaptic markers in postmortem cortical tissue from heterozygous female CDD patients.

This integrated approach was designed to determine whether inhibitory dysfunction in CDKL5 deficiency reflects a static deficit or a dynamic, region-specific remodeling of inhibitory circuits that may underlie seizure susceptibility in mosaic conditions.

MATERIALS AND METHODS

Animals

Male and female *Cdkl5* knockout (KO) mice, originally generated by Amendola et al. (Amendola et al., 2014), and wild-type (WT) littermates were used in this study. Experimental animals were obtained by crossing *Cdkl5* +/- females with either *Cdkl5* +/-Y or *Cdkl5* -/Y males. Genotyping was performed by PCR on genomic DNA as previously described [2]. The day of birth was designated as postnatal day 0 (P0), and animals 24 hours old were considered postnatal day 1 (P1). After weaning (P21-P23), mice were group-housed (three to five per cage) under a 12-hour light/dark cycle in a temperature- and humidity-controlled environment, with food and water available ad libitum. Animal health and welfare were monitored daily by institutional veterinary staff. All experimental procedures complied with Italian and European Community regulations on the use of laboratory animals and were approved by the University of Bologna Bioethical Committee. Every effort was made to minimize animal suffering and to reduce the number of animals used. Two age groups were examined: 3-month-old and 12-month-old mice. Independent cohorts of animals were used for electrophysiology, immunohistochemistry, and transcriptomic analyses, unless otherwise specified. The number of animals (*n*) used for each experiment is reported in the corresponding figure legends. In total, 63 mice were used in this study (*Cdkl5* +/+ *n* = 24; *Cdkl5* +/- *n* = 27; *Cdkl5* -/- *n* = 4; *Cdkl5* +/-Y *n* = 4; *Cdkl5* -/Y *n* = 4).

Human postmortem brain tissue

Flash-frozen human brain tissue blocks from two female CDD patients and neurotypical controls were obtained from the Harvard Brain Tissue Resource Center (Belmont, MA, USA) and the University of Maryland Brain Tissue Repository (Baltimore, MD, USA), respectively, via the NIH NeuroBioBank. CDD patient samples were derived from a 5.7-year-old female carrying a frameshift mutation (c.2153_2154dupTG) in exon 15 of the CDKL5 gene, resulting in a premature stop codon, and from a 30-year-old female with a deletion of exons 1-3 in CDKL5. Control samples consisted of age- and sex-matched individuals (4 and 29 years old) with no known neurological or genetic disorders, matched to CDD patients by sex, age, and brain hemisphere. Due to the limited availability of human tissue, this analysis should be considered descriptive and not intended for statistical generalization across subjects.

Patch-clamp recordings

Animals were euthanized by cervical dislocation and decapitated, then the head was submerged in ice-cold, oxygenated cutting solution and the brain was quickly dissected. For 3-month-old mice, the cutting solution contained (in mM): sucrose 248, HEPES 20, glucose 10, MgCl_2 10, Na^+ ascorbate 5, Na^+ pyruvate 3, KCl 2, CaCl_2 0.5, pH adjusted to 7.4 with NaOH. For 12-month-old mice, the protective cutting approach described in [68] was applied, using a solution containing (in mM): N-methyl-D-glucamine (NMDG) 92, NaHCO_3 30, glucose 25, HEPES 20, MgSO_4 10, KCl 2.5, Na^+ ascorbate 5, Na^+ pyruvate 3, thiourea 2, NaH_2PO_4 1.25, CaCl_2 0.5, pH adjusted to 7.4 with HCl. 300- μm -thick coronal brain sections were cut using a vibratome (Leica VT1200S; Leica Biosystems, Buccinasco (MI), Italy), and transferred to a recovery chamber containing oxygenated artificial cerebrospinal fluid (aCSF) composed of (in mM) NaCl 119, HEPES 10, glucose 10, NaHCO_3 6.2, KCl 2.5, CaCl_2 2, MgCl_2 1.2, NaH_2PO_4 1, Na^+ ascorbate 0.4, pH adjusted to 7.4 with NaOH. After being

maintained at 32°C for 30 min, the chamber was moved to room temperature and recovery lasted for an additional 60 min before recordings were started under continuous perfusion with oxygenated aCSF. Borosilicate glass micropipettes were fabricated from 1B150F-4 capillaries (WPI, Sarasota, FL, USA) using a PC-100 vertical puller (Narishige International, London, UK) and had a 4- to 6-M Ω resistance when filled with an internal solution containing (in mM) CsCl 135, HEPES 10, phosphocreatine 5, Mg²⁺-ATP 2.5, MgCl₂ 2, Na⁺-GTP 0.25, EGTA 0.1, pH adjusted to 7.35 with CsOH [60]. To isolate spontaneous inhibitory postsynaptic currents (sIPSCs), the NMDA receptor antagonist D-2-amino-5-phosphonovalerate (D-AP5, 50 μ M; HelloBio, Dublin, Ireland) and the AMPA receptor antagonist 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX, 10 μ M; Hello Bio, Dublin, Ireland) were used. Neurons from layers II-III of the somatosensory cortex and from the pyramidal layer of CA1 field of the hippocampus of wild-type and *Cdk15* heterozygous female mice were approached under differential interference contrast illumination using a 63 $\times$ immersion objective and recorded in whole-cell configuration. At least 3 min were allowed for complete cytosol dialysis, then sIPSCs were recorded while holding the neuron at -70 mV [60], using a MultiClamp 700A amplifier and a Digidata 1550B card, controlled via Clampex 10 software (Molecular Devices, San Jose, CA, USA). Data analysis was performed using Clampfit 11 (Molecular Devices, San Jose, CA, USA) using a template-based automated detection of events [60].

Immunofluorescence staining

Mouse brain tissue

Mice were anesthetized with isoflurane (2% in pure oxygen) and sacrificed via cervical dislocation. Brains were quickly removed and cut along the midline. Left hemispheres were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 48 h, then transferred to 15-20% sucrose in PBS for an additional 24 h, frozen in dry ice, and stored at -80°C for subsequent

immunofluorescence analyses. Fixed hemispheres were cut using a freezing microtome (Microm GmbH, Walldorf, Germany) into 30- μ m-thick coronal sections, which were serially collected in 96-well plates containing a solution composed of 30% glycerol, 30% ethylene glycol, 0.02% sodium azide in 0.1 M PBS. For each animal, a total of six free-floating sections from the hippocampal formation were selected for immunofluorescence analysis, corresponding to one section every eight throughout the rostro-caudal extent of the hippocampus.

Sections were blocked for 1 h at room temperature (RT) in PBS containing 0.1% Triton X-100 and 3% bovine serum albumin (BSA), and then incubated 16 h at 4°C in PBS containing 0.1% Triton X-100 and 1% BSA with one of the following primary antibodies: vesicular glutamate transporter 1 (VGLUT1), vesicular GABA transporter (VGAT), postsynaptic density protein 95 (PSD-95), gephyrin, parvalbumin, calbindin, somatostatin, allograft inflammatory factor 1 (AIF-1), glial fibrillary acidic protein (GFAP), or neuronal nuclei (NeuN). The following day, sections were incubated for 2 h at RT in PBS containing 0.1% Triton X-100 and 1% BSA with the appropriate species-specific secondary antibodies. Details of all primary and secondary antibodies are provided in Table S1. After washing, sections were mounted onto Superfrost® Plus microscope slides (Thermo Fisher Scientific, Waltham, MA, USA) and counterstained with DAPI (40',6-diamidino-2-phenylindole)-Fluoromount-G® (SouthernBiotech, Birmingham, AL, USA).

Double-immunolabeled sections were used for colocalization analysis in order to visualize *bonafide* inhibitory synapses identified by presynaptic VGAT-positive terminals juxtaposed to postsynaptic gephyrin-positive clusters. For double immunofluorescence staining, sections were blocked for 1 h at RT in PBS containing 0.1% Triton X-100 and 3% BSA, and then incubated 16 h at 4°C in PBS containing 0.1% Triton X-100 and 1% BSA with a combination of primary antibodies against VGAT and gephyrin. The following day, sections were incubated for 2 h at RT in PBS

containing 0.1% Triton X-100 and 1% BSA with the appropriate species-specific secondary antibodies, and processed as described above.

Human brain tissue

20- μ m-thick cryosections were obtained from tissue blocks of two cortical regions: primary visual cortex (Brodmann Area 17, BA17) and prefrontal cortex (Brodmann Area 9, BA9). Sections were mounted on glass slides and stored at -80°C until use. Prior to immunostaining, sections were immersed in methanol for 3 min and rinsed for 5 min with 0.01 M PBS, as previously described [29]. Non-specific antibody binding was avoided by incubating sections in 10% normal donkey serum (NDS) with 0.5% Triton X-100 in PBS for 1 hour at RT. Sections were then incubated 16 h at RT with a primary antibody against VGAT, diluted in 3% NDS and 0.5% Triton X-100 in PBS. After three rinses in PBS, sections were incubated for 1 h at RT with an appropriate species-matched secondary antibody in 3% normal goat serum and 0.5% Triton X-100 in PBS. Sections were then rinsed in PBS and coverslipped with anti-fade mounting medium (Agilent Technologies, Santa Clara, CA, USA). Details of primary and secondary antibodies are provided in Table S1.

Image Acquisition and Measurements

Fluorescence images of mouse brain sections immunostained for parvalbumin, calbindin, somatostatin, AIF-1, GFAP, or NeuN were acquired using a Nikon Eclipse TE 2000-S fluorescence microscope equipped with a DS-Qi2 digital SLR camera (Nikon Instruments Inc., Tokyo, Japan). Fluorescence images of mouse brain sections immunostained for VGAT, VGLUT1, PSD-95, or gephyrin were acquired using a Nikon Ti-E fluorescence microscope connected to an A1R confocal system (Nikon Instruments Inc., Tokyo, Japan). Fluorescence images of human brain sections immunostained for VGAT were acquired using a ZEISS LSM 900 laser scanning confocal microscope

mounted on an Axio Imager.Z2 platform (Zeiss, Oberkochen, Germany). Thresholds and acquisition parameters were kept constant across all experimental groups.

Quantification of single-marker puncta

For mouse brain tissue analysis, three to four sections per animal were analyzed. For each section, three images were acquired from layers II-III of the somatosensory cortex and from the stratum oriens of the hippocampus using a 60× oil immersion objective (NA = 1.40; zoom factor = 6; Nikon Instruments Inc., Tokyo, Japan). Individual puncta immunoreactive for VGLUT1, VGAT, PSD-95, or gephyrin were manually counted using Image Pro Plus software (version 4.5; Media Cybernetics, Rockville, MD, USA). Counts were performed across 5 regions of interest (ROIs; 6 × 6 μm each) per image, and puncta density was expressed as the number of immunoreactive puncta per 100 μm².

For human brain tissue analysis, five serial optical sections at 0.5 μm z-step intervals were acquired from cortical layer II-III of BA17 and BA9 using a 100× oil immersion objective (NA = 1.40; Zeiss, Oberkochen, Germany). Individual puncta immunoreactive for VGAT were manually counted using the point tool in ImageJ (NIH, Bethesda, MD, USA). Only puncta present in at least two consecutive optical sections were included in the analysis. Counts were performed across 8 regions of interest (ROIs; 9 × 9 μm each) per section, and puncta density was expressed as the number of immunoreactive puncta per 100 μm².

VGAT-Gephyrin colocalization analysis

To evaluate whether presynaptic VGAT-positive terminals corresponded to structurally complete inhibitory synapses, a colocalization analysis between VGAT and gephyrin was performed on double-immunolabeled mouse brain sections obtained from the same animals used for single-marker analysis. Confocal images were acquired from layers II-III of the somatosensory cortex and from the stratum oriens of the hippocampus, using the same imaging parameters described above.

Image stacks were subjected to deconvolution to improve spatial resolution and facilitate the identification of closely apposed puncta. VGAT-positive puncta, gephyrin-positive puncta, and VGAT-gephyrin double-positive puncta were manually quantified using Image Pro Plus software (version 4.5; Media Cybernetics, Rockville, MD, USA). All image acquisition and analyses were performed using identical settings across experimental groups and blinded to genotype and age. Colocalization was defined as spatial apposition between VGAT-positive puncta and gephyrin-positive clusters within the same optical plane or in adjacent z-sections following deconvolution. The percentage of VGAT puncta colocalized with gephyrin relative to the total number of VGAT puncta was calculated. Dual immunostaining was used exclusively to evaluate VGAT-gephyrin colocalization, whereas the quantification of individual synaptic puncta was performed on separate single-marker immunostainings. Due to the use of distinct staining procedures and image-processing workflows, the two datasets were analyzed and interpreted independently.

Cell density

Starting from 20× magnification images of mouse brain sections, the number of AIF-1-positive cells in the hippocampus and somatosensory cortex was manually counted using the point tool in Image Pro Plus software (version 4.5; Media Cybernetics, Silver Spring, MD, USA), and cell density was expressed as AIF-1-positive cells/mm². Similarly, the densities of mature neurons (NeuN-positive cells) and astrocytes (GFAP-positive cells) in the hippocampus were also determined manually and expressed as cells/mm². Parvalbumin-, calbindin-, and somatostatin-positive interneurons were counted separately in each layer of the somatosensory cortex and in the hippocampus and expressed as cells/mm². Six sections per animal were analyzed.

Morphometric analysis of microglial cells

Starting from 20× magnification images of mouse brain sections, microglial soma size of AIF1-immunostained cortical and hippocampal sections was measured using the measurement tool in

Image Pro Plus software (version 4.5; Media Cybernetics, Rockville, MD, USA). Cell body area was manually delineated and expressed in μm^2 . Only fully visible, non-overlapping microglial cells were included in the analysis. Approximately 120 cells per animal were analyzed.

Western blotting

Synaptosomal fractions were prepared from the brains of 12-month-old wild-type and *Cdkl5* heterozygous female mice by differential centrifugation as previously described [38], with minor modifications. Briefly, whole-brain tissue excluding the cerebellum and olfactory bulbs was homogenized in ice-cold sucrose-HEPES buffer containing protease inhibitors and subjected to sequential centrifugation steps to obtain a crude synaptosomal pellet. The synaptosomal fraction was then lysed in ice-cold RIPA buffer supplemented with protease inhibitor cocktail and PMSF to extract synaptic proteins. Protein concentration was determined using the Bradford assay [9]. Equal amounts of protein (5 μg) were separated by SDS-PAGE on Bolt™ 4-12% Bis-Tris Plus gels (Life Technologies Corporation, Carlsbad, CA, USA) and transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Membranes were blocked in Tris-buffered saline (TBS) containing 4% non-fat dry milk for 1 h at RT and incubated 16 h at 4°C in TBS containing 5% BSA with primary antibodies. After washing, membranes were incubated with appropriate HRP-conjugated secondary antibodies for 2 h at RT in TBS containing 2% non-fat dry milk. Primary and secondary antibodies are listed in Table S1. Immunoreactive bands were visualized using enhanced chemiluminescence and imaged with a ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA), which automatically detects and highlights saturated pixels. Densitometric analysis was performed using Image Lab™ software (Bio-Rad, Hercules, CA, USA). Images acquired with exposure times that generated protein signals out of a linear range were not

considered for quantitative analysis. Protein levels were normalized to total protein loading as determined by Ponceau S staining.

Transcriptome Analysis via polyA⁺-mRNA sequencing

Total RNA was isolated from frozen right hippocampi of 3- and 12-month-old wild-type and *Cdk15* heterozygous female mice using the TRI Reagent method (Sigma-Aldrich, St. Louis, MO, USA). RNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and its integrity was evaluated with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Only samples with an RNA integrity number (RIN) ≥ 7 were used for library preparation.

Indexed libraries were prepared from 500 ng of total RNA using the Illumina Stranded mRNA Prep kit (Illumina Inc., San Diego, CA, USA). Library size was defined using the Agilent High Sensitivity assay onto an Agilent TapeStation instrument (Agilent Technologies, Santa Clara, CA, USA) and quantified using the dsDNA High Sensitivity Assay for Qubit 3.0 (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were sequenced (paired-end, 100 $\times$ 2, total 200 cycles) at a concentration of 750 pM on the NextSeq 2000 platform (Illumina Inc., San Diego, CA, USA). Raw RNAseq reads were quality assessed and trimmed for sequencing adaptors using TrimGalore (v0.6.5) with the default Phred score. Quality control, reads mapping to the mouse reference genome (GRCm38/mm10), duplicate removal, sorting and indexing, were carried out using the DRAGEN Bio-IT Platform (Illumina Inc., San Diego, CA, USA).

Raw gene counts generated by DRAGEN were used for differential gene expression analysis using DESeq2 [42], implemented in the usegalaxy.eu web platform [25]. DESeq2 applies size factor normalization (median-of-ratios method) to estimate normalized gene expression values.

Differentially expressed genes were used to generate volcano plots and heatmaps using the “ggplot2” package in R.

Statistical Analysis

The distributions of sIPSC interevent interval and amplitude were compared between wild-type and *Cdk15* +/- mice using the two-sample Kolmogorov-Smirnov test, performed with OriginLab Pro 2025 (OriginLab Corp., Northampton, MA, USA). For these analyses, cumulative distributions of sIPSC interevent interval and amplitude were compared using Kolmogorov-Smirnov test, while cells and animals were considered the biological units. Consistency across cells and animals was verified, and the number of cells and animals per group is reported in the figure legends.

Statistical analyses for immunofluorescence and western blot data were performed using GraphPad Prism (version 8.0; GraphPad Software, San Diego, CA, USA). Prior to analysis, all datasets were screened for outliers using the ROUT method (Q = 1%). For datasets involving comparisons between two groups, a two-tailed unpaired Student's *t*-test was applied; normality was assessed in all cases using the Shapiro-Wilk test, and since all datasets passed the normality assumption, parametric tests were used throughout. For datasets involving two independent variables, a two-way ANOVA was performed, followed by Tukey's or Sidak's post hoc test for multiple comparisons. When multiple measurements were obtained from the same biological sample across one of the independent variables, as in the case of cell counts across cortical layers, where all layers were derived from the same subject, a two-way repeated measures ANOVA followed by Sidak's post hoc test was used instead to account for the non-independence of observations.

For transcriptomic analysis, RNA sequencing data were analyzed using DESeq2 [42] in Galaxy Europe. Three separate analyses were performed. To identify genes differentially expressed between genotypes at each individual timepoint, two pairwise comparisons were carried out:

heterozygous versus wild-type mice at 3 months of age, and heterozygous versus wild-type mice at 12 months of age, using a design including genotype as the sole factor (design formula: $\sim \text{genotype}$). To identify genes whose age-dependent expression trajectories differ between genotypes, a third analysis was performed using a full factorial design including genotype, age, and their interaction (design formula: $\sim \text{age} * \text{genotype}$), in which the age $\times$ genotype interaction term was tested using the Wald test. In this model, the age $\times$ genotype interaction term was used to identify genes showing age-dependent genotype effects. DESeq2 models count data using a negative binomial generalized linear model while accounting for biological variability and library size normalization. In all analyses, p -values were adjusted for multiple testing using the Benjamini-Hochberg procedure (false discovery rate, FDR).

The specific statistical test applied to each dataset, the number of samples (n), and the results of the statistical comparisons are reported in the respective figure legends. A summary of all statistical analyses, including test statistics and p -values, is provided in Table S2 (excluding transcriptomic analyses, which are detailed in Tables S3-S5). A p -value or adjusted p -value < 0.05 was considered statistically significant.

RESULTS

Age-specific alterations in inhibitory neurotransmission in young and middle-aged heterozygous

Cdkl5 +/- female mice

To investigate the functional properties of inhibitory synapses in the mosaic *Cdkl5*-deficient brain, we performed whole-cell patch-clamp recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) from pyramidal neurons in the somatosensory cortex (layers II-III) and hippocampal CA1 region in acute brain slices of heterozygous *Cdkl5* +/- and wild-type female mice at 3 and 12 months of age.

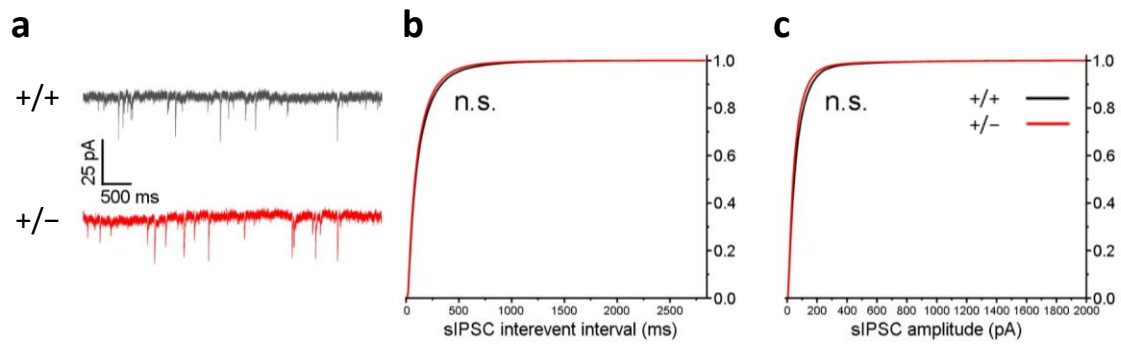
At 3 months, no significant differences in sIPSC frequency or amplitude were detected in cortical neurons between *Cdkl5* +/- mice and wild-type controls (Fig. 1a-c), indicating that inhibitory synaptic transmission in this region is largely preserved at this stage. In contrast, hippocampal CA1 pyramidal neurons from *Cdkl5* +/- females exhibited a significant increase in both sIPSC frequency and amplitude compared to wild-type animals (Fig. 1d-f), consistent with an early enhancement of inhibitory synaptic drive.

By 12 months, a marked and region-specific remodeling of inhibitory transmission emerged. In the somatosensory cortex, *Cdkl5* +/- females displayed a significant increase in both sIPSC frequency and amplitude relative to age-matched wild-type controls (Fig. 1g-i), indicating a delayed enhancement of inhibitory neurotransmission in this region. Conversely, in the hippocampus, *Cdkl5* +/- mice showed a significant reduction in sIPSC frequency compared to wild-type animals, while sIPSC amplitude remained unchanged (Fig. 1j-l), consistent with a reduction in inhibitory input without evidence for changes in postsynaptic response amplitude.

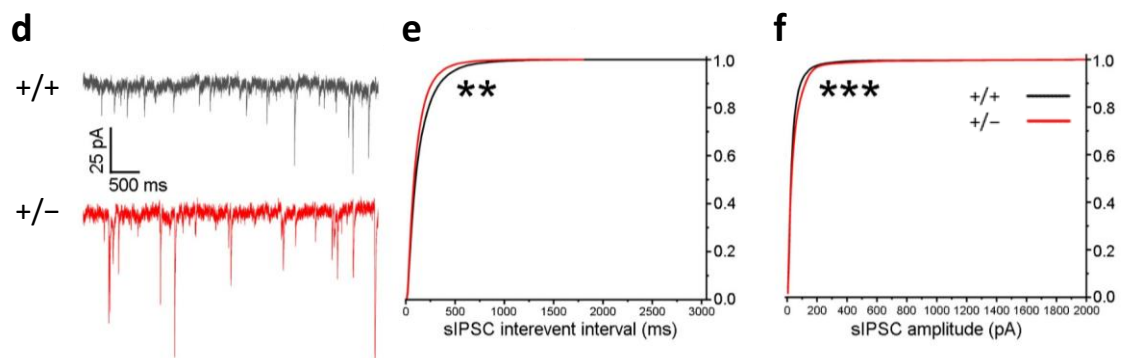
Notably, cortical and hippocampal circuits exhibited divergent trajectories across age: while hippocampal inhibition transitioned from early enhancement to later reduction, cortical inhibition showed a delayed increase emerging at 12 months. This divergence highlights a differential temporal vulnerability of inhibitory circuits across brain regions.

Overall, these findings demonstrate an age- and region-dependent remodeling of inhibitory synaptic transmission in *Cdkl5* +/- females. Hippocampal circuits display a biphasic trajectory, whereas cortical circuits exhibit a delayed functional enhancement. This dissociation suggests that mosaic CDKL5 deficiency differentially impacts inhibitory network dynamics across brain regions and may contribute to progressive circuit instability and altered excitation-inhibition balance over time.

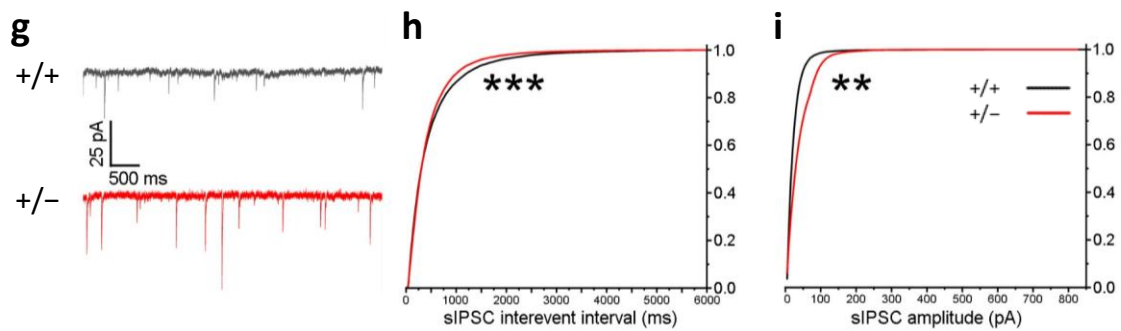
Cortex 3M



Hippocampus 3M



Cortex 12M



Hippocampus 12M

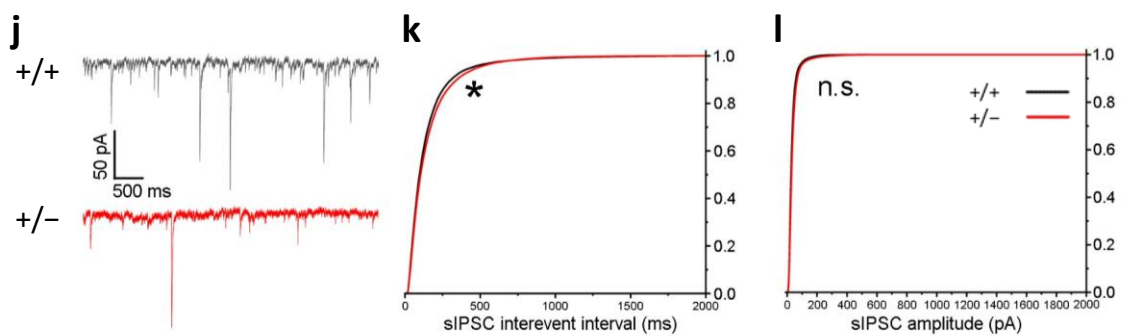


Fig. 1. Spontaneous inhibitory postsynaptic currents (sIPSCs) in the somatosensory cortex and hippocampus of young and middle-aged heterozygous *Cdkl5* +/- female mice.

(a) Representative traces of sIPSC recordings in the somatosensory cortex of 3-month-old (3M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice. (b,c) Cumulative distributions of sIPSC interevent interval (b) and amplitude (c) in the somatosensory cortex of 3-month-old wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice (*Cdkl5* +/+ : 33,978 events from 10 cells, 3 mice; *Cdkl5* +/- : 66,461 events from 18 cells, 4 mice). (d) Representative traces of sIPSC recordings in hippocampal CA1 pyramidal neurons of 3-month-old (3M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice. (e,f) Cumulative distributions of sIPSC interevent interval (e) and amplitude (f) in hippocampal CA1 pyramidal neurons of 3-month-old wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice (*Cdkl5* +/+ : 17,450-17,479 events from 6-8 cells, 3 mice; *Cdkl5* +/- : 36,627-36,674 events from 8 cells, 4 mice). (g) Representative traces of sIPSC recordings in the somatosensory cortex of 12-month-old (12M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice. (h,i) Cumulative distributions of sIPSC interevent interval (h) and amplitude (i) in the somatosensory cortex of 12-month-old wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice (*Cdkl5* +/+ : 19,885-19,923 events from 14 cells, 3 mice; *Cdkl5* +/- : 19,336-19,365 events from 12 cells, 3 mice). (j) Representative traces of sIPSC recordings in hippocampal CA1 pyramidal neurons of 12-month-old (12M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice. (k,l) Cumulative distributions of sIPSC interevent interval (k) and amplitude (l) in hippocampal CA1 pyramidal neurons of 12-month-old wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice (*Cdkl5* +/+ : 34,504-34,635 events from 8 cells, 3 mice; *Cdkl5* +/- : 60,910-60,975 events from 12 cells, 4 mice). Statistical significance was assessed by Kolmogorov-Smirnov test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Quantification of excitatory and inhibitory synaptic markers in young and middle-aged heterozygous *Cdkl5* +/- female mice

To determine whether the functional alterations in inhibitory synaptic transmission observed in heterozygous *Cdkl5* females (Fig. 1) were associated with structural changes at the synaptic level, we quantified excitatory and inhibitory synaptic puncta density in cortical and hippocampal sections from young (3 months; 3M) and middle-aged (12 months; 12M) wild-type and heterozygous mice. Presynaptic and postsynaptic markers of excitatory (VGLUT1, PSD-95) and inhibitory (VGAT, gephyrin) synapses were analyzed by confocal microscopy and quantitative image analysis.

Quantification of VGLUT1-positive presynaptic puncta revealed no major genotype-dependent differences at either age in the cortex or hippocampus (Fig. 2a,c). A modest age-related increase in VGLUT1 puncta density was observed in both regions, reaching statistical significance in the cortex

of heterozygous *Cdkl5* females and in the hippocampus of wild-type mice (Fig. 2a), indicating limited and region-dependent modulation of excitatory presynaptic markers.

In contrast, VGAT-positive puncta displayed pronounced age- and region-specific alterations (Fig. 2b,c). In the cortex, no differences were detected between genotypes at 3 months. By 12 months, wild-type mice exhibited a clear age-dependent increase in VGAT puncta density, whereas this increase was absent in heterozygous mice, resulting in significantly lower VGAT density compared to age-matched controls. In the hippocampus, heterozygous females showed a significant increase in VGAT puncta density at 3 months compared to wild-type controls, consistent with the enhanced inhibitory synaptic activity observed at this stage (Fig. 1d-f). By 12 months, however, VGAT puncta density was markedly reduced in the hippocampus of heterozygous mice, while remaining stable in wild-type animals (Fig. 2b).

Importantly, this age-dependent reduction was specific to heterozygous females. In contrast, 12-month-old *Cdkl5* knockout males ($-/Y$) and homozygous females ($-/-$) displayed increased VGAT puncta density compared to their respective wild-type controls in both cortex and hippocampus (Supplementary Fig. 1), indicating that complete *Cdkl5* loss does not recapitulate the progressive decline observed under mosaic conditions. These findings highlight mosaic CDKL5 deficiency as a key determinant of inhibitory synaptic remodeling.

Overall, changes in VGAT puncta density closely paralleled the electrophysiological findings, with increased density at 3 months corresponding to enhanced inhibitory transmission and reduced density at 12 months associated with decreased hippocampal inhibitory input. Together, these results support a model of dynamic, age-dependent remodeling of inhibitory synapses rather than a uniform progressive loss.

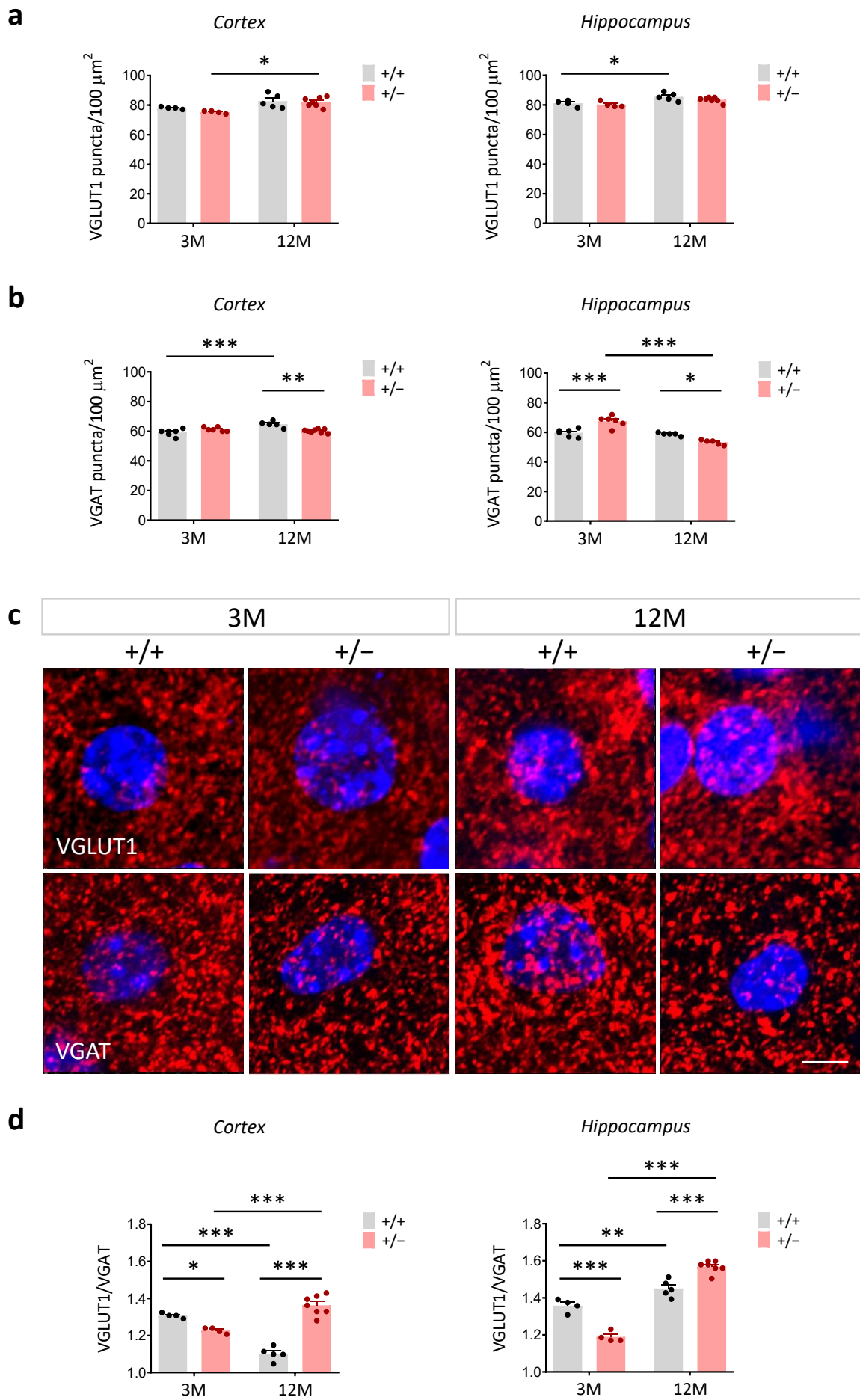


Fig. 2. Density of VGLUT1- and VGAT-immunoreactive synaptic puncta in the somatosensory cortex and hippocampus of young and middle-aged heterozygous *Cdkl5* +/- female mice.

(a) Density of vesicular glutamate transporter 1 (VGLUT1)-immunoreactive puncta in layers II-III of the somatosensory cortex (left histogram) and in the stratum oriens of the CA1 hippocampal field (right histogram) in 3-month-old (3M) and 12-month-old (12M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice (3M: *Cdkl5* +/+ *n* = 4, *Cdkl5* +/- *n* = 4; 12M: *Cdkl5* +/+ *n* = 5, *Cdkl5* +/- *n* = 7). (b) Density of vesicular GABA transporter (VGAT)-immunoreactive puncta in layers II-III of the somatosensory cortex (left histogram) and in the stratum oriens of the CA1 hippocampal field (right histogram) in 3-month-old (3M) and 12-month-old (12M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice (3M: *Cdkl5* +/+ *n* = 6, *Cdkl5* +/- *n* = 6; 12M: *Cdkl5* +/+ *n* = 5, *Cdkl5* +/- *n* = 5-8). (c) Representative confocal images of brain sections immunostained for VGLUT1 (upper panels) or VGAT (lower panels) in layers II-III of the somatosensory cortex from one mouse per experimental group. Scale bar = 5 μ m. (d) Ratio of VGLUT1 to VGAT puncta density in layers II-III of the somatosensory cortex (left histogram) and in the stratum oriens of the CA1 hippocampal field (right histogram) in 3-month-old (3M) and 12-month-old (12M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice (3M: *Cdkl5* +/+ *n* = 4, *Cdkl5* +/- *n* = 4; 12M: *Cdkl5* +/+ *n* = 5, *Cdkl5* +/- *n* = 7). The VGLUT1/VGAT ratio was calculated for each individual mouse. Data in (a), (b), and (d) are presented as mean $\pm$ SEM. Statistical significance was assessed by two-way ANOVA followed by Tukey's post hoc test. * p < 0.05, ** p < 0.01, *** p < 0.001.

As a descriptive index of the relative abundance of excitatory and inhibitory presynaptic markers, we calculated the VGLUT1/VGAT puncta density ratio (Fig. 2d). At 3 months, both cortex and hippocampus of heterozygous females showed a reduced ratio compared to wild-type controls, consistent with relatively increased inhibitory labeling. By 12 months, this ratio increased in both regions, suggesting a relative shift in the balance of presynaptic markers.

Analysis of postsynaptic compartments revealed a marked reduction in PSD-95-positive puncta in heterozygous females compared to wild-type controls in both cortex and hippocampus at both ages (Fig. 3a,b), indicating a persistent decrease in excitatory postsynaptic marker density. In contrast, gephyrin-positive puncta exhibited a region-specific pattern. While an overall age-dependent reduction was observed across genotypes, no genotype-dependent differences were detected in the cortex at either age (Fig. 3c,d). In the hippocampus, however, heterozygous females consistently showed reduced gephyrin puncta density compared to wild-type controls at both ages (Fig. 3c,d), indicating a sustained impairment in inhibitory postsynaptic organization.

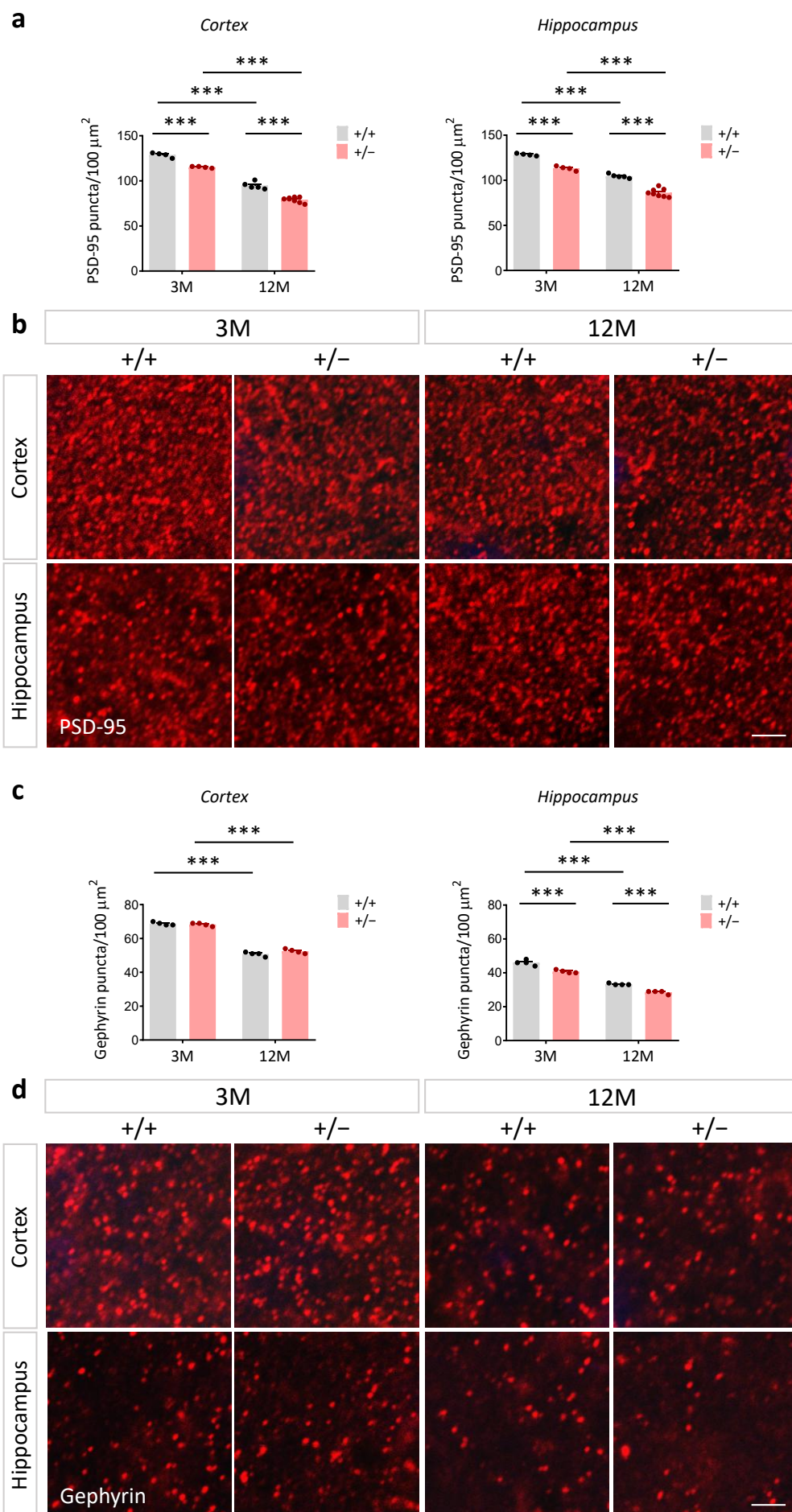


Fig. 3. Density of PSD-95- and gephyrin-immunoreactive synaptic puncta in the somatosensory cortex and hippocampus of young and middle-aged heterozygous *Cdkl5* +/- female mice.

(a) Density of postsynaptic density protein 95 (PSD-95)-immunoreactive puncta in layers II-III of the somatosensory cortex (left histogram) and in the stratum oriens of the CA1 hippocampal field (right histogram) of 3-month-old (3M) and 12-month-old (12M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice (3M: *Cdkl5* +/+ *n* = 4; *Cdkl5* +/- *n* = 4; 12M: *Cdkl5* +/+ *n* = 5; *Cdkl5* +/- *n* = 8). (b) Representative confocal images of brain sections immunostained for PSD-95 in layers II-III of the somatosensory cortex (upper panels) and in the stratum oriens of the CA1 hippocampal field (lower panels) from one mouse per experimental group. Scale bar = 3 μ m. (c) Density of gephyrin-immunoreactive puncta in layers II-III of the somatosensory cortex (left histogram) and in the stratum oriens of the CA1 hippocampal field (right histogram) of 3-month-old (3M) and 12-month-old (12M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice (3M: *Cdkl5* +/+ *n* = 4; *Cdkl5* +/- *n* = 4; 12M: *Cdkl5* +/+ *n* = 4; *Cdkl5* +/- *n* = 4). (d) Representative confocal images of brain sections immunostained for gephyrin in layers II-III of the somatosensory cortex (upper panels) and in the stratum oriens of the CA1 hippocampal field (lower panels) from one mouse per experimental group. Scale bar = 3 μ m. Data in (a) and (c) are presented as mean $\pm$ SEM. Statistical significance was assessed by two-way ANOVA followed by Tukey's post hoc test. *** *p* < 0.001.

To further assess synaptic alterations, we analyzed protein expression in synaptosomal fractions from 12-month-old mice (Fig. 4). Western blot analysis confirmed an approximately 50% reduction in *Cdkl5* protein levels in heterozygous females, consistent with mosaic expression. A significant decrease in synaptophysin was observed, whereas PSD-95 and VGAT showed non-significant downward trends and VGLUT1 remained unchanged. These biochemical data are broadly consistent with the structural findings, although they should be interpreted with caution due to the regional heterogeneity of the synaptosomal preparations.

Reduced VGAT/gephyrin colocalization in the cortex and hippocampus of heterozygous *Cdkl5* +/- female mice across age

Given the region-specific differences observed in inhibitory synaptic structure and function, we next investigated the organization of inhibitory synapses by analyzing the colocalization between the presynaptic marker VGAT and the postsynaptic scaffold protein gephyrin in both cortex and hippocampus.

Quantification of VGAT/gephyrin double-positive puncta revealed a significant reduction in the proportion of VGAT puncta juxtaposed to gephyrin in heterozygous *Cdkl5* females compared with wild-type controls at both ages in the cortex (Fig. 5a,b). In heterozygous mice, the percentage of colocalized puncta was reduced to approximately 77% and 75% of wild-type levels at 3 and 12 months, respectively (Fig. 5a,b), indicating a persistent impairment in inhibitory synapse organization.

A similar reduction in VGAT/gephyrin colocalization was observed in the hippocampus of heterozygous females at both ages, where heterozygous females showed approximately 68% and 76% of wild-type levels at 3 and 12 months, respectively (Fig. 5c), indicating that this structural alteration is not region-specific but represents a general feature of the *Cdkl5* heterozygous condition.

Together, these findings identify an early-onset and persistent defect in the structural organization of inhibitory synapses that is maintained across age. Notably, in the cortex this structural deficit coexists with increased inhibitory synaptic transmission observed at 12 months (Fig. 1g-i), indicating a dissociation between synaptic organization and functional output.

These results suggest that structural disruption of inhibitory synapses is a stable feature of mosaic CDKL5 deficiency, whereas functional output is shaped by additional region- and age-dependent mechanisms.

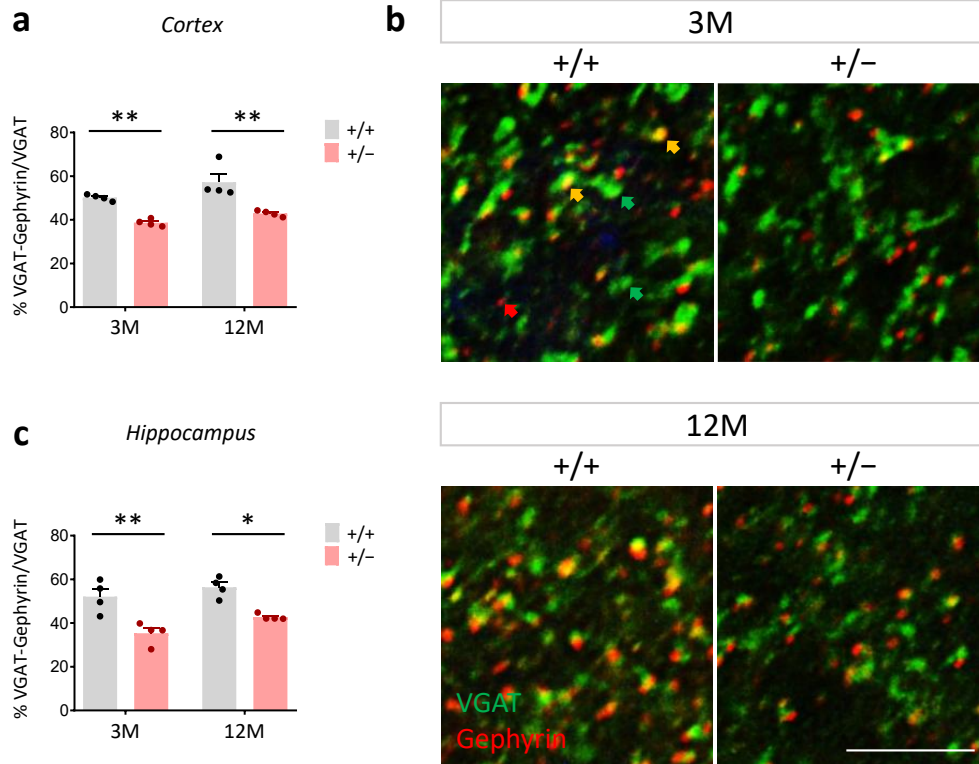


Fig. 5. Colocalization of VGAT- and gephyrin-immunoreactive synaptic puncta in the somatosensory cortex and hippocampus of young and middle-aged heterozygous *Cdkl5* +/- female mice.

(a,c) Percentage of vesicular GABA transporter (VGAT) puncta colocalized with gephyrin relative to the total number of VGAT puncta in layers II-III of the somatosensory cortex (a) and in the stratum oriens of the CA1 hippocampal field (c) of 3-month-old (3M) and 12-month-old (12M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice (3M: *Cdkl5* +/+ *n* = 4; *Cdkl5* +/- *n* = 4; 12M: *Cdkl5* +/+ *n* = 4; *Cdkl5* +/- *n* = 4). (b) Representative confocal images of brain sections immunostained for VGAT (green) and gephyrin (red) in layers II-III of the somatosensory cortex from one mouse per experimental group. Yellow arrows indicate VGAT puncta colocalized with gephyrin; green arrows indicate VGAT puncta not colocalized with gephyrin; red arrows indicate gephyrin puncta not colocalized with VGAT. Scale bar = 5 μ m. Data in (a) and (c) are presented as mean $\pm$ SEM. Statistical significance was assessed by two-way ANOVA followed by Tukey's post hoc test. * p < 0.05, ** p < 0.01.

Quantification of inhibitory interneurons in young and middle-aged heterozygous *Cdkl5* +/- female mice

To assess whether the observed alterations in inhibitory synapses could be attributed to changes in interneuron populations, we quantified parvalbumin (PV)-positive interneurons, a major class of fast-spiking GABAergic cells critically involved in regulating cortical and hippocampal excitability [33,

49]. PV-positive cell density was analyzed in both cortex and hippocampus of *Cdk15* heterozygous and wild-type female mice at 3 and 12 months of age (Fig. 6a).

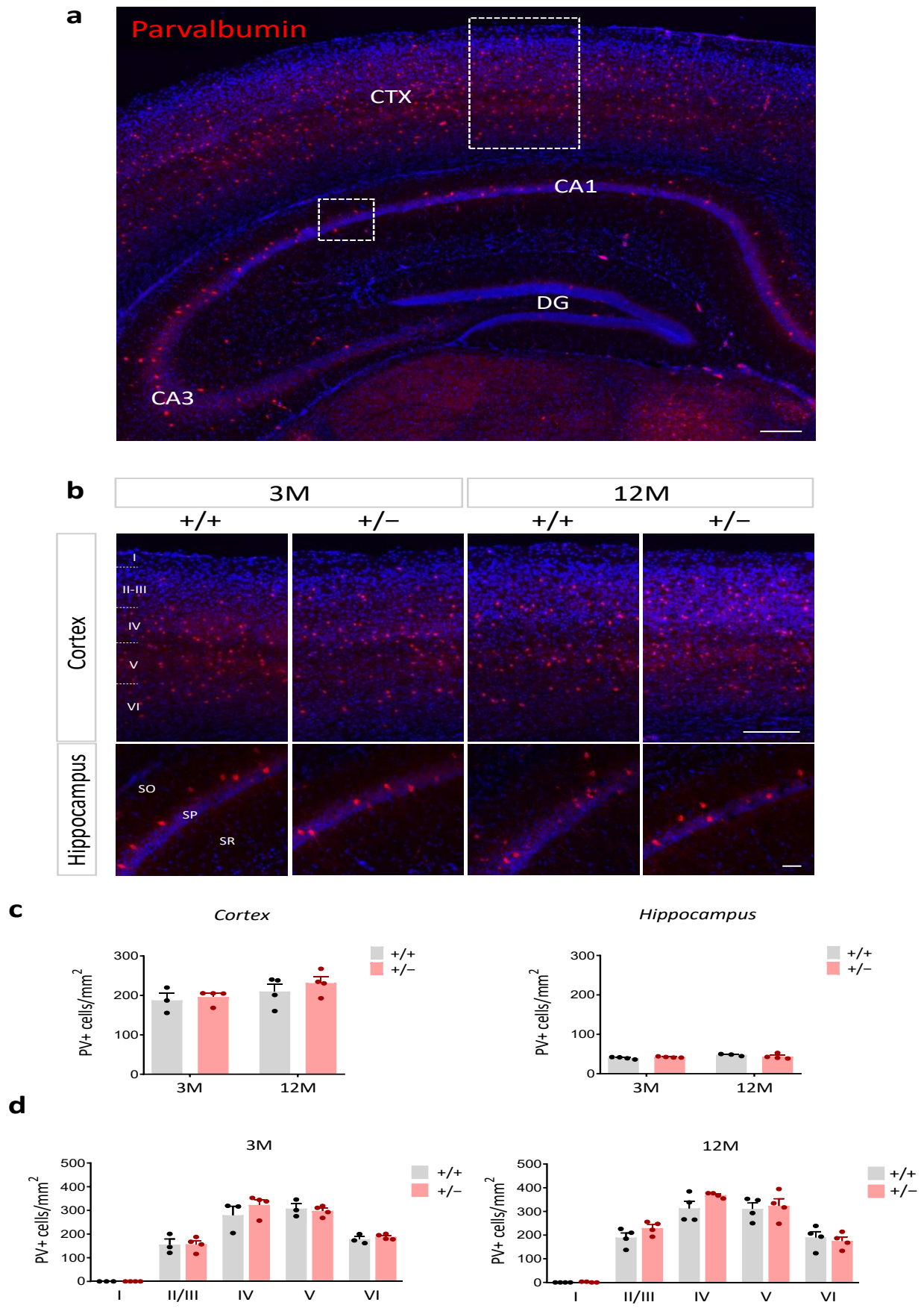


Fig. 6. Density of parvalbumin-positive interneurons in the somatosensory cortex and hippocampus of young and middle-aged heterozygous *Cdkl5* +/- female mice.

(a) Representative fluorescence image of a brain section immunostained for parvalbumin (PV) showing the cortical and hippocampal regions analyzed for PV-positive cell density (areas outlined by dashed rectangles). Scale bar = 200 μ m. (b) Representative fluorescence images of brain sections immunostained for PV in the somatosensory cortex (upper panels, scale bar = 200 μ m) and hippocampus (lower panels, scale bar = 50 μ m) from one mouse per experimental group. (c) Density of PV-positive interneurons in the somatosensory cortex (left histogram) and hippocampus (right histogram) of 3-month-old (3M) and 12-month-old (12M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice. For the cortex: 3M *Cdkl5* +/+ (n = 3), *Cdkl5* +/- (n = 4); 12M *Cdkl5* +/+ (n = 4), *Cdkl5* +/- (n = 4). For the hippocampus: 3M *Cdkl5* +/+ (n = 4), *Cdkl5* +/- (n = 4); 12M *Cdkl5* +/+ (n = 3), *Cdkl5* +/- (n = 4). (d) Density of PV-positive interneurons across somatosensory cortical layers in 3-month-old (3M, left histogram) and 12-month-old (12M, right histogram) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice, from the same animals shown in (c). Data in (c) and (d) are presented as mean $\pm$ SEM. Statistical significance was assessed by two-way ANOVA followed by Tukey's post hoc test for panel (c), and by two-way repeated measures ANOVA followed by Sidak's post hoc test for panel (d). Abbreviations: CTX, cortex; CA1, hippocampal CA1 field; CA3, hippocampal CA3 field; DG, dentate gyrus; I-VI, cortical layers; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum of the hippocampal CA1 region.

No significant differences between genotypes or across ages were detected in either region (Fig. 6b,c). Laminar analysis of PV interneuron density across the cortex also revealed no genotype- or age-dependent differences (Fig. 6d; Supplementary Fig. 2a). Overall, these findings indicate that inhibitory synaptic alterations are not associated with a loss or major redistribution of PV-expressing interneurons.

To determine whether additional interneuron populations contribute to the observed phenotype, we next examined calbindin (CB)-positive interneurons (Fig. 7a), a distinct class of GABAergic cells involved in shaping cortical and hippocampal network activity [7, 30, 45]. Similar to PV-positive interneurons, overall CB-positive cell density did not differ between genotypes or across ages in either brain region (Fig. 7b,c). Cortical laminar distribution was also largely comparable between groups (Fig. 7d; Supplementary Fig. 2b), although a layer-specific increase was detected in cortical layer V at 12 months (Fig. 7d). These results further support the absence of major alterations in interneuron number or distribution.

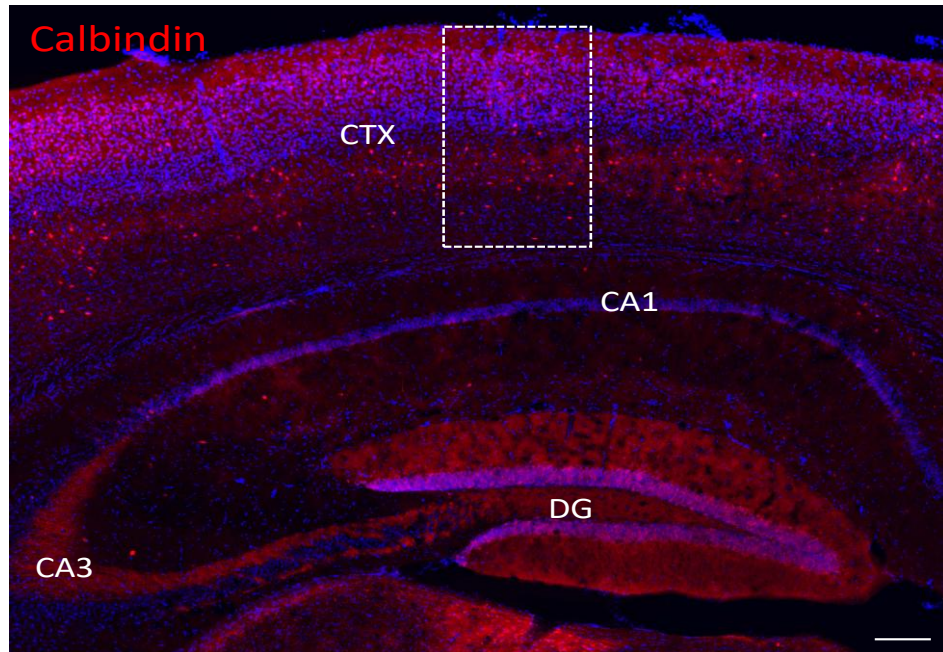
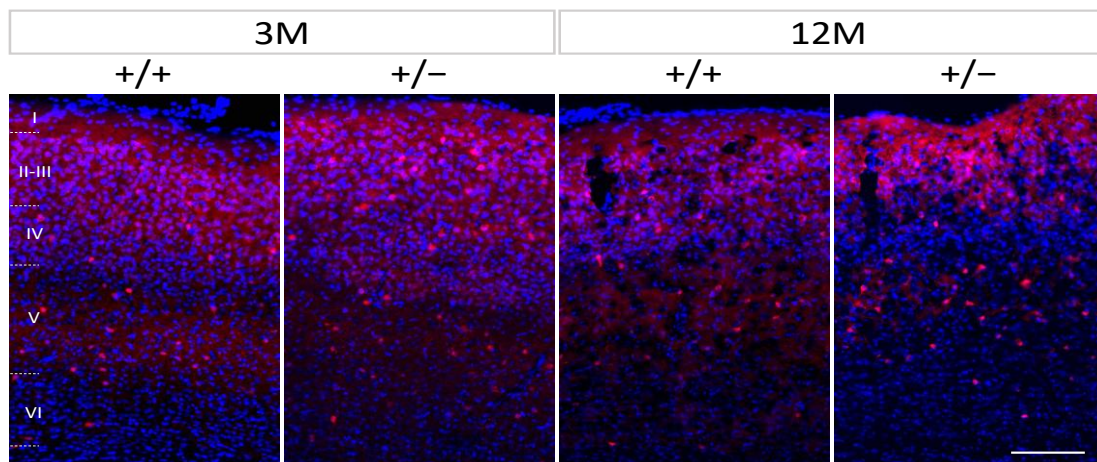
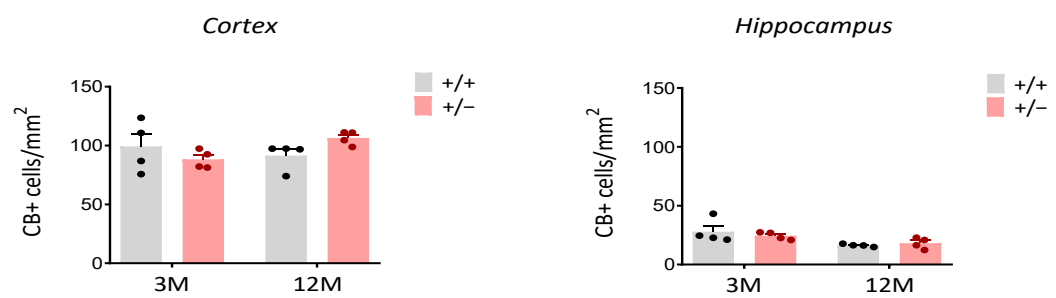
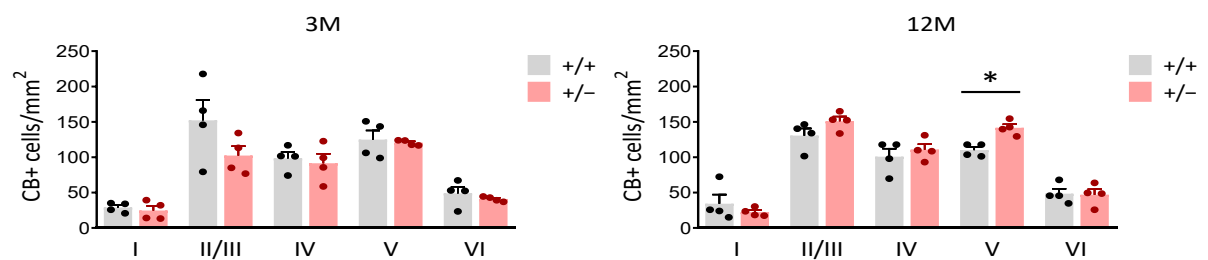
a**b****c****d**

Fig. 7. Density of calbindin-positive interneurons in the somatosensory cortex and hippocampus of young and middle-aged heterozygous *Cdkl5* +/- female mice.

(a) Representative fluorescence image of a brain section immunostained for calbindin (CB) showing the cortical region analyzed for CB-positive cell density (area outlined by a dashed rectangle). Scale bar = 200 μ m. (b) Representative fluorescence images of cortical sections immunostained for CB from one mouse per experimental group. Scale bar = 150 μ m. (c) Density of CB-positive interneurons in the somatosensory cortex (left histogram) and hippocampus (right histogram) of 3-month-old (3M) and 12-month-old (12M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice (3M: *Cdkl5* +/+ $n = 4$, *Cdkl5* +/- $n = 4$; 12M: *Cdkl5* +/+ $n = 4$, *Cdkl5* +/- $n = 4$). (d) Density of CB-positive interneurons across somatosensory cortical layers in 3-month-old (3M, left histogram) and 12-month-old (12M, right histogram) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice, from the same animals shown in (c). Data in (c) and (d) are presented as mean $\pm$ SEM. Statistical significance was assessed by two-way ANOVA followed by Tukey's post hoc test for panel (c), and by two-way repeated measures ANOVA followed by Sidak's post hoc test for panel (d). * $p < 0.05$. Abbreviations: CTX, cortex; CA1, hippocampal CA1 field; CA3, hippocampal CA3 field; DG, dentate gyrus; I-VI, cortical layers.

We also analyzed somatostatin (SST)-positive interneurons, a key subclass of dendrite-targeting GABAergic neurons involved in regulating excitatory input and cortical processing [69, 72]. Quantification of SST-positive cells in 12-month-old animals revealed no significant differences between heterozygous and wild-type mice in either cortex or hippocampus, nor any alterations in their laminar distribution (Supplementary Fig. 2c-e).

Taken together, these findings indicate that the reduction in inhibitory synaptic markers observed in *Cdkl5* heterozygous females is not attributable to changes in the abundance or distribution of major interneuron subclasses. Instead, they support the interpretation that inhibitory dysfunction arises from synaptic and circuit-level alterations rather than from interneuron loss.

Evaluation of microglial and astrocyte populations in young and middle-aged heterozygous *Cdkl5* +/- female mice

To investigate whether age-dependent alterations in inhibitory circuitry in *Cdkl5* heterozygous females were associated with changes in glial populations, we analyzed microglial and astrocyte markers in the cortex and hippocampus at 3 and 12 months of age.

Immunolabeling for AIF-1 revealed significant genotype-dependent differences at 3 months (Supplementary Fig. 3). At this stage, heterozygous females displayed increased microglial cell body size and density in both cortex and hippocampus compared to wild-type controls, consistent with a more reactive phenotype, as previously reported [26]. By 12 months, wild-type mice exhibited an age-dependent increase in both microglial soma size and cell number, progressively narrowing the gap with heterozygous animals. Nevertheless, genotype-dependent differences in microglial soma size persisted at 12 months, with heterozygous females still showing larger microglial cell bodies than wild-type littermates in both cortex and hippocampus (Supplementary Fig. 3a,c). In contrast, differences in microglial cell number were largely resolved by this age in both regions, with no significant differences between genotypes at 12 months (Supplementary Fig. 3b,c). Quantitative analysis of the heterozygous-to-wild-type ratio confirmed that the magnitude of the genotype effect decreased significantly over time for soma size in both regions and for cell density in the cortex, indicating that microglial alterations emerging early in heterozygous females are progressively matched by age-related changes in wild-type animals (Fig. 8a).

Astrocyte density was assessed by glial fibrillary acidic protein (GFAP) immunostaining in the hippocampus. At 3 months, no significant differences in GFAP-positive astrocyte density were observed between genotypes (Fig. 8b,d). However, heterozygous females exhibited a significant age-dependent reduction in GFAP-positive cell number, resulting in a significant difference between genotypes at 12 months of age (Fig. 8b,d). Notably, homozygous *Cdkl5* knockout ($-/-$) females showed a comparable reduction relative to wild-type controls ($81.14 \pm 3.5\%$, $p < 0.0001$, t -test; data not shown), suggesting that this phenotype may reflect a consequence of *Cdkl5* loss of function not strictly dependent on mosaic expression. Ratio analysis further supported an age-dependent decline in GFAP-positive cell density in heterozygous mice (Fig. 8c).

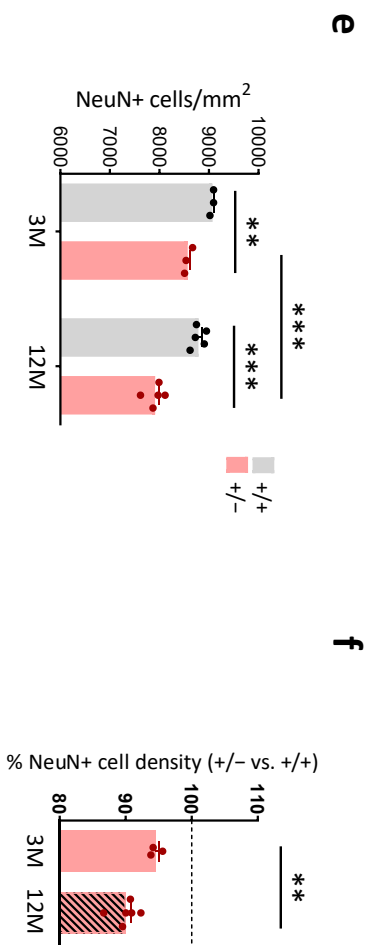
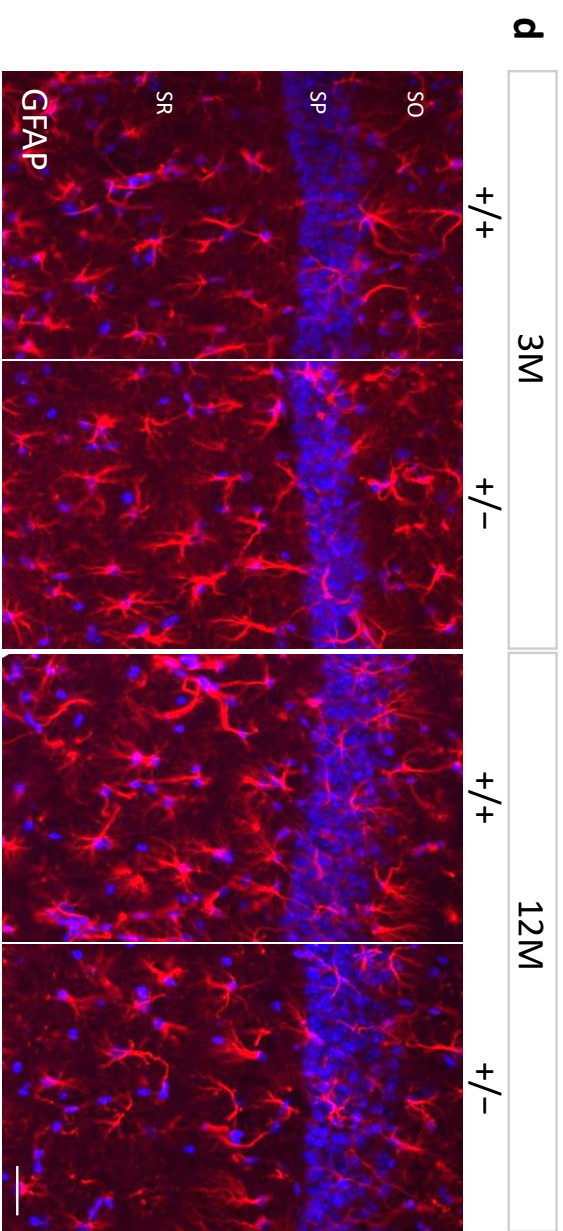
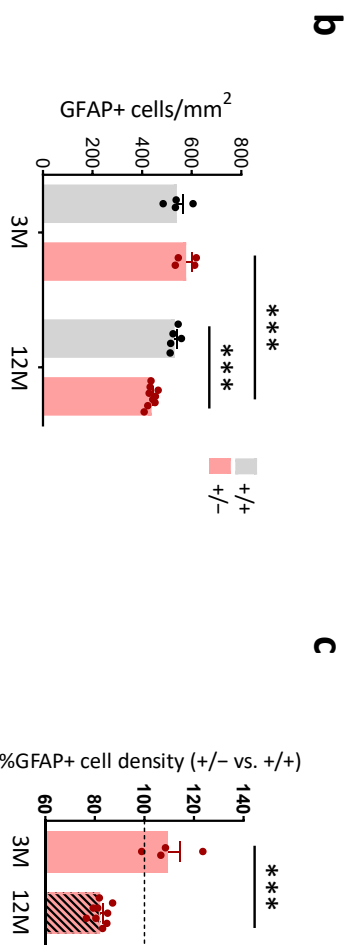
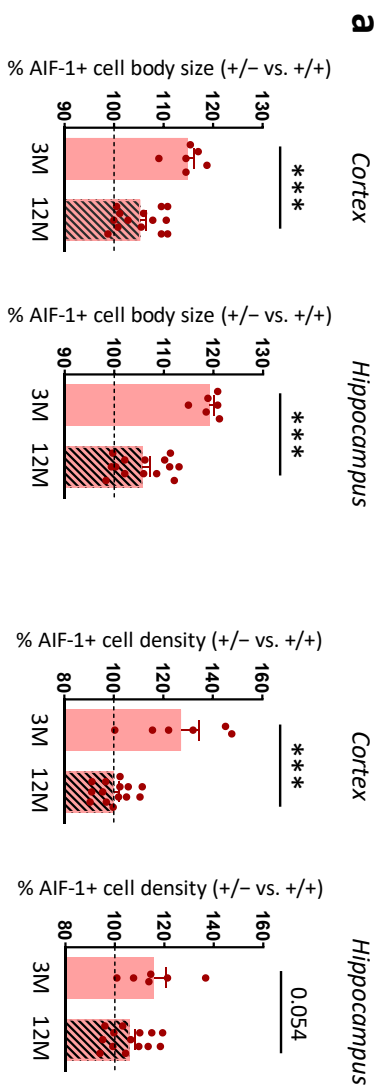


Fig. 8. Glial cell density and morphology in the somatosensory cortex and hippocampus of young and middle-aged heterozygous *Cdkl5* +/- female mice.

(a) Microglial cell body size (left histograms) and cell density (right histograms) in the somatosensory cortex and hippocampus of 3-month-old (3M) and 12-month-old (12M) *Cdkl5* heterozygous (+/-) female mice expressed as percentage of the wild-type (+/+) mean (3M: *Cdkl5* +/+ *n* = 6; *Cdkl5* +/- *n* = 6; 12M: *Cdkl5* +/+ *n* = 14; *Cdkl5* +/- *n* = 14). (b) Density of glial fibrillary acidic protein (GFAP)-positive cells in the hippocampal stratum radiatum of 3-month-old (3M) and 12-month-old (12M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice (3M: *Cdkl5* +/+ *n* = 4; *Cdkl5* +/- *n* = 4; 12M: *Cdkl5* +/+ *n* = 5; *Cdkl5* +/- *n* = 9). (c) GFAP-positive cell density in the hippocampal stratum radiatum of 3-month-old (3M) and 12-month-old (12M) *Cdkl5* heterozygous (+/-) female mice, expressed as percentage of the mean wild-type (+/+) cell density, from the same animals shown in (b). (d) Representative fluorescence images of hippocampal sections immunostained for GFAP from one mouse per experimental group. Scale bar = 30 μ m. (e) Density of NeuN-positive cells in the hippocampal CA1 region of 3-month-old (3M) and 12-month-old (12M) wild-type (+/+) and *Cdkl5* heterozygous (+/-) female mice (3M: *Cdkl5* +/+ *n* = 3; *Cdkl5* +/- *n* = 3; 12M: *Cdkl5* +/+ *n* = 5; *Cdkl5* +/- *n* = 5). (f) NeuN-positive cell density in the hippocampal CA1 region of 3-month-old (3M) and 12-month-old (12M) *Cdkl5* heterozygous (+/-) female mice, expressed as percentage of the mean wild-type (+/+) cell density (3M: *Cdkl5* +/+ *n* = 3; *Cdkl5* +/- *n* = 3; 12M: *Cdkl5* +/+ *n* = 5; *Cdkl5* +/- *n* = 6). The percentage values shown in (a), (c), and (f) were calculated by dividing each individual value from *Cdkl5* heterozygous mice by the mean value of the corresponding wild-type group and multiplying by 100. Data in (a-c), (e), and (f) are presented as mean $\pm$ SEM. Statistical significance was assessed by two-tailed Student's *t*-test for panels (a), (c), and (f), and by two-way ANOVA followed by Tukey's post hoc test for panels (b) and (e). ** *p* < 0.01, *** *p* < 0.001. Abbreviations: SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum.

To assess neuronal integrity, NeuN-positive cells were quantified in the CA1 region of the hippocampus. Heterozygous females exhibited a significant reduction in neuronal density as early as 3 months compared to wild-type controls (Fig. 8e). This deficit became more pronounced at 12 months, reflecting a progressive, age-dependent decline in NeuN-positive cells in heterozygous, but not wild-type, animals (Fig. 8e). Ratio analysis confirmed a time-dependent reduction in NeuN-positive cells in heterozygous mice relative to controls (Fig. 8f), supporting a role for CDKL5 in maintaining neuronal integrity during aging [27].

Overall, these findings indicate that *Cdkl5* heterozygous females exhibit early microglial activation, followed by age-dependent astrocyte reduction and progressive reduction in NeuN-positive cell density in the hippocampus. While these alterations may contribute to circuit vulnerability, they do not directly account for the observed synaptic phenotype, supporting the interpretation that

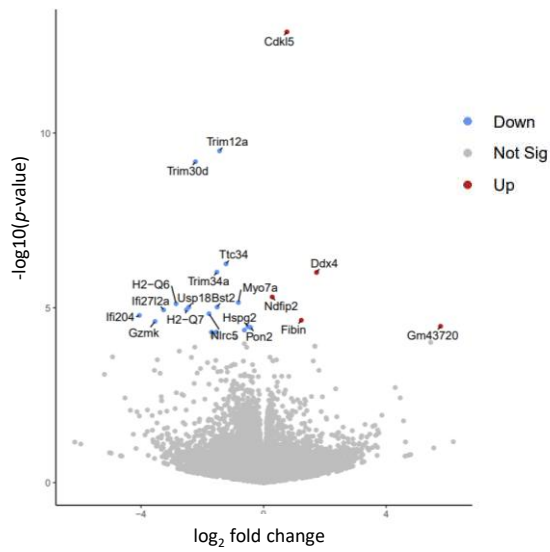
inhibitory dysfunction primarily arises from synaptic and circuit-level remodeling rather than from major changes in cell populations.

Divergent age-dependent transcriptional programs are associated with inhibitory synapse remodeling in *Cdkl5* heterozygous females

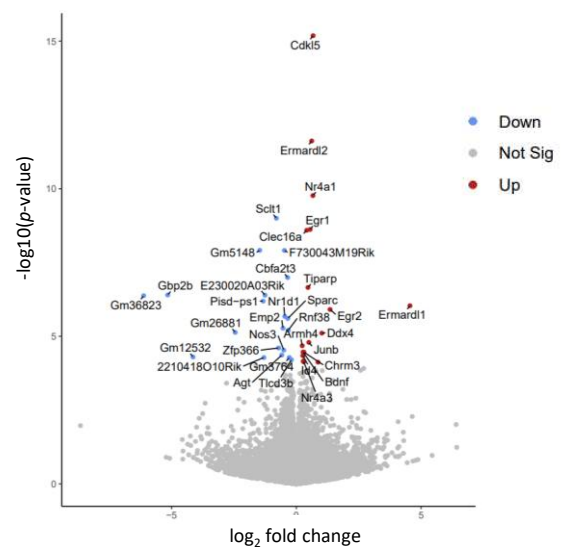
To determine whether the age-dependent alterations in inhibitory synaptic transmission and structure observed in heterozygous *Cdkl5* mice were associated with molecular changes, we performed transcriptomic profiling of hippocampal tissue from wild-type and heterozygous females at 3 and 12 months of age (Supplementary Fig. 4a). The hippocampus was selected based on its pronounced functional and structural remodeling across age.

At 3 months, differential expression analysis revealed a relatively limited set of transcriptional changes, with 5 upregulated and 18 downregulated genes in heterozygous mice compared to wild-type controls (Fig. 9a; Supplementary Fig. 4b; Table S3). This early transcriptional signature was dominated by a coordinated downregulation of interferon-stimulated genes (ISGs) and MHC-I-related genes, including members of the *Trim* family (*Trim12a*, *Trim30d*, *Trim34a*, *Trim30a*), and immune response regulators (*Usp18*, *Bst2*, *Ifi2712a*, *Ifi204*, *Nlrc5*, *Parp12*, *Tgtp1*). These changes indicate a reduction in interferon/MHC-I-associated signaling rather than activation of inflammatory pathways. Given the established role of neuronal MHC-I signaling in synaptic refinement and plasticity [56, 66], this profile is consistent with an early alteration of immune-synaptic regulatory mechanisms. Notably, this molecular state occurs in parallel with increased inhibitory synaptic drive, raising the possibility that reduced MHC-I signaling is associated with altered synaptic refinement and circuit organization.

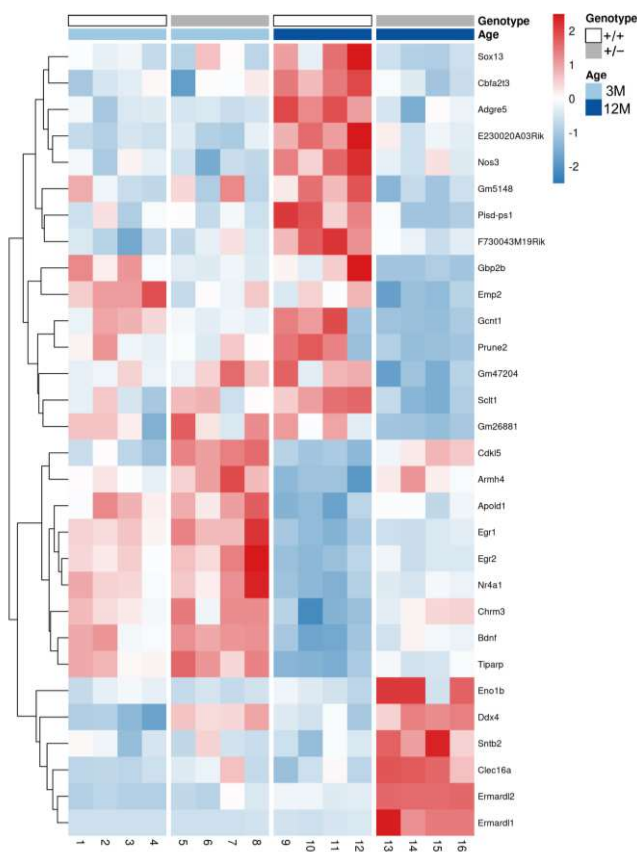
a **3M**
(*Cdkl5* +/- vs. *Cdkl5* +/+)



b **12M**
(*Cdkl5* +/- vs. *Cdkl5* +/+)



c Heatmap of genes with age-dependent genotype effect



d

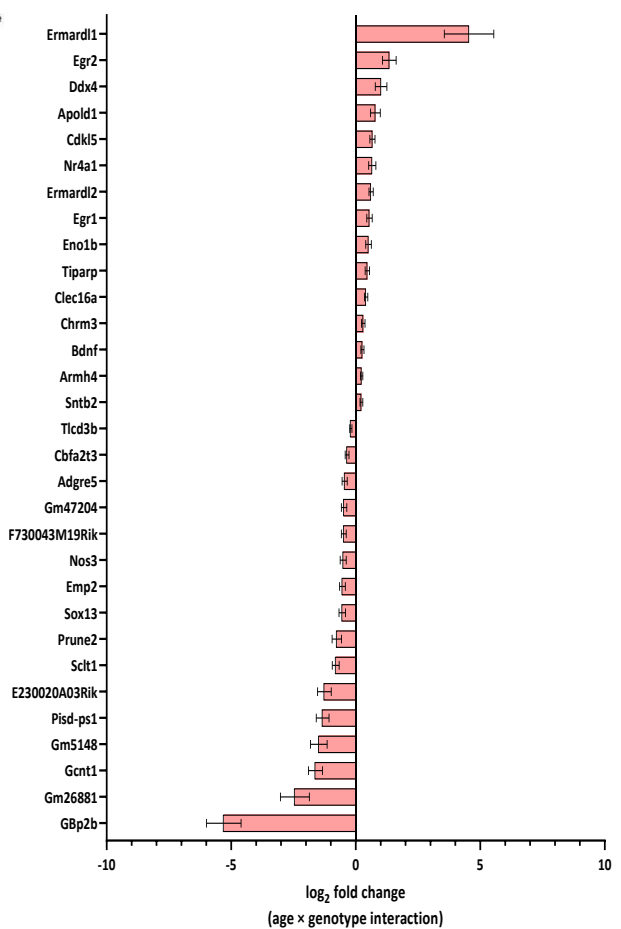


Fig. 9. Transcriptome analysis of hippocampal gene expression in young and middle-aged heterozygous *Cdkl5* +/- female mice.

(a, b) Volcano plots showing differential gene expression between *Cdkl5* heterozygous (+/-) and wild-type (+/+) mice at 3 months (3M, a) and 12 months (12M, b) of age. The x-axis represents the $\log_2$ fold change ($\log_2FC$) and the y-axis the $-\log_{10}$ adjusted *p*-value (*p*-adj). Significantly upregulated genes in *Cdkl5* +/- mice ($\log_2FC > 0$, *p*-adj < 0.05) are shown in red; significantly downregulated genes in *Cdkl5* +/- mice ($\log_2FC < 0$, *p*-adj < 0.05) are shown in blue; non-significant genes are shown in grey. Selected genes are labelled. (c) Heatmap showing normalized expression levels of genes with a significant age $\times$ genotype interaction (*p*-adj < 0.05, DESeq2). Each column represents an individual animal; each row represents a gene. Red indicates positive interaction $\log_2$ fold changes, corresponding to genes whose genotype-dependent expression differences increase with age in *Cdkl5* +/- mice relative to wild-type controls, whereas blue indicates negative interaction $\log_2$ fold changes, corresponding to genes whose genotype-dependent differences decrease with age. Samples are grouped by genotype and age. (d) Differential expression analysis by DESeq2 with age $\times$ genotype interaction term. Shown are $\log_2FC \pm SEM$ for genes with *p*-adj < 0.05. A positive $\log_2FC$ indicates that expression in *Cdkl5* heterozygous mice increases relative to wild-type with age; a negative $\log_2FC$ indicates that it decreases.

Among the few upregulated genes at this stage, *Cdkl5* expression appeared increased (Fig. 9a; Supplementary Fig. 4b; Table S3). However, this result should be interpreted with caution, as transcript detection depends on exon targeting: in the Amendola et al. model [2], deletion of exon 4 abolishes exon-specific detection while other exons remain detectable, consistent with escape from nonsense-mediated decay. Thus, increased *Cdkl5* signal at the RNA level does not imply restoration of functional protein.

At 12 months, the transcriptional profile became more pronounced and qualitatively distinct, with 17 upregulated and 20 downregulated genes in heterozygous mice relative to wild-type controls (Fig. 9b; Supplementary Fig. 4c; Table S4). Strikingly, the broad ISG transcriptional signature that dominated the 3-month profile was absent at 12 months, with only two immune-related genes reaching significance: *Gbp2b*, which was markedly downregulated, and *Clec16a*, which was upregulated. Notably, the predominant upregulated signature at 12 months comprised activity-dependent and immediate-early transcriptional programs, including *Egr1*, *Egr2*, *Nr4a1*, *Nr4a3*, and *Bdnf*, indicating increased neuronal activity-related signaling. In parallel, downregulated genes were

associated with vascular, structural, and homeostatic support pathways, including *Agt*, *Nos3*, *Sparc*, *Sc1t1*, and *Emp2*. Together, these changes define a transcriptional profile in which increased activity-related signaling is accompanied by reduced expression of genes involved in structural, and vascular support.

Importantly, this molecular profile is observed in parallel with functional and structural alterations in the hippocampus, including reduced inhibitory synaptic input, decreased VGAT puncta density, and persistent deficits in gephyrin-positive postsynaptic structures (Fig. 1j-l; Fig. 2b,c; Fig. 3c,d), suggesting that transcriptional changes occur in a context of impaired inhibitory synaptic integrity.

To further investigate genotype-specific aging trajectories, we analyzed the age $\times$ genotype interaction using a full factorial DESeq2 model (Fig. 9c,d; Table S5). This analysis identified 31 genes whose age-dependent expression trajectories differed significantly between wild-type and heterozygous mice (Fig. 9c,d). Among genes with a positive interaction term, indicating enhanced modulation in heterozygous animals with age, were activity-dependent genes (*Egr1*, *Egr2*, *Nr4a1*, *Bdnf*), and synaptic modulators (*Chrm3*, *Sntb2*). Conversely, genes with a negative interaction term, indicating reduced or blunted age-dependent regulation in heterozygous mice, included cellular structure-associated genes (*Emp2*, *Gcnt1*, *Sc1t1*), and immune-related genes (*Gbp2b*, *Adgre5*).

Overall, these findings indicate that the heterozygous hippocampus follows a biphasic molecular trajectory across age, characterized by early alterations in immune-synaptic signaling and a later shift toward activity-dependent transcription coupled with reduced structural and homeostatic support. This transition parallels the progression from early enhanced inhibitory activity to later synaptic remodeling and reduced inhibitory input observed in heterozygous *Cdkl5* females.

Analysis of age-dependent changes in VGAT puncta density in postmortem cortical tissue from CDD patients

To assess the translational relevance of the age-dependent alterations in inhibitory synaptic organization observed in the *Cdkl5* mouse model, we performed an analysis of VGAT-positive presynaptic puncta in postmortem cortical tissue from two CDD patients and neurotypical controls matched for age. Importantly, both CDD cases analyzed were heterozygous female individuals, consistent with the predominant clinical presentation of the disorder and directly comparable to the mosaic *Cdkl5* +/- female mouse model.

Analyses were performed in layers II-III of the prefrontal and visual cortex (Brodmann Areas 9 and 17, respectively), two cortical regions characterized by distinct developmental trajectories. Due to the limited availability of human tissue, one CDD case was examined for each age group (4-5 and 29-30 years). For each subject, VGAT puncta density was quantified across multiple regions of interest (ROIs) obtained from at least 2-3 sections per region, and comparisons were performed within each age group.

In the prefrontal cortex, ROI-level analysis suggested an age-related alteration in VGAT puncta density. At younger age, the CDD sample exhibited higher VGAT puncta density compared to the corresponding control, whereas in adult samples VGAT puncta density appeared reduced relative to the matched control (Fig. 10a,b). Notably, control samples showed an increase in VGAT puncta density from childhood to adulthood, whereas the CDD samples displayed the opposite pattern.

In the visual cortex, no apparent difference in VGAT puncta density was observed between the young CDD case and the corresponding control (4-5-year-old; Fig. 10a,c). In contrast, a reduction was observed in the adult CDD sample relative to the age-matched control (29-30-year-old Fig. 10a,c).

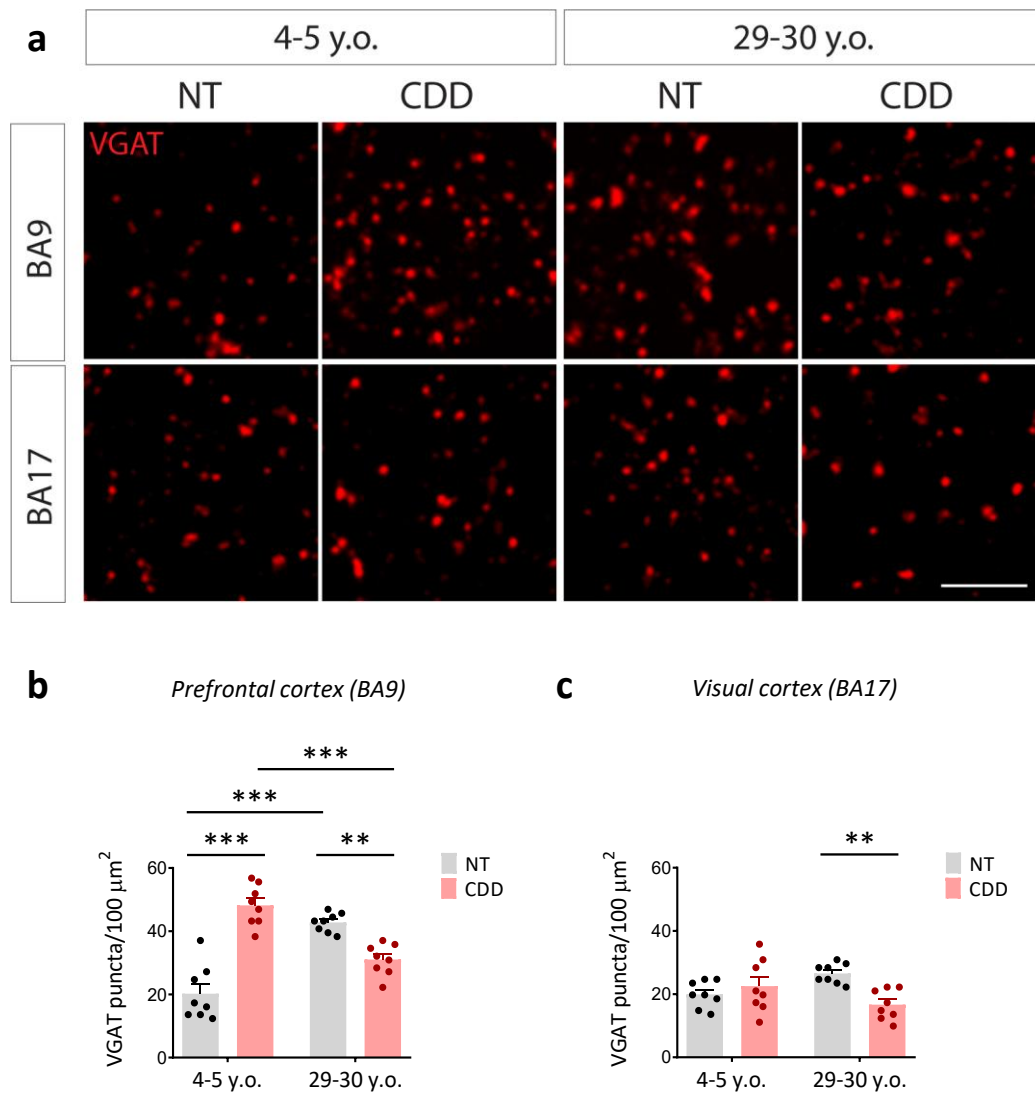


Fig. 10. Density of VGAT-immunoreactive synaptic puncta in postmortem cortical tissue from CDD patients.

(a) Representative confocal images of human brain sections immunostained for vesicular GABA transporter (VGAT) in prefrontal cortex (Brodmann Area 9, BA9) and primary visual cortex (Brodmann Area 17, BA17) from 4-5-year-old (4-5 y.o.) and 29-30-year-old (29-30 y.o.) neurotypical controls (NT) and CDD patients (CDD). Scale bar = 5 μm . (b,c) Density of VGAT-immunoreactive puncta in cortical areas BA9 (b) and BA17 (c) from the same subjects as in (a). Data in (b) and (c) are presented as mean $\pm$ SEM. Each dot represents a single region of interest (ROI), with multiple ROIs sampled from one subject per group. Statistical comparisons were performed at the ROI level using two-way ANOVA followed by Tukey's post hoc test (** $p < 0.01$, *** $p < 0.001$) and should be interpreted as descriptive, as each group included a single biological subject.

Although based on a limited number of biological cases, these findings are consistent with an altered developmental trajectory of inhibitory presynaptic organization in the cortex of CDD patients. In particular, the pattern of relatively increased inhibitory presynaptic labeling at earlier stages

followed by reduced density in adulthood parallels the age-dependent remodeling observed in the mouse model. These observations should be interpreted with caution but provide preliminary support for the possibility that inhibitory circuit alterations associated with mosaic CDKL5 deficiency may also occur in the human brain and follow a non-linear trajectory across age.

DISCUSSION

Our findings reveal that mosaic CDKL5 deficiency reshapes inhibitory synaptic regulation in a dynamic and region-dependent manner in heterozygous *Cdkl5* +/- female mice. Rather than reflecting a uniform progressive loss of inhibition, inhibitory circuit alterations followed distinct trajectories across brain regions. In the hippocampus, inhibitory synaptic transmission displayed a biphasic profile, characterized by early enhancement followed by a later reduction in synaptic input. In contrast, cortical circuits showed persistent structural disorganization accompanied by delayed enhancement of inhibitory synaptic activity. Together, these results indicate that mosaic CDKL5 deficiency induces a non-linear reorganization of inhibitory networks that evolves across age and brain region.

At 3 months of age, *Cdkl5* +/- mice exhibited increased spontaneous inhibitory synaptic currents (sIPSCs) in the hippocampus, together with a transient increase in VGAT-positive presynaptic puncta. This early enhancement of inhibitory tone may reflect an adaptive attempt to stabilize network excitability during a vulnerable phase of circuit maturation. Alternatively, it may indicate aberrant inhibitory circuit assembly or impaired synaptic refinement, leading to an excess of functionally active inhibitory contacts. In contrast, cortical inhibitory transmission was not significantly altered at this age, consistent with the absence of detectable changes in VGAT or gephyrin puncta density in this region. This regional dissociation suggests that inhibitory circuit

remodeling emerges earlier in the hippocampus than in the cortex, possibly reflecting differences in developmental timing, plasticity demands, or local circuit architecture.

By 12 months of age, the two regions displayed divergent functional profiles. In the hippocampus, inhibitory synaptic input was reduced, as indicated by decreased sIPSC frequency together with lower VGAT puncta density and persistently reduced gephyrin-positive postsynaptic puncta. Because gephyrin labeling reflects the organization of the inhibitory postsynaptic scaffold rather than GABA_A receptor abundance or composition, these data should be interpreted as evidence of impaired inhibitory synaptic architecture, not as a direct measure of postsynaptic receptor content. In the cortex, by contrast, both sIPSC frequency and amplitude were increased, indicating enhanced inhibitory synaptic transmission despite structural disorganization. This indicates that structural disruption of inhibitory synapses does not translate linearly into reduced inhibitory output, particularly in cortical circuits. Importantly, these region-specific alterations occurred in the absence of detectable changes in the number or laminar distribution of parvalbumin-, calbindin-, or somatostatin-positive interneurons [72], suggesting that this functional enhancement does not arise from an increased number of inhibitory neurons, but rather from changes in synaptic or circuit-level properties. These may include altered release probability, changes in intrinsic excitability, or compensatory strengthening of remaining inhibitory contacts [12, 39, 54, 77]. This dissociation supports the idea that inhibitory dysfunction arises primarily from synaptic and circuit-level remodeling rather than interneuron loss, as previously demonstrated in a rat model of posttraumatic neocortical epileptogenesis [28].

Notably, despite the reduction in sIPSC frequency at 12 months, sIPSC amplitude did not show a parallel decline in hippocampal pyramidal neurons. This pattern suggests that the activity or number of inhibitory inputs are reduced, whereas the average postsynaptic response is preserved. We speculate that these data indicate, in *Cdkl5* +/- mice, a presynaptic reduction in inhibitory

transmission which is not associated with a compensatory increase in postsynaptic response. Similar dissociations between synapse number and synaptic strength have been described in contexts of inhibitory imbalance and epileptogenesis [13, 22, 70], and may contribute to the progressive destabilization of hippocampal network function. Additional insight into inhibitory synapse organization emerged from VGAT/gephyrin colocalization analysis. In both cortex and hippocampus, the proportion of VGAT-positive puncta apposed to gephyrin was reduced in *Cdkl5* +/- mice at both ages, indicating a persistent impairment in the structural coupling between presynaptic inhibitory terminals and postsynaptic scaffolding sites. This alteration likely reflects a reduced fraction of structurally defined inhibitory synapses rather than a simple decrease in presynaptic or postsynaptic elements alone. However, because VGAT/gephyrin apposition provides a structural proxy rather than a direct measure of synaptic efficacy, these findings should be interpreted as evidence of altered inhibitory synapse organization. Importantly, this structural abnormality was observed regardless of the direction of functional change: it coexisted with enhanced cortical inhibitory transmission at 12 months and with divergent hippocampal functional states across age. These findings indicate that defective inhibitory synapse organization is a stable feature of mosaic CDKL5 deficiency, whereas functional output is shaped by additional region- and age-dependent mechanisms.

The hippocampus emerged as a particularly vulnerable region. Gephyrin-positive puncta were persistently reduced in this structure at both ages, consistent with previous findings in *Cdkl5* -/Y males [14]. In addition, hippocampal inhibitory transmission followed a clear biphasic trajectory, shifting from early enhancement to late reduction, whereas cortical circuits showed a distinct pattern characterized by structural disorganization together with delayed enhancement of inhibitory activity. This vulnerability is further supported by the hippocampal transcriptional profile, which suggests a transition from altered immune-synaptic signaling at early stages to increased

activity-dependent transcription and reduced homeostatic support at later stages. Regional vulnerability may therefore reflect differences in activity-dependent plasticity requirements, metabolic demand, glial-neuronal interactions, or local circuit architecture. Given the central role of hippocampal inhibitory circuits in regulating seizure threshold [17, 65, 79], their progressive destabilization is likely to contribute to network vulnerability and may be relevant to epileptogenesis in this model.

Glial alterations may further contribute to this interpretation. At 3 months, *Cdkl5* +/- females exhibited increased microglial cell body size and density [26], indicating an early change in microglial state. However, because inflammatory signaling was not directly assessed in this study, these findings should not be interpreted solely as evidence of increased neuroinflammation. One possibility is that an altered microglial state may influence synaptic remodeling processes, including synapse pruning. In this context, dysregulated microglial-mediated synapse elimination has been shown to contribute to excitation-inhibition imbalance and network hyperexcitability [18, 76].

By 12 months, microglial differences between genotypes were attenuated, arguing against a model in which progressive microglial activation alone drives the late synaptic phenotype. In contrast, GFAP-positive astroglial cells showed delayed vulnerability, with reduced GFAP-positive cell density in the hippocampus of *Cdkl5* +/- females at 12 months, a phenotype also observed in *Cdkl5* -/- females. Because GFAP immunolabeling reflects a specific astroglial state rather than the full astrocyte population, this result should be interpreted as an alteration in GFAP-positive astroglial cells rather than definitive evidence of global astrocyte loss. Nevertheless, astrocytes play central roles in neurotransmitter uptake, extracellular potassium buffering, and synaptic stabilization [1, 59, 73]. Thus, altered astroglial support may impair synapse maintenance and contribute to circuit vulnerability. The temporal convergence between reduced GFAP-positive astroglial density and late

hippocampal synaptic impairment suggests that glial dysfunction may participate in the progressive destabilization of inhibitory networks.

The hippocampal transcriptomic analysis provides a molecular framework to interpret the age-dependent remodeling of inhibitory circuits observed in heterozygous *Cdkl5* mice. Although these data do not establish causality, they support the existence of temporally distinct transcriptional states, suggesting that inhibitory circuit dysfunction emerges through stage-specific mechanisms rather than a single progressive process.

At early stages, the transcriptional profile is dominated by a coordinated downregulation of interferon stimulated genes (ISGs) and MHC-I-related genes. Beyond their canonical immune functions, these pathways are increasingly recognized as key regulators of synaptic refinement and activity-dependent circuit maturation. In particular, neuronal MHC-I signaling has been shown to influence synaptic pruning and plasticity, thereby shaping circuit connectivity during development [8, 34]. The reduction of this pathway is therefore consistent with an early alteration of immune-synaptic regulatory mechanisms rather than a classical inflammatory response. In this context, decreased MHC-I signaling has been associated with impaired synaptic elimination and increased synaptic connectivity, suggesting that this early transcriptional state may be associated with altered synaptic refinement and circuit organization. Notably, these molecular changes occur in parallel with increased inhibitory synaptic drive in the hippocampus, indicating that early circuit alterations precede the emergence of compensatory transcriptional programs.

At later stages, the molecular landscape shifts toward a distinct configuration characterized by increased expression of activity-dependent genes. Immediate early genes such as *Egr1*, *Egr2*, *Nr4a1*, and *Nr4a3* are well-established markers of neuronal activation and activity-dependent transcriptional responses [71, 78]. Importantly, *Bdnf* is also upregulated, further supporting

activation of plasticity-related pathways. Together, these changes indicate sustained engagement of activity-dependent transcriptional programs in the heterozygous hippocampus.

In parallel, genes involved in vascular regulation (*Nos3*, *Agt*), and structural support (*Sparc*, *Emp2*) are downregulated. The neurovascular system plays a critical role in coupling metabolic supply to neuronal activity [35], while extracellular matrix components contribute to synaptic stabilization and plasticity [16, 21]. The coexistence of increased activity-related transcription with reduced structural and metabolic support suggests a progressive mismatch between neuronal activity demands and the mechanisms required to sustain synaptic integrity.

Importantly, this transcriptional configuration parallels the functional and structural phenotype observed in the hippocampus at 12 months, characterized by reduced inhibitory synaptic input, decreased VGAT puncta density, and persistent deficits in gephyrin-positive postsynaptic structures. Rather than reflecting a simple loss of inhibition, this pattern is consistent with a state of circuit instability in which synaptic function cannot be effectively maintained. Similar imbalances between neuronal activity and homeostatic mechanisms have been implicated in conditions of network hyperexcitability and epileptogenesis [67, 70, 74].

Notably, core synaptic marker transcripts, including *Slc32a1* (VGAT), *Gphn* (Gephyrin), *Slc17a7* (VGLUT1), and *Dlg4* (PSD-95), remain largely unchanged at both 3 and 12 months. This indicates that the observed alterations in inhibitory synaptic structure and function are unlikely to be driven by transcriptional regulation of synaptic components, and instead reflect post-transcriptional or post-translational mechanisms, such as changes in synaptic protein trafficking, clustering, or stability. This interpretation is consistent with the absence of significant differences in overall PSD-95, VGAT, and VGLUT1 levels we found in Western blotting analyses on synaptosomes, as well as with the known role of CDKL5 as a synaptic kinase [15, 40, 81], regulating inhibitory synapse organization at the post-translational level.

Analysis of the age $\times$ genotype interaction further supports this interpretation, demonstrating that transcriptional trajectories in heterozygous mice do not simply represent an accelerated or attenuated version of normal aging, but rather a divergent age-dependent program. Activity-dependent genes, including *Egr1*, *Egr2*, *Nr4a1*, and *Bdnf*, exhibit enhanced modulation in heterozygous animals, whereas pathways involved in vascular, structural, and metabolic homeostasis show reduced adaptive responses.

In addition, a subset of genes related to synaptic modulation was also affected, including *Chrm3*, which regulates inhibitory synaptic transmission through modulation of GABAergic interneurons [11], and *Sntb2*, a component of the dystrophin-associated protein complex implicated in inhibitory postsynaptic organization [10]. Although modest in magnitude, these changes suggest that synaptic regulatory mechanisms may contribute to the remodeling of inhibitory circuits.

This divergence indicates that aging in the heterozygous condition is associated with a progressive uncoupling between neuronal activity and homeostatic support mechanisms, ultimately contributing to the vulnerability of inhibitory circuits.

Together, these findings support a two-stage model of inhibitory circuit destabilization in *Cdkl5* heterozygous females. An early phase is characterized by altered immune-synaptic regulatory pathways, which may affect synaptic refinement and circuit maturation. This is followed by a later phase in which increased activity-dependent transcription is accompanied by reduced structural, vascular, and metabolic support, potentially favoring progressive synaptic instability. This dynamic framework provides a mechanistic basis for the age-dependent remodeling of inhibitory circuits observed in this model and highlights the importance of considering temporal evolution when investigating the pathophysiology of CDKL5 deficiency.

These results have translational relevance for CDKL5 Deficiency Disorder (CDD), as the vast majority of affected individuals are heterozygous females in whom random X-chromosome inactivation

generates mosaic CDKL5 expression [19]. Thus, the *Cdkl5* +/- female mouse closely models not only the genetic condition but also the mosaic cellular architecture characteristic of most CDD patients. The age-dependent and region-specific inhibitory trajectories observed in *Cdkl5* +/- mice may therefore mirror important aspects of CDD progression. In particular, the possibility that inhibitory dysfunction is dynamic rather than static may help explain clinical variability and age-related symptom evolution.

Although males are typically hemizygous for CDKL5 mutations, somatic mosaicism has been reported in some cases [6, 44, 57, 61]. In the *Cdkl5* -/Y mouse model, the absence of comparable dynamic inhibitory remodeling suggests that complete loss of CDKL5 may produce a more stable, though maladaptive, network state, whereas mosaicism may introduce progressive instability. This distinction may have important translational implications for understanding sex differences and phenotypic heterogeneity in CDD.

Our analysis of postmortem cortical tissue from CDD patients provides preliminary, hypothesis-generating translational support for this model. Importantly, both CDD cases analyzed were heterozygous females, consistent with the typical genetic presentation of the disorder and directly comparable to the *Cdkl5* +/- mouse model examined in this study. Region of interest (ROI)-level analysis suggested higher VGAT puncta density in the prefrontal cortex at 4 years and reduced density at 30 years, whereas control samples showed an age-related increase. In the visual cortex, inhibitory presynaptic puncta appeared preserved in early childhood but reduced in adulthood. Although limited by the availability of only one CDD case per age group, the convergence between murine and human age-dependent patterns supports the hypothesis that CDD may involve progressive dysregulation of inhibitory circuit maturation rather than a fixed synaptic deficit. Intriguingly, in further support of a developmental trajectory specifically affecting inhibitory signalling, we previously showed that excitatory connectivity is already affected in the cerebral

cortex of the early childhood CDD patient and remains reduced in adulthood [29]. These observations should be interpreted with caution, but they suggest that distinct inhibitory circuit alterations associated with mosaic CDKL5 deficiency may also occur in the human brain and follow a non-linear trajectory across age.

A critical aspect to consider is the temporal dissociation between the mouse model and the human condition. In CDD patients, epileptic seizures typically emerge within the first months of life [4, 20, 32, 41], whereas spontaneous epileptic spasms in heterozygous *Cdkl5* +/- female mice arise later in adulthood [47]. This discrepancy suggests that early circuit dysfunction in patients may reflect developmental processes that are only partially recapitulated in the murine model. Alternatively, the early enhancement of inhibitory signaling observed in young *Cdkl5* +/- mice may represent a pre-epileptic or maladaptive network state, but its relationship to seizure onset remains to be directly tested. Species-specific differences in cortical maturation, circuit scaling, and developmental timing may further contribute to the different temporal dynamics observed between mice and humans. Therefore, while the model captures the mosaic architecture and progressive inhibitory remodeling characteristic of CDD, the temporal dynamics of seizure onset should be interpreted cautiously when extrapolating to patients.

Several limitations should be considered. First, electrophysiological recordings were not directly correlated with seizure occurrence in the same animals, and therefore the relationship between inhibitory remodeling and epileptogenesis remains inferential. Second, sIPSC recordings do not identify the interneuron subclasses or circuit motifs responsible for the observed changes. Third, the study did not directly map CDKL5-positive and CDKL5-negative cells within the same circuits, limiting conclusions regarding cell-autonomous and non-cell-autonomous mechanisms. Fourth, transcriptomic profiling was restricted to the hippocampus and therefore does not capture molecular trajectories in cortical regions. Finally, the human postmortem analysis was limited to

very few biological cases and should be considered exploratory. Despite these limitations, the convergence of electrophysiological, structural, cellular, and transcriptional data supports a model in which mosaic CDKL5 deficiency progressively remodels inhibitory circuits in an age- and region-dependent manner.

Taken together, our data support a model in which mosaic CDKL5 deficiency does not simply reduce inhibition, but progressively reshapes inhibitory circuit organization through age- and region-dependent trajectories. These findings suggest that inhibitory dysfunction in CDKL5 deficiency is not defined by a fixed deficit, but by an evolving imbalance between synaptic structure, activity, and support mechanisms. Early alterations in synaptic organization and immune-synaptic regulatory pathways are followed by divergent regional outcomes, including late hippocampal vulnerability and cortical functional reorganization. This progressive mismatch between synaptic structure, glial support, and activity-dependent molecular programs may contribute to network instability in heterozygous females. These findings identify mosaicism as a central determinant of CDKL5-related circuit dysfunction and suggest that therapeutic strategies should consider not only cell type and brain region, but also disease stage.

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Institutional Review Board Statement: The animal study protocol complied with EU Directive 2010/63/EU and with Italian law (DL 26, 4 March 2014) and was approved by the National Bioethical Committee (n° 375/2024-PR), approved on 23 April 2024.

Informed Consent Statement: Not applicable

Data Availability Statement: The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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References

- 1 Allen NJ, Eroglu C (2017) Cell Biology of Astrocyte-Synapse Interactions. *Neuron* 96: 697-708 Doi 10.1016/j.neuron.2017.09.056
- 2 Amendola E, Zhan Y, Mattucci C, Castroflorio E, Calcagno E, Fuchs C, Lonetti G, Silingardi D, Vyssotski AL, Farley Det al (2014) Mapping pathological phenotypes in a mouse model of CDKL5 disorder. *PLoS One* 9: e91613 Doi 10.1371/journal.pone.0091613
- 3 Archer HL, Evans J, Edwards S, Colley J, Newbury-Ecob R, O'Callaghan F, Huyton M, O'Regan M, Tolmie J, Sampson Jet al (2006) CDKL5 mutations cause infantile spasms, early onset seizures, and severe mental retardation in female patients. *J Med Genet* 43: 729-734 Doi 10.1136/jmg.2006.041467
- 4 Bahi-Buisson N, Kaminska A, Boddaert N, Rio M, Afenjar A, Gerard M, Giuliano F, Motte J, Heron D, Morel MAet al (2008) The three stages of epilepsy in patients with CDKL5 mutations. *Epilepsia* 49: 1027-1037 Doi 10.1111/j.1528-1167.2007.01520.x
- 5 Bahi-Buisson N, Nectoux J, Rosas-Vargas H, Milh M, Boddaert N, Girard B, Cances C, Ville D, Afenjar A, Rio Met al (2008) Key clinical features to identify girls with CDKL5 mutations. *Brain* 131: 2647-2661 Doi 10.1093/brain/awn197
- 6 Bartnik M, Derwinska K, Gos M, Obersztyn E, Kolodziejska KE, Erez A, Szpecht-Potocka A, Fang P, Terczynska I, Mierzewska Het al (2011) Early-onset seizures due to mosaic exonic deletions of CDKL5 in a male and two females. *Genet Med* 13: 447-452 Doi 10.1097/GIM.0b013e31820605f5
- 7 Bjerke IE, Yates SC, Laja A, Witter MP, Puchades MA, Bjaalie JG, Leergaard TB (2021) Densities and numbers of calbindin and parvalbumin positive neurons across the rat and mouse brain. *iScience* 24: 101906 Doi 10.1016/j.isci.2020.101906
- 8 Boulanger LM (2009) Immune proteins in brain development and synaptic plasticity. *Neuron* 64: 93-109 Doi 10.1016/j.neuron.2009.09.001
- 9 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254 Doi 10.1006/abio.1976.9999
- 10 Brunig I, Suter A, Knuesel I, Luscher B, Fritschy JM (2002) GABAergic terminals are required for postsynaptic clustering of dystrophin but not of GABA(A) receptors and gephyrin. *J Neurosci* 22: 4805-4813 Doi 10.1523/JNEUROSCI.22-12-04805.2002
- 11 Cea-del Rio CA, Lawrence JJ, Tricoire L, Erdelyi F, Szabo G, McBain CJ (2010) M3 muscarinic acetylcholine receptor expression confers differential cholinergic modulation to neurochemically distinct hippocampal basket cell subtypes. *J Neurosci* 30: 6011-6024 Doi 10.1523/JNEUROSCI.5040-09.2010
- 12 Cherubini E, Conti F (2001) Generating diversity at GABAergic synapses. *Trends Neurosci* 24: 155-162 Doi 10.1016/s0166-2236(00)01724-0
- 13 Conti S, Spalletti C, Pasquini M, Giordano N, Barsotti N, Mainardi M, Lai S, Giorgi A, Pasqualetti M, Micera Set al (2021) Combining robotics with enhanced serotonin-driven cortical plasticity improves post-stroke motor recovery. *Prog Neurobiol* 203: 102073 Doi 10.1016/j.pneurobio.2021.102073

- 14 De Rosa R, Valastro S, Cambria C, Barbiero I, Puricelli C, Tramarin M, Randi S, Bianchi M, Antonucci F, Kilstrup-Nielsen C (2022) Loss of CDKL5 Causes Synaptic GABAergic Defects That Can Be Restored with the Neuroactive Steroid Pregnenolone-Methyl-Ether. *Int J Mol Sci* 24: Doi 10.3390/ijms24010068
- 15 Della Sala G, Putignano E, Chelini G, Melani R, Calcagno E, Michele Ratto G, Amendola E, Gross CT, Giustetto M, Pizzorusso T (2016) Dendritic Spine Instability in a Mouse Model of CDKL5 Disorder Is Rescued by Insulin-like Growth Factor 1. *Biol Psychiatry* 80: 302-311 Doi 10.1016/j.biopsych.2015.08.028
- 16 Dityatev A, Schachner M, Sonderegger P (2010) The dual role of the extracellular matrix in synaptic plasticity and homeostasis. *Nat Rev Neurosci* 11: 735-746 Doi 10.1038/nrn2898
- 17 Drexel M, Romanov RA, Wood J, Weger S, Heilbronn R, Wulff P, Tasan RO, Harkany T, Sperk G (2017) Selective Silencing of Hippocampal Parvalbumin Interneurons Induces Development of Recurrent Spontaneous Limbic Seizures in Mice. *J Neurosci* 37: 8166-8179 Doi 10.1523/JNEUROSCI.3456-16.2017
- 18 Fan J, Dong X, Tang Y, Wang X, Lin D, Gong L, Chen C, Jiang J, Shen W, Xu A et al (2023) Preferential pruning of inhibitory synapses by microglia contributes to alteration of the balance between excitatory and inhibitory synapses in the hippocampus in temporal lobe epilepsy. *CNS Neurosci Ther* 29: 2884-2900 Doi 10.1111/cns.14224
- 19 Fehr S, Leonard H, Ho G, Williams S, de Klerk N, Forbes D, Christodoulou J, Downs J (2015) There is variability in the attainment of developmental milestones in the CDKL5 disorder. *J Neurodev Disord* 7: 2 Doi 10.1186/1866-1955-7-2
- 20 Fehr S, Wilson M, Downs J, Williams S, Murgia A, Sartori S, Vecchi M, Ho G, Polli R, Psoni S et al (2013) The CDKL5 disorder is an independent clinical entity associated with early-onset encephalopathy. *Eur J Hum Genet* 21: 266-273 Doi 10.1038/ejhg.2012.156
- 21 Frischknecht R, Gundelfinger ED (2012) The brain's extracellular matrix and its role in synaptic plasticity. *Adv Exp Med Biol* 970: 153-171 Doi 10.1007/978-3-7091-0932-8_7
- 22 Fritschy JM (2008) Epilepsy, E/I Balance and GABA(A) Receptor Plasticity. *Front Mol Neurosci* 1: 5 Doi 10.3389/neuro.02.005.2008
- 23 Fuchs C, Gennaccaro L, Trazzi S, Bastianini S, Bettini S, Lo Martire V, Ren E, Medici G, Zoccoli G, Rimondini R et al (2018) Heterozygous CDKL5 Knockout Female Mice Are a Valuable Animal Model for CDKL5 Disorder. *Neural Plast* 2018: 9726950 Doi 10.1155/2018/9726950
- 24 Fuchs C, Trazzi S, Torricella R, Viggiano R, De Franceschi M, Amendola E, Gross C, Calza L, Bartesaghi R, Ciani E (2014) Loss of CDKL5 impairs survival and dendritic growth of newborn neurons by altering AKT/GSK-3beta signaling. *Neurobiol Dis* 70: 53-68 Doi 10.1016/j.nbd.2014.06.006
- 25 Galaxy C (2022) The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update. *Nucleic Acids Res* 50: W345-W351 Doi 10.1093/nar/gkac247
- 26 Galvani G, Mottolese N, Gennaccaro L, Loi M, Medici G, Tassinari M, Fuchs C, Ciani E, Trazzi S (2021) Inhibition of microglia overactivation restores neuronal survival in a mouse model of CDKL5 deficiency disorder. *J Neuroinflammation* 18: 155 Doi 10.1186/s12974-021-02204-0

- 27 Gennaccaro L, Fuchs C, Loi M, Pizzo R, Alvente S, Berteotti C, Lupori L, Sagona G, Galvani G, Gurgone A et al (2021) Age-Related Cognitive and Motor Decline in a Mouse Model of CDKL5 Deficiency Disorder is Associated with Increased Neuronal Senescence and Death. *Aging Dis* 12: 764-785 Doi 10.14336/AD.2020.0827
- 28 Gu F, Parada I, Shen F, Li J, Bacci A, Graber K, Taghavi RM, Scalise K, Schwartzkroin P, Wenzel J et al (2017) Structural alterations in fast-spiking GABAergic interneurons in a model of posttraumatic neocortical epileptogenesis. *Neurobiol Dis* 108: 100-114 Doi 10.1016/j.nbd.2017.08.008
- 29 Gurgone A, Pizzo R, Raspanti A, Chiantia G, Devi S, Comai D, Morello N, Pilotto F, Gnani S, Lupori L et al (2022) mGluR5 PAMs rescue cortical and behavioural defects in a mouse model of CDKL5 deficiency disorder. *Neuropsychopharmacology*: Doi 10.1038/s41386-022-01412-3
- 30 Harris EP, Abel JM, Tejada LD, Rissman EF (2016) Calbindin Knockout Alters Sex-Specific Regulation of Behavior and Gene Expression in Amygdala and Prefrontal Cortex. *Endocrinology* 157: 1967-1979 Doi 10.1210/en.2016-1055
- 31 Hector RD, Kalscheuer VM, Hennig F, Leonard H, Downs J, Clarke A, Benke TA, Armstrong J, Pineda M, Bailey ME et al (2017) CDKL5 variants: Improving our understanding of a rare neurologic disorder. *Neurol Genet* 3: e200 Doi 10.1212/NXG.0000000000000200
- 32 Hong W, Haviland I, Pestana-Knight E, Weisenberg JL, Demarest S, Marsh ED, Olson HE (2022) CDKL5 Deficiency Disorder-Related Epilepsy: A Review of Current and Emerging Treatment. *CNS Drugs* 36: 591-604 Doi 10.1007/s40263-022-00921-5
- 33 Hu H, Gan J, Jonas P (2014) Interneurons. Fast-spiking, parvalbumin(+) GABAergic interneurons: from cellular design to microcircuit function. *Science* 345: 1255-1263 Doi 10.1126/science.1255263
- 34 Huh GS, Boulanger LM, Du H, Riquelme PA, Brotz TM, Shatz CJ (2000) Functional requirement for class I MHC in CNS development and plasticity. *Science* 290: 2155-2159 Doi 10.1126/science.290.5499.2155
- 35 Iadecola C (2017) The Neurovascular Unit Coming of Age: A Journey through Neurovascular Coupling in Health and Disease. *Neuron* 96: 17-42 Doi 10.1016/j.neuron.2017.07.030
- 36 Jakimiec M, Paprocka J, Smigiel R (2020) CDKL5 Deficiency Disorder-A Complex Epileptic Encephalopathy. *Brain Sci* 10: Doi 10.3390/brainsci10020107
- 37 Kalscheuer VM, Tao J, Donnelly A, Hollway G, Schwinger E, Kubart S, Menzel C, Hoeltzenbein M, Tommerup N, Eyre H et al (2003) Disruption of the serine/threonine kinase 9 gene causes severe X-linked infantile spasms and mental retardation. *Am J Hum Genet* 72: 1401-1411 Doi 10.1086/375538
- 38 Kamat PK, Kalani A, Tyagi N (2014) Method and validation of synaptosomal preparation for isolation of synaptic membrane proteins from rat brain. *MethodsX* 1: 102-107 Doi 10.1016/j.mex.2014.08.002
- 39 Kannan M, Gross GG, Arnold DB, Higley MJ (2016) Visual Deprivation During the Critical Period Enhances Layer 2/3 GABAergic Inhibition in Mouse V1. *J Neurosci* 36: 5914-5919 Doi 10.1523/JNEUROSCI.0051-16.2016

- 40 La Montanara P, Rusconi L, Locarno A, Forti L, Barbiero I, Tramarin M, Chandola C, Kilstrup-Nielsen C, Landsberger N (2015) Synaptic synthesis, dephosphorylation, and degradation: a novel paradigm for an activity-dependent neuronal control of CDKL5. *J Biol Chem* 290: 4512-4527 Doi 10.1074/jbc.M114.589762
- 41 Leonard H, Downs J, Benke TA, Swanson L, Olson H, Demarest S (2022) CDKL5 deficiency disorder: clinical features, diagnosis, and management. *Lancet Neurol* 21: 563-576 Doi 10.1016/S1474-4422(22)00035-7
- 42 Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15: 550 Doi 10.1186/s13059-014-0550-8
- 43 Martinez D, Jiang E, Zhou Z (2024) Overcoming genetic and cellular complexity to study the pathophysiology of X-linked intellectual disabilities. *J Neurodev Disord* 16: 5 Doi 10.1186/s11689-024-09517-0
- 44 Mei D, Darra F, Barba C, Marini C, Fontana E, Chiti L, Parrini E, Dalla Bernardina B, Guerrini R (2014) Optimizing the molecular diagnosis of CDKL5 gene-related epileptic encephalopathy in boys. *Epilepsia* 55: 1748-1753 Doi 10.1111/epi.12803
- 45 Molinari S, Battini R, Ferrari S, Pozzi L, Killcross AS, Robbins TW, Jouvenceau A, Billard JM, Dutar P, Lamour Yet al (1996) Deficits in memory and hippocampal long-term potentiation in mice with reduced calbindin D28K expression. *Proc Natl Acad Sci U S A* 93: 8028-8033 Doi 10.1073/pnas.93.15.8028
- 46 Mottolese N, Iannibelli F, Candini G, Trebbi F, Loi M, Bove AM, Medici G, Xiong ZQ, Ciani E, Trazzi S (2025) Adult-Onset Deletion of CDKL5 in Forebrain Glutamatergic Neurons Impairs Synaptic Integrity and Behavior in Mice. *Int J Mol Sci* 26: Doi 10.3390/ijms26146626
- 47 Mulcahey PJ, Tang S, Takano H, White A, Davila Portillo DR, Kane OM, Marsh ED, Zhou Z, Coulter DA (2020) Aged heterozygous Cdkl5 mutant mice exhibit spontaneous epileptic spasms. *Exp Neurol* 332: 113388 Doi 10.1016/j.expneurol.2020.113388
- 48 Munoz IM, Morgan ME, Peltier J, Weiland F, Gregorczyk M, Brown FC, Macartney T, Toth R, Trost M, Rouse J (2018) Phosphoproteomic screening identifies physiological substrates of the CDKL5 kinase. *EMBO J*: Doi 10.15252/embj.201899559
- 49 Nahar L, Delacroix BM, Nam HW (2021) The Role of Parvalbumin Interneurons in Neurotransmitter Balance and Neurological Disease. *Front Psychiatry* 12: 679960 Doi 10.3389/fpsy.2021.679960
- 50 Okuda K, Takao K, Watanabe A, Miyakawa T, Mizuguchi M, Tanaka T (2018) Comprehensive behavioral analysis of the Cdkl5 knockout mice revealed significant enhancement in anxiety- and fear-related behaviors and impairment in both acquisition and long-term retention of spatial reference memory. *PLoS One* 13: e0196587 Doi 10.1371/journal.pone.0196587
- 51 Olson HE, Daniels CI, Haviland I, Swanson LC, Greene CA, Denny AMM, Demarest ST, Pestana-Knight E, Zhang X, Moosa ANet al (2021) Current neurologic treatment and emerging therapies in CDKL5 deficiency disorder. *J Neurodev Disord* 13: 40 Doi 10.1186/s11689-021-09384-z
- 52 Olson HE, Demarest ST, Pestana-Knight EM, Swanson LC, Iqbal S, Lal D, Leonard H, Cross JH, Devinsky O, Benke TA (2019) Cyclin-Dependent Kinase-Like 5 Deficiency Disorder: Clinical Review. *Pediatr Neurol* 97: 18-25 Doi 10.1016/j.pediatrneurol.2019.02.015

- 53 Pizzo R, Gurgone A, Castroflorio E, Amendola E, Gross C, Sassoe-Pognetto M, Giustetto M (2016) Lack of Cdkl5 Disrupts the Organization of Excitatory and Inhibitory Synapses and Parvalbumin Interneurons in the Primary Visual Cortex. *Front Cell Neurosci* 10: 261 Doi 10.3389/fncel.2016.00261
- 54 Rannals MD, Kapur J (2011) Homeostatic strengthening of inhibitory synapses is mediated by the accumulation of GABA(A) receptors. *J Neurosci* 31: 17701-17712 Doi 10.1523/JNEUROSCI.4476-11.2011
- 55 Ricciardi S, Ungaro F, Hambrock M, Rademacher N, Stefanelli G, Brambilla D, Sessa A, Magagnotti C, Bachi A, Giarda E et al (2012) CDKL5 ensures excitatory synapse stability by reinforcing NGL-1-PSD95 interaction in the postsynaptic compartment and is impaired in patient iPSC-derived neurons. *Nat Cell Biol* 14: 911-923 Doi 10.1038/ncb2566
- 56 Shatz CJ (2009) MHC class I: an unexpected role in neuronal plasticity. *Neuron* 64: 40-45 Doi 10.1016/j.neuron.2009.09.044
- 57 Siri B, Varesio C, Freri E, Darra F, Gana S, Mei D, Porta F, Fontana E, Galati G, Solazzi R et al (2021) CDKL5 deficiency disorder in males: Five new variants and review of the literature. *Eur J Paediatr Neurol* 33: 9-20 Doi 10.1016/j.ejpn.2021.04.007
- 58 Sivilia S, Mangano C, Beggiato S, Giuliani A, Torricella R, Baldassarro VA, Fernandez M, Lorenzini L, Giardino L, Borelli A et al (2016) CDKL5 knockout leads to altered inhibitory transmission in the cerebellum of adult mice. *Genes Brain Behav* 15: 491-502 Doi 10.1111/gbb.12292
- 59 Sofroniew MV, Vinters HV (2010) Astrocytes: biology and pathology. *Acta Neuropathol* 119: 7-35 Doi 10.1007/s00401-009-0619-8
- 60 Spalletti C, Scalera M, Mori E, Haddad S, Mainardi M, Cangi D, Pillai V, Parmigiani E, Landi S, Caleo M et al (2025) Inhibitory circuit dysfunction as a potential contributor to cortical reorganization in Glioblastoma progression. *Neurobiol Dis* 213: 106997 Doi 10.1016/j.nbd.2025.106997
- 61 Stosser MB, Lindy AS, Butler E, Retterer K, Piccirillo-Stosser CM, Richard G, McKnight DA (2018) High frequency of mosaic pathogenic variants in genes causing epilepsy-related neurodevelopmental disorders. *Genet Med* 20: 403-410 Doi 10.1038/gim.2017.114
- 62 Tao J, Van Esch H, Hagedorn-Greiwe M, Hoffmann K, Moser B, Raynaud M, Sperner J, Fryns JP, Schwinger E, Gecz J et al (2004) Mutations in the X-linked cyclin-dependent kinase-like 5 (CDKL5/STK9) gene are associated with severe neurodevelopmental retardation. *Am J Hum Genet* 75: 1149-1154 Doi 10.1086/426460
- 63 Tassinari M, Uguagliati B, Trazzi S, Cerchier CB, Cavina OV, Mottolese N, Loi M, Candini G, Medici G, Ciani E (2023) Early-onset brain alterations during postnatal development in a mouse model of CDKL5 deficiency disorder. *Neurobiol Dis* 182: 106146 Doi 10.1016/j.nbd.2023.106146
- 64 Terzic B, Cui Y, Edmondson AC, Tang S, Sarmiento N, Zaitseva D, Marsh ED, Coulter DA, Zhou Z (2021) X-linked cellular mosaicism underlies age-dependent occurrence of seizure-like events in mouse models of CDKL5 deficiency disorder. *Neurobiol Dis* 148: 105176 Doi 10.1016/j.nbd.2020.105176

- 65 Testa G, Mainardi M, Olimpico F, Pancrazi L, Cattaneo A, Caleo M, Costa M (2019) A triheptanoin-supplemented diet rescues hippocampal hyperexcitability and seizure susceptibility in FoxG1(+/-) mice. *Neuropharmacology* 148: 305-310 Doi 10.1016/j.neuropharm.2019.01.005
- 66 Thams S, Oliveira A, Cullheim S (2008) MHC class I expression and synaptic plasticity after nerve lesion. *Brain Res Rev* 57: 265-269 Doi 10.1016/j.brainresrev.2007.06.016
- 67 Tien NW, Kerschensteiner D (2018) Homeostatic plasticity in neural development. *Neural Dev* 13: 9 Doi 10.1186/s13064-018-0105-x
- 68 Ting JT, Daigle TL, Chen Q, Feng G (2014) Acute brain slice methods for adult and aging animals: application of targeted patch clamp analysis and optogenetics. *Methods Mol Biol* 1183: 221-242 Doi 10.1007/978-1-4939-1096-0_14
- 69 Tremblay R, Lee S, Rudy B (2016) GABAergic Interneurons in the Neocortex: From Cellular Properties to Circuits. *Neuron* 91: 260-292 Doi 10.1016/j.neuron.2016.06.033
- 70 Turrigiano G (2012) Homeostatic synaptic plasticity: local and global mechanisms for stabilizing neuronal function. *Cold Spring Harb Perspect Biol* 4: a005736 Doi 10.1101/cshperspect.a005736
- 71 Tyssowski KM, Gray JM (2019) The neuronal stimulation-transcription coupling map. *Curr Opin Neurobiol* 59: 87-94 Doi 10.1016/j.conb.2019.05.001
- 72 Urban-Ciecko J, Barth AL (2016) Somatostatin-expressing neurons in cortical networks. *Nat Rev Neurosci* 17: 401-409 Doi 10.1038/nrn.2016.53
- 73 Verkhratsky A, Nedergaard M (2018) Physiology of Astroglia. *Physiol Rev* 98: 239-389 Doi 10.1152/physrev.00042.2016
- 74 Vitureira N, Letellier M, Goda Y (2012) Homeostatic synaptic plasticity: from single synapses to neural circuits. *Curr Opin Neurobiol* 22: 516-521 Doi 10.1016/j.conb.2011.09.006
- 75 Wang HT, Zhu ZA, Li YY, Lou SS, Yang G, Feng X, Xu W, Huang ZL, Cheng X, Xiong ZQ (2021) CDKL5 deficiency in forebrain glutamatergic neurons results in recurrent spontaneous seizures. *Epilepsia* 62: 517-528 Doi 10.1111/epi.16805
- 76 Wang X, Zhou H, Zhai Y, Zhang Y, Cheng Y, Yuan Y, Cao W, Gao A, Liu M, Wang Q et al (2026) TREM2-mediated microglial phagocytosis of inhibitory synapses contributes to prolonged FS-induced epileptogenesis. *Cell Death Discov*: Doi 10.1038/s41420-026-03118-7
- 77 Welle TM, Smith KR (2025) Release your inhibitions: The cell biology of GABAergic postsynaptic plasticity. *Curr Opin Neurobiol* 90: 102952 Doi 10.1016/j.conb.2024.102952
- 78 Yap EL, Greenberg ME (2018) Activity-Regulated Transcription: Bridging the Gap between Neural Activity and Behavior. *Neuron* 100: 330-348 Doi 10.1016/j.neuron.2018.10.013
- 79 Ye H, Kaszuba S (2017) Inhibitory or excitatory? Optogenetic interrogation of the functional roles of GABAergic interneurons in epileptogenesis. *J Biomed Sci* 24: 93 Doi 10.1186/s12929-017-0399-8
- 80 Yennawar M, White RS, Jensen FE (2019) AMPA Receptor Dysregulation and Therapeutic Interventions in a Mouse Model of CDKL5 Deficiency Disorder. *J Neurosci* 39: 4814-4828 Doi 10.1523/JNEUROSCI.2041-18.2019

- 81 Zhu YC, Li D, Wang L, Lu B, Zheng J, Zhao SL, Zeng R, Xiong ZQ (2013) Palmitoylation-dependent CDKL5-PSD-95 interaction regulates synaptic targeting of CDKL5 and dendritic spine development. *Proc Natl Acad Sci U S A* 110: 9118-9123 Doi 10.1073/pnas.1300003110
- 82 Zhu YC, Xiong ZQ (2019) Molecular and Synaptic Bases of CDKL5 Disorder. *Dev Neurobiol* 79: 8-19 Doi 10.1002/dneu.22639

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