ARTICLE OPEN Synaptic vulnerability to amyloid-β and tau pathologies differentially disrupts emotional and memory neural circuits

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Alzheimer's disease (AD) is characterized by memory loss and neuropsychiatric symptoms associated with cerebral amyloid- β (A β) and tau pathologies, but whether and how these factors differentially disrupt neural circuits remains unclear. Here, we investigated the vulnerability of memory and emotional circuits to A β and tau pathologies in mice expressing mutant human amyloid precursor protein (APP), Tau or both APP/Tau in excitatory neurons. APP/Tau mice develop age- and sex-dependent A β and phosphorylated tau pathologies, the latter exacerbated at early stages, in vulnerable brain regions. Early memory deficits were associated with hippocampal tau pathology in Tau and APP/Tau mice, whereas anxiety and fear appeared linked to intracellular A β in the basolateral amygdala (BLA) of APP and APP/Tau mice. Transcriptome hippocampal profiling revealed gene changes affecting myelination and RNA processing in Tau mice, and inflammation and synaptic-related pathways in APP/Tau mice at 6 months. At 9 months, we detected common and region-specific changes in astrocytic, microglia and 63 AD-associated genes in the hippocampus and BLA of APP/Tau mice. Spatial learning deficits were associated with synaptic tau accumulation and synapse disruption in the hippocampus of Tau and APP/Tau mice, whereas emotional disturbances were linked to A β pathology but not synaptic tau in the BLA. Interestingly, A β and tau exhibited synergistic detrimental effects in long-term potentiation (LTP) in the hippocampus but they counteract with each other to mitigate LTP impairments in the amygdala. These findings indicate that A β and tau pathologies cause region-specific effects and synergize to induce synaptic dysfunction and immune responses, contributing to the differing vulnerability of memory and emotional neural circuits in AD.

Molecular Psychiatry (2025) 30:2966-2979; https://doi.org/10.1038/s41380-025-02901-9

INTRODUCTION

Alzheimer's disease (AD), the major cause of memory loss in the elderly, is accompanied by early neuropsychiatric symptoms that act as early risk factors for conversion to dementia [1, 2]. Cognitive and emotional disturbances are associated with pathological changes in the hippocampus and amygdala at early AD stages [3], but how these brain regions cooperate to cause emotional-related memory changes in AD is unclear. Age and sex affect differentially regional accumulation of amyloid- β (A β) and tau pathologies, and cognitive and mood disturbances (depression, anxiety, fear, apathy) [4-7]. In AD mouse models, females with enhanced neuropathology are first affected by anxiety and cognitive deficits [8–14]. Young 3xTg-AD females show higher anxiety and reduced memory retention compared to males [13], whereas sexual dimorphism in behavior, neuropathology and inflammatory molecules are also evident in a tauopathy mouse model [14]. However, how AB and tau interact to cause cognitive and neuropsychiatric symptoms remain unclear, in part because emotional factors are largely underrepresented in basic, pathological and clinical studies [15].

Recent evidence in human demonstrates dissociation effects but also a crosstalk of pathological tau- and amyloid-related neural circuit dysconnectivity in memory impairments of AD patients [16-21]. Spatiotemporal pathological changes of tau and Aß are linked with cognitive decline in aging, and they cause selective regional vulnerability associated with differential gene profiles and cognitive dysfunction in AD [22, 23]. Several studies indicate that AB and/or amyloid plagues increase tau phosphorvlation, aggregation and seeding [24-28], and tau inactivation ameliorates Aβ-induced memory deficits independently of amyloid pathology [29, 30]. Importantly, Aβ and tau induce synapse dysfunction and loss [31], a pathological feature that tightly correlates with cognitive decline [32-34]. Synaptic tau induces synapse dysfunction, instability and loss [35-38], and it dominates over AB on neural circuit disruption [39, 40]. AB and tau affect both presynaptic and postsynaptic mechanisms impairing excitatory glutamatergic transmission [35, 41, 42], but whether synaptic tau is responsible and/or synergize with Aβ to induce synapse pathology and behavioral changes is unclear. Moreover, the mechanisms of neuropathological crosstalk between Aß

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Received: 19 April 2024 Revised: 22 December 2024 Accepted: 16 January 2025 Published online: 30 January 2025

and tau leading to synaptic dysfunction and vulnerability of emotional and memory neural circuits are still unknown.

To better elucidate the cellular mechanisms and factors contributing to disruption of cognitive- and emotion-related neural circuits in AD, we generated novel double APP/Tau transgenic mice that recapitulate early synaptic, behavioral, and transcriptional alterations linked to AD pathophysiology. Interestingly, whereas spatial learning and memory deficits were associated with synaptic tau pathology in the hippocampus, emotional disturbances were linked to AB in the basolateral amygdala (BLA). Transcriptional profiling revealed hippocampal gene signatures enriched in inflammatory and synaptic pathways affected by early concomitant AB/tau pathology but not by AB or tau alone. At late stages, specific and coordinated transcriptional responses, including astrocytic, microglia and AD risk genes identified by genome-wide association studies (GWAS) occur in the hippocampus and amygdala of APP/ Tau mice. Our study reveals that AB and tau affect differentially emotional and memory neural circuits by exerting distinct effects at the transcriptional, functional and behavioral levels.

MATERIALS AND METHODS

Behavioral, biochemical, transcriptomic, immunohistiochemical and electrophysiological methods are extensively described in Supplementary Information.

Mice

Control (WT), APP, Tau and APP/Tau transgenic mice were obtained by crossing heterozygous APP_{Sw,Ind} (line J9; C57BL/6) and Tau P301S (line PS19, JAX #008169; C57BL/6) mice [36, 43], and housed under standard conditions ($n = 4-6/cage; 22 \pm 2$ °C, 12 h light:dark cycle). Sex, age and genotypes are indicated in the figure legends. Experimental procedures were approved by the Animal and Human Ethical Committee (CEEAH) of the Universitat Autònoma de Barcelona and local government (CEEAH/DMAH: 2895/10571, 4750/10839) following European Union regulations (2010/63/EU).

Behavioral tests

General and anxiety-like behaviors were studied in the open field and dark/light box (DLB) tests [44, 45] (see Supplementary Information). For cued fear conditioning (CFC), 6 month-old mice were exposed in context A to a conditioned sound stimulus (CS, 2800 Hz, 80 dB; 30 s) followed by an electric footshock (unconditioned stimulus, US, 0.8 mA; 2 s). Freezing behavior was automatically recorded (Video Freeze Software, Med Associates) immediately after the shock (2 min) and again 24 h later in a novel chamber (context B) before (pre-CS; 2 min) and during (CS; 3 min) tone presentation [45]. Mice at 9–10 months were conditioned with two CS-US pairings (context A; US, 1 mA; 2 s). Freezing behavior was recorded during the 2-min interval between pairings and immediately after the shock. At 24 h, 3-min pre-CS period was followed by 16 CS presentations (30 s each, 5-s inter-CS) in context B [46].

Immunohistochemistry

Deparaffinized coronal brain sections (5 µm) were antigen-retrieved with citrate buffer (10 mM, pH 6.0) for tau or with formic acid (60%, 5 min) for Aβ or Aβ/tau, and incubated with anti-phosphorylated (p)tau (Ser202/Thr205, AT8, 1:50; Ser202, CP13, 1:50) or anti-Aβ (6E10, 1:1000) antibodies before biotin-conjugated anti-mouse secondary antibodies (1:200), DAB peroxidase staining (Vector laboratories) and imaging (Nikon Eclipse 80i microscope). For astrocytic and microglial stainings, deparaffined coronal sections (5 µm) were antigen-retrieved (citrate buffer) and incubated with anti-GFAP (Dako Z0334; 1:500) or anti-Iba1 (Wako 019-19741, 1:250) antibodies followed by AlexaFluor-488/594-conjugated goat IgGs (1:400) and Hoechst (1:5000) before imaging (Confocal Zeiss LSM 700 microscope). Immunofluorescence staining with APP/Aβ, anti-Aβ42, pTau and neuronal expansion methods are described in Supplementary Information.

Bulk RNA transcriptional profiling

Bulk RNA-sequencing (RNA-seq) of hippocampus and BLA from 6 and 9 month-old females was performed on an Illumina NextSeq 6000/2000. RNA-seq data analyses, including alignment to the reference genome and

differential expression analysis were performed using QuasR/Rhisat2 and DESeq2 packages, and Gene ontology (GO) and functional enrichment analyses using enrichR in Bioconductor [48–50].

Statistical analysis

Statistical analysis was performed using parametric one- or two-way Analysis of Variance (ANOVA) or non-parametric Kruskal-Wallis tests according to D'Agostino-Pearson omnibus normality test (Prism software, GraphPad 8.0.2). For multiple comparisons, we used Sidak's or Tukey's post hocs for parametric tests and Dunn's post hoc for non-parametric test. Parametric unpaired two-tailed Student's t-test or non-parametric Mann-Whitney test, according to D'Agostino-Pearson omnibus normality test, were used when two groups were compared. *P* values less than 0.05 were considered significant. Adjusted *P* values (padj) less than 0.1 were considered significant in the transcriptomic analysis. The sample size was calculated using the "Power and Precision" software to ensure adequate power while adhering to the 3Rs principle. Randomization was performed by an independent investigator who assigned animal codes and sample order, ensuring unbiased allocation and blindness to the genotypes during data acquisition. Grubbs' test was used to identify outliers.

RESULTS

$A\beta$ and tau accumulation in excitatory neurons results in agedependent cerebral AD pathology

To investigate the specific contribution of A β and tau on memory and emotional disturbances, we crossed APP_{Sw.Ind} and Tau P301S transgenic mice to generate double APP/Tau transgenic mice that express human APP and Tau translated transcripts in hippocampal excitatory CaMKIIa but not inhibitory parvalbumin neurons (Supplementary Fig. 1A). Immunofluorescence analysis revealed high localization of hAPP/AB (6E10) and total/phosphorylated (p) Tau in excitatory neurons (CaMKIIa, vGlut1, L-glutamate) but not inhibitory interneurons (GAD-67, parvalbumin, somatostatin) in APP/Tau hippocampus, and some colocalization of AB with GAD-67 in the BLA (Supplementary Fig. 1B). APP and APP/Tau mice at 6 months show elevated human APP (~2 fold, APP CTF antibody;~17 fold, 6E10), APP α -CTFs and intraneuronal AB42 (MOAB-2) but unchanged APP B-CTFs (Supplementary Fig. 2A, B). Tau was similar in Tau and APP/Tau mice (~25 fold) but phosphorylated (p)Tau (Ser202/Thr205) levels were significantly increased in APP/Tau mice (Supplementary Fig. 2A; P < 0.01).

APP and APP/Tau mice show similar intracellular Aβ staining in CA1/ CA3 hippocampus with no staining in the entorhinal cortex (EC) and BLA at 6 months (Kruskal-Wallis test, CA1: P < 0.0001; CA3: P < 0.0001; EC: P > 0.05; BLA: P > 0.05; Fig. 1A). At 9 months, male and female APP and APP/Tau mice show similar number of Aβ-positive neurons and amyloid plagues in hippocampal and cortical regions, except for an increase of AB-positive neurons in the EC of females (Two-way ANOVA, EC, genotype effect: F (3, 37) = 12.78, P < 0.0001; sex effect: F (1, 37) = 10.10, P < 0.01; interaction: F (3, 37) = 3.43, P < 0.05; Fig. 1B; Supplementary Fig. 3). The number of pTau (Ser202/Thr205)-positive neurons was significantly increased in APP/Tau mice at 6 months (Kruskal-Wallis test, CA1: P < 0.001; CA3: P < 0.001; one-way ANOVA, EC: P < 0.001; BLA: P < 0.001; Fig. 1A). At 9 months, pTau (Ser202, CP13) was prominently increased in neuronal cell bodies and fibers of hippocampus and cortex of male and female Tau and APP/Tau mice (~20-40 fold; Fig. 1B, Supplementary Fig. 3), whereas, compared to males, APP/Tau females show elevated pTau in CA3 (Two-way ANOVA, CA3, genotype effect: F (3, 35) = 43.57, P < 0.0001; sex effect: F (1, 35) = 7.87, P < 0.01) (Fig. 1B). Interestingly, APP/Tau mice develop prominent hippocampal atrophy at 9 months (P < 0.01 - 0.0001; Supplementary Fig. 2C). These results demonstrate that APP/Tau mice develop age-dependent AB and tau pathologies with no major sex differences.

Hippocampal-dependent learning/memory deficits in young Tau and APP/Tau mice

Analysis of general behavior in the open field revealed no significant differences among genotypes at 6 months in total



Fig. 1 Aβ potentiates early tau pathology in AD vulnerable brain regions in APP/Tau mice. Coronal brain sections of control (WT), APP, Tau and APP/Tau mice at 6 months (**A**) and 9 months (**B**) were stained with anti-human A β /APP (6E10; top) and pTau (Ser202, AT-8 (**A**) or CP13 (**B**); bottom) antibodies. Left images: Representative low and high (insets) magnified images of A β - and pTau-stained neurons in CA1 and CA3 hippocampus, entorhinal cortex (EC) and basolateral amygdala (BLA). Amyloid plaques in a 9 month-old APP/Tau mouse are visualized in the top right inset of CA3 region. Objective: 20x. Scale bar: 50 µm. <u>Right diagrams</u>: Quantification of A β -positive cells in WT (white symbols), APP (red symbols), Tau (blue symbols), and APP/Tau (grey symbols) mice. Data represent mean number ± SEM of APP/A β -and pTau-positive cells/µm² (×10⁻⁵) (n = 3-4 slices/mouse). Number of mice (male/female), 6 months: WT (2/6), APP (1/5), Tau (0/5) and APP/Tau (3/5); 9 months: WT (6/6), APP (5/6), Tau (5/5) and APP/Tau (4-5/6-7). Statistical analysis was performed using one-way ANOVA or non-parametric Kruskal-Wallis tests according to the D'Agostino-Pearson omnibus normality test, followed by Tukey's or Dunn's post hoc test, respectively (**A**), and two-way ANOVA followed by Sidak's (sex comparison) and Tukey's (genotypes comparison within each sex) multiple comparison tests (**B**). *P < 0.05, **P < 0.01, ***P < 0.001, ***P < 0.001 vs WT or the indicated group.

travelled distance, time inactive and percentage of time in the center (C) and periphery (P) at days 1 and 2 (Supplementary Fig. 4A). DLB and cued fear conditioning (CFC) tests revealed no significant changes in anxiety, neophobia, and fear memory among the transgenic lines at this age (Kruskal-Wallis test and two-way ANOVA, P > 0.05; Fig. 2A, B). In the Morris water maze (MWM), all groups showed similar swimming speeds (P > 0.05) and decreased latencies during spatial training (Two-way ANOVA, training effect: F (4, 390) = 37.40, P < 0.0001),

although Tau and APP/Tau mice exhibited significantly longer latencies starting at day 2 (Genotype effect: F (3, 390) = 25.95, P < 0.0001; Fig. 2C, D). In the probe trial, control mice displayed a preference for the target quadrant (P < 0.05), whereas APP, Tau and APP/Tau mice showed reduced target quadrant occupancies (Two-way ANOVA, interaction effect: F (3, 156) = 5.86, P < 0.001) (Fig. 2C, D). This result suggests that spatial learning deficits are associated with hippocampal tau pathology in Tau and APP/Tau mice.

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Fig. 2 Differential Aβ and tau effects on spatial memory and emotional disturbances in AD transgenic mice. A–D Behavior of AD transgenic mice at 6 months. A Number of entries and latencies to light zone in the DLB test. **B** Associative memory in the fear conditioning test. Freezing responses in the CFC during conditioning (day 1, left) and training (day 2, right) of WT, APP, Tau and APP/Tau mice. **C** Spatial memory training in the MWM for 5 days. **D** % Time in target quadrant vs others on day 5 probe trial in the MWM. Heat maps show decreased occupancies and trajectories of transgenic mice in the target quadrant (marked in white). Data represent mean ± SEM. For **A**, **B** number of mice (male/female): WT (4/6), APP (5/5), Tau (2/8) and APP/Tau (4-5/4). For **C**, **D** number of mice (male/female): WT (12/15), APP (11/6), Tau (7/8) and APP/Tau (9/14). **E–H** Behavior of AD transgenic mice at 9 months. **E**, **G** Anxiety behavior of male (**E**) and female (**G**) mice in the dark/light test. **F**, **H** Associative memories of male (**F**) and female (**H**) mice in the CFC test. Freezing responses during conditioning (day 1) and training (CS 1: conditioned stimulus 1) or extinction (CS 2–8) on day 2 in the CFC. The mean ± SEM of time freezing (%) during CS (period from 2 to 8) is represented at the right. In all cases, data represent mean ± SEM. Number of mice (male/female); wt (14/8, white bars/symbols) APP (9/8, red bars/symbols), Tau (10/9, blue bars/symbols) and APP/Tau (7/8, grey bars/symbols). Statistical analysis was determined by one-way or non-parametric Kruskal-Wallis tests followed by Tukey's or Dunn's post hoc test (**A**, **E**, **G**) or two-way ANOVA (**B–D**, **F**, **H**) followed by Tukey's post hoc test for genotype comparisons. **P* < 0.05, **P* < 0.001, ****P* < 0.0001 vs the indicated group or control mice (**C**, **F**, **H**).

Age-dependent emotional disturbances in APP and APP/ Tau mice

General behavior evaluated in the open field revealed no significant differences in travelled distances in all male and female groups at 9–10 months, except for increased activity on day 1 in APP/Tau females, indicating similar ambulatory locomotor activities (Supplementary Fig. 4B). In the DLB test, 9 month-old APP and APP/Tau mice of both sexes, but not Tau mice, showed reduced entries (One-way ANOVA, male: F (3, 36) = 6.88, P < 0.001; females: F (3, 29) = 5.16, P < 0.01), and higher latencies (except male APP/Tau mice; male: F

(3, 36) = 2.98, P < 0.05; females: F (329) = 4.47, P < 0.05) into the light compartment (Fig. 2E, G), suggesting increased anxiety in APP and APP/Tau mice. In CFC, innate freezing responses before shock (neophobia) and immediately after shock are similar in female groups (Two-way ANOVA, genotype effect: P > 0.05), whereas males show a main significant effect of genotype (F (3, 108) = 5.23, P < 0.01) and treatment (F (2, 108) = 15.70, P < 0.0001) (Fig. 2F, H). At 24 h, all groups showed similar and significant CS-induced freezing responses indicating fear memory consolidation. However, there was a significant effect of genotype and tone



during extinction training (males: genotype effect: F (3, 324) = 9.57, P < 0.0001; tone effect: F (8, 324) = 9.51, P < 0.0001; females: genotype effect: F (3, 306) = 12.56, P < 0.0001; tone effect: F (8, 306) = 11.48, P < 0.0001) (Fig. 2F, H). Particularly, APP and APP/Tau mice of both sexes show enhanced freezing responses

compared to control and Tau mice (One-way ANOVA, male: F (3, 24) = 7.00, *P* < 0.01; females: F (3, 24) = 12.51, *P* < 0.0001) (Fig. 2F, H). These results indicate that fear-related emotional symptoms are associated with A β accumulation in the amygdala of APP and APP/Tau mice.

Fig. 3 Differential transcriptional profiles and synapse pathology in the hippocampus of AD transgenic mice. A Venn and gene ontology diagrams illustrating the differentially expressed genes and enriched cellular pathways altered in the hippocampus of 6 month-old female APP (n = 8), Tau (n = 8) and APP/Tau mice (n = 10) compared with controls (WT, n = 11). **B** Biochemical analysis of pre- and post-synaptic proteins and tau (D1M9X antibody) in hippocampal lysates and purified synaptosomes of WT, APP, Tau and/or APP/Tau mice at 6 months (n = 8-11 mice/group). Data represent mean levels (fold change) normalized to β -tubulin ± SEM. Number of mice (male/female): 11 WT (6/5, white symbols), 8 APP (5/3, red symbols), 8 Tau (5/3, blue symbols) and 9 APP/Tau (5/4, grey symbols). **C** Confocal microscope images of cleared expanded hippocampal and BLA sections (expansion factor 3.63x) of 6 month-old WT and APP/Tau mice showing colocalization (yellow; quantification at the right graph) of postsynaptic Homer1 (green) and pTau (Ser 202; red) in the CA1 hippocampus but not in the BLA of APP/Tau mice. Right top insets are magnified images of the indicated squared regions. Scale bars: 10 µm and 36.30 µm (expanded). Statistics were determined by one-way ANOVA or Kruskal-Wallis tests followed by Dunnet's or Dunn's multiple comparisons tests according to the normality test, respectively (**B**) or by two-tailed Student's t-test (**C**). *P < 0.05, **P < 0.01, ***P < 0.001 vs non-transgenic control group.

Early $A\beta$ /tau-induced transcriptional responses in the hippocampus are related to synapse disruption and inflammation

To identify transcriptional responses related to biological pathways affected by early AB and tau pathologies, we performed genome-wide bulk RNAseg analysis followed by pathway enrichment analysis in the hippocampus of littermate female WT, APP, Tau and APP/Tau mice at 6 months. Gene expression analysis revealed differentially expressed genes (DEGs) in the hippocampus of APP (94: 39 up, 55 down), Tau (1551: 743 up, 808 down) and APP/Tau (1129: 808 up, 321 down) mice (Fig. 3A). In APP mice, we found significant upregulation of genes related to metabolic processes (Idh1, Ugt2b34) and oxidative stress (Prdx1, Gstm1), the latter shared with APP/Tau mice but not Tau mice. GO analysis revealed that the top significantly enriched pathways of the upregulated genes were related to gliogenesis and axon ensheathment/myelination in Tau mice (Bmp2, Dlx2, Cst7, Itgax, Opalin, Tenm4), and immune responses in APP/Tau mice (Aif1, C3, Cd9, Clu, Cnp, Cxcl5, Sox10). By contrast, downregulated genes were related to RNA processing/splicing in Tau mice (Cdk9, Celf3, Celf5, Mettl3, Srsf7, Ybx2), and synapse organization, transporters and receptors in APP/Tau mice (Bdnf, Cacnb3, Fqf13, Dlq4, Negr1, Nptxr). The common upregulated (178) and downregulated (54) genes shared between Tau and APP/Tau mice were related to myelination/glial differentiation and synapse organization/transmission pathways, respectively (not shown). Consistently, cellular pathways related to immune/leukocyte responses (upregulation) and synapse function/ion transporters/neurotransmitter receptors (downregulation) were altered in APP/Tau hippocampus at 6 and 9 months of age (Supplementary Fig. 5A). These results indicate that AB and tau cooperate to induce synaptic disruption and immune responses at the transcriptional level.

We next examined pre- and post-synaptic proteins and tau in purified hippocampal synaptosomes and/or brain sections of 6 month-old mice. Biochemical analysis revealed no major changes in synaptic proteins in hippocampal lysates of transgenic mice despite ~45% of APP/Tau mice showed decrease of these proteins (Fig. 3B). In purified hippocampal synaptosomes, we found decrease of Homer-1 in APP mice, and synaptophysin, Homer-1 and PSD95 in Tau mice, whereas all analyzed synaptic proteins and β-actin were decreased in APP/Tau mice (Fig. 3B). Tissue expansion revealed high colocalization of pTau (Ser 202) with the glutamatergic postsynaptic protein Homer1 in the hippocampus (53%) but not in the BLA (15%) of APP/Tau mice (Fig. 3C). Changes in pre- and post-synaptic proteins occur in parallel with synaptic tau accumulation (Fig. 3B), which suggests that synaptic pathological tau contributes to hippocampal synapse pathology in APP/Tau mice.

Tau and Aβ cooperate to induce differential brain regional effects in synaptic plasticity

We next investigated the functional effects of neuronal amyloid and tau pathologies on hippocampal and amygdalar synaptic transmission and plasticity. Input-output curves in Schaffer collateral CA3/CA1 synapses were similarly decreased in all mutant transgenic female mice at 6 months (Two-way ANOVA, stimulus effect: F (8, 180) = 19.90, *P* < 0.0001; genotype effect: F (3, 180) = 34.34, *P* < 0.0001; interaction effect: F (24,180) = 2.24, *P* < 0.01; Fig. 4A). Short-term synaptic plasticity (STP), and long-term potentiation (LTP) induction, measured as early-LTP (60 min) and late-LTP (120 min) were significantly impaired in APP/Tau hippocampus compared with the rest of groups (One-way ANOVA, STP: F (3, 24) = 4.06, *P* < 0.05; E-LTP: F (3, 24) = 3.41, *P* < 0.05; L-LTP: F (3, 24) = 7.00, *P* < 0.01; Fig. 4A–C). Paired-pulse facilitation (PPF) ratio was reduced in APP and APP/Tau mice (F (3, 21) = 5.37, *P* < 0.01; Fig. 4D). Whole-cell patch-clamp recordings revealed impaired NMDA responses and NMDA/AMPA ratio in APP/Tau mice (F (3, 36) = 3.44, *P* < 0.01; Fig. 4E). These results demonstrate that tau and Aβ cooperate to disrupt hippocampal synaptic plasticity through postsynaptic mechanisms.

In thalamic-LA synapses, basal synaptic transmission was similarly decreased in APP, Tau and APP/Tau mice at 6 months (Two-way ANOVA, stimulus effect: F (12,481) = 3.45, P < 0.0001; genotype effect: F (3,481) = 10.11, P < 0.0001; Fig. 4F). Field recordings revealed decreased STP induction in APP, Tau and APP/Tau mice (F (3, 34) = 9.01, P < 0.001), and E-LTP and L-LTP deficits in APP and Tau mice but not in APP/Tau mice (E-LTP: F (3, 34) = 7.61, P < 0.001; L-LTP: F (3, 34) = 11.29, P < 0.0001; Fig. 4G, H). Basal PPF was reduced in APP mice and recovered in APP/Tau mice (F (3, 36) = 5.13, P < 0.01; Fig. 4I). NMDA/AMPA ratios were not significantly affected in the transgenic groups (F (3, 40) = 0.41, P > 0.05; Fig. 4J). Together, these findings indicate that, in contrast to the hippocampus, A β and tau counteract each other to maintain synaptic plasticity in the amygdala of young mice.

Coordinated and divergent transcriptional changes related to inflammation and AD-associated genes occur in the hippocampus and BLA of APP/Tau mice

To identify gene signatures and networks differentially affected by late AB/tau pathology in the hippocampus and BLA, we next assessed DEGs of female WT and APP/Tau mice at 9 months. We identified 5985 hippocampal genes (3260 up, 2725 down) and 2168 BLA genes (1263 up, 905 down) deregulated in APP/Tau mice, with a high number of DEGs (1423: 24% hippocampus, 65% BLA) coordinately deregulated in both regions (Fig. 5A, B). The most significant enriched pathways affected in both regions were related to neutrophil immune response (upregulation: Arhgap9, Cd33, Cd68, Itgam, Lamp1, Nfam1, Stk10, Ticam2) and neurotransmission/glutamate receptor (downregulation: Camk2a, Dlgap1/4, Gria2/3, Grin1, Homer1, Lrrk2, Mapk8ip2, Mink1, Shank1, Syn1) (Fig. 5C). By contrast, the most significantly divergent enriched pathways of upregulated genes in APP/Tau mice were fatty acid metabolism (Echs1, Eci1, Echdc3, Hadha, Hadhb, Hsd17b4/10) in the hippocampus, and AB binding, GTPase regulation and oxidoreductase/NAD (Adrb2, Bdh2, Hspg2, Itgb2, Ncf1/2/4, Rnls, Sirt2, Sirt5, Tlr2) in the BLA (Fig. 5C; Table 1). In addition, genes highly expressed in reactive astrocytes (Gfap, ApoE) and diseaseassociated microglia (DAM) (Ccl6, Cd33, Csf1, Cst7, Cx3cr1, Itgax, Trem2, Tyrobp) were upregulated in hippocampus and/or BLA of APP/Tau mice (Supplementary Table 1). Accordingly, GFAP and



Fig. 4 Brain regional specific effects of tau on Aβ-induced synaptic plasticity impairments. A, F Input-output curves showing EPSC amplitudes (**A**) and fEPSP slope (**F**) *vs* the applied stimulus intensity in CA3/CA1 hippocampal (**A**) and thalamus-lateral amygdala (LA) (**F**) synapses of control (WT, white), APP (red), Tau (blue), and APP/Tau (grey) mice. **B, G** Traces (top) and time course (bottom) of fEPSPs before and after LTP induction in CA3-CA1 hippocampal (**B**) and thalamus-LA synapses (**G**). **C, H** Histograms showing short-term synaptic plasticity (STP), early long-term potentiation (E-LTP), late LTP (L-LTP) in CA3-CA1 hippocampal (**C**) and thalamus-LA synapses (**H**). **D, I** Paired-pulse facilitation (PPF) does not change after LTP induction in the hippocampus (**D**) or amygdala (**I**), and baseline PPFs of APP are decreased in the hippocampus and amygdala compared to controls. However, PPF of APP/Tau mice is significantly decreased only in hippocampas. **E, J** NMDA/AMPA ratio was decreased in hippocampal neurons of APP/Tau mice compared to control, APP, and Tau mice (**E**). In amygdala (**J**), NMDA/AMPA ratio was not affected in the transgenic mice. Data represent mean ± SEM from electrophysiological recordings in CA3-CA1 malysis was determined by one-way ANOVA or Kruskal-Wallis tests according to the D'Agostino-Pearson omnibus normality test, followed by Tukey's or Dunn's post hoc test, respectively (**D, E, I, J**) or two-way ANOVA (**A, F**) followed by Tukey's post-hoc test. **P* < 0.05, **P* < 0.01, ****P* < 0.001, ****P* < 0.0001 *vs* the indicated group.

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lba1 stainings were significantly elevated in the CA1/CA3 hippocampus and BLA of APP/Tau mice (One-way ANOVA, F(3, 22) = 3.51, P < 0.05; BLA: F(3, 22) = 5.44, P < 0.01; F(3, 20) = 3.82, P < 0.05; BLA: F(3, 22) = 4.10, P < 0.05) (Fig. 5E). Importantly, 63 mouse orthologs of human AD-associated genes

previously identified in GWAS (e.g, APOE, BIN1, CD33, CLU, MS4A4A, PICALM, PLCG2, PTK2B, SLC24A4, SORL1, TREM2, USP6NL) were deregulated in the hippocampus and/or BLA of female APP/Tau mice (Fig. 5C; Table 1) [51, 52]. Intriguingly, AD-associated genes related to synapse function and ion transport (NCS1, NKAIN2,

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Fig. 5 Bulk RNAseq reveals differential and common gene expression signatures associated with AD in hippocampus and BLA of APP/ Tau mice. A Number and Venn diagram illustrating the total, up and down differentially expressed transcripts in the hippocampus (HPC) and BLA of 9 month-old female APP/Tau mice (n = 9) compared to controls (WT, n = 11 for HPC; n = 10 for BLA). **B** Volcano plots showing the fold change of genes differentially expressed in the hippocampus and BLA of APP/Tau mice (cut-off: *P* value < 0.01, log2FC > 0.5). Some significant deregulated AD risk genes are indicated (see also Table 1). **C** Functional set enrichment of differentially expressed genes in APP/Tau hippocampus and BLA. The plots show the top significantly enriched pathways from the GWAS catalog (2019) (left) and GO Biological Process/ Molecular Function (2021) (right) databases. Human orthologs were used in the analysis with GWAS catalog database. **D** Venn diagram illustrating the AD-associated genes identified previously by GWAS shared in the hippocampus of 6 and 9 month-old female APP/Tau and/or Tau mice. **E** Confocal microscope images (left) and quantification (right graphs) of percentage of GFAP (magenta; top images) and Iba1 (magenta; bottom images) areas, and Hoechst (blue) staining in CA1 hippocampus and BLA sections of female 9 month-old WT (n = 7), APP (n = 7), Tau (n = 4-5) and APP/Tau (n = 6-7) mice. Insets are magnified images of the indicated regions. Scale bars: 30 µm. Statistical analysis was determined by one-way ANOVA followed by Tukey's post-hoc test. ^{*}P < 0.05, [#]P = 0.06 vs the indicated control group.

PDE7B, SLC24A4, SLC4A8, TSPAN13) were specifically downregulated in APP/Tau hippocampus at 9 months. In addition, 23 of these genes were distinctly altered in the hippocampus of APP, Tau and APP/Tau mice at 6 months (Supplementary Fig. 5B). Remarkably, ten mouse orthologs of these human AD-related genes (*AHNAK, ARHGAP20, BCL3, CELF1, CLU, INPP5D, NKAIN2, PDE7B, STK32B, TREM2*) were shared and changed in the same direction in the hippocampus of APP/Tau mice at 6 and 9 months but not in Tau mice (Fig. 5D; Supplementary Table 2). These results suggest that coordinated specific transcriptional responses related to inflammation and synapse function occur in response to A β /tau pathology in the hippocampus and BLA.

DISCUSSION

Recent compelling evidence indicates that AB and tau pathologies exert synergistic effects on synaptic dysfunction and memory loss, suggesting that therapeutic approaches targeting only one of these factors may not be sufficient for achieving clinical benefits [31, 53]. Accordingly, immunotherapy clinical trials targeting AB or tau showed promising biomarker reductions but limited cognitive benefits [54, 55], likely because multiple pathological factors contribute to the disease process [56]. Discerning the pathological mechanisms of Aß and tau crosstalk is critical to develop effective AD therapies. Importantly, understanding how AB and tau pathologies are mechanistically linked in specific brain circuits may help to elucidate their role in cognitive and neuropsychiatric symptoms. Here, we show that APP/Tau mice replicate the key pathological features of AD, including early intracellular AB and potentiation of tau pathology, synapse dysfunction, inflammation, neuron loss and deregulation of disease-related gene signatures. Comprehensive analysis of single and double transgenic mice, indicate that AB enhances neuronal pathological tau at early disease stages, coinciding with tau-induced disruption of synaptic function, memory, and altered transcriptional profiles related to inflammation and synaptic pathways. Conversely, altered emotional responses were linked to the presence of A β in the amygdala at late pathological stages.

Our study reveals distinct pathological vulnerability of memory and emotional circuits, with the presence of pathological tau in hippocampal glutamatergic neurons linked to spatial memory deficits, and AB accumulation in the BLA associated with fear emotional disturbances. Hippocampal tau pathology is a strong predictor of memory decline by disconnecting neural networks [18, 57], and synergizes with A β to disrupt hippocampal function and memory performance in older adults [16, 17]. The regionspecific susceptibility to pathology may be exacerbated by the effect of AB on the progression of neuronal tau pathology, as previously shown in double APP-V717I/Tau-P301L mice [58], which can be mediated by cell- or non-cell-autonomous (e.g. microglia) effects [59]. As reported in AD [32, 33, 41, 60], synaptic tau was associated with reduced glutamatergic synaptic proteins and impaired hippocampal-dependent learning in both Tau and APP/ Tau mice. This similar phenotype occurs despite significant differences in early hippocampal synaptic plasticity, inflammation, and dysregulation of transcriptional programs, suggesting that alternative cellular and molecular mechanisms converge to induce hippocampal dysfunction in Tau and APP/Tau mice. Based on our transcriptomic analysis, these alternative mechanisms may implicate molecular changes in RNA processing/splicing and synaptic genes/pathways linked to dementia and intellectual disability (e.g. *Bdnf, Cacnb3, Dlg4, Fgf13...*) in Tau and APP/Tau mice, respectively [61–63].

The presence of Aβ pathology in the EC and BLA coincided with anxiety and emotional disturbances in 9-10 month-old APP and APP/Tau mice, further emphasizing the detrimental effects of A β on emotional and mood behaviors in AD [45, 64, 65]. Amvgdala pathology affects key cellular pathways related to inflammation, neurotransmission, AB binding, GTPase activity, and oxidoreductase/NAD+ activity. Given the link among emotional symptoms, inflammation, and neuronal excitability, it is plausible that pharmacological interventions aimed to enhance GABAergic neurotransmission or to mitigate inflammation and oxidative stress could represent promising therapeutic strategies for neuropsychiatric symptoms in dementia [45, 64]. Notably, tau counteracted the AB-induced synaptic transmission and plasticity deficits in the amygdala at early stages. The mechanisms underlying taumediated protection of synaptic function remain unknown; however, it is intriguing that this coincides with the absence of synaptic tau in this region. As the pathology advances, deficits in this neural circuitry may arise alongside dysregulation of glutamatergic synaptic genes, as observed in APP knock-in mice [66]. Notably, female APP/Tau mice at 9 months exhibited a regionspecific increase of A_β in EC and pTau in CA3 compared to males. This finding is particularly significant considering that women are more susceptible to emotional disturbances and experience faster cognitive decline. The molecular factors driving sex-specific differences in emotional and memory changes remain poorly understood and warrant further investigation. A potential explanation may be the distinct cellular vulnerability of women to pathological changes, including A_β and/or neurofibrillary tangles [67-70].

The temporal and regional associations of AB and tau pathologies with specific behavioral changes do not exclude the possibility that both factors may contribute to memory and emotional decline [17]. Besides the strong correlation between cerebral AB deposition and anxiety in elderly non-demented individuals [71, 72], tau and A\u03c3/tau pathologies are closely associated with neuropsychiatric symptoms in dementia [73, 74]. Considering that anxiety is reversed by anti-tau therapeutic treatments [75-77], the apparent lack of tau effects on emotional symptoms could be explained by dominant effects of AB over tau. differences in cell-specific transgene expression and/or agedependent pathological differences in APP/Tau mice. In this context, gene profiling analysis identified critical genes related to inflammation and synaptic function in two vulnerable brain regions in AD, which is particularly relevant considering the strong correlation between inflammatory and synaptic genes and the progression of AD [78–80]. Nonetheless, our transcriptomic

Table 1.	AD-associated genes differentially	expressed in the	hippocampus and	basolateral amygdala c	of APP/Tau mice.
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Biological pathway/gene name	Gene symbol	HPC		BLA		Biological function
		Log ₂ FC	padj	Log ₂ FC	padj	
Cell adhesion						
CD33 molecule	CD33	0.909	0.0002	0.811	0.0112	Carbohydrate and protein binding
Cell adhesion associated, oncogene regulated [*]	CDON	-0.407	0.0129	0.044	0.8796	Protein binding
FERM domain containing kindlin 2**	FERMT2	0.072	0.2276	0.160	0.0355	Actin, integrin, and protein kinase binding
Spondin 1	SPON1	-0.195	0.0208	-0.468	0.0499	Extracellular matrix structural constituent
Cell differentiation						
Proline rich coiled-coil 2C*	PRRC2C	-0.157	0.0357	-0.200	0.2183	RNA binding
Zinc finger CW-type and PWWP domain containing 1*	ZCWPW1	0.220	0.0625	-0.085	0.6838	Methylated histone and zinc ion binding
Cell signaling						
CD2 associated protein [*]	CD2AP	0.159	0.0409	0.194	0.2039	Protein binding
GRB2 associated binding protein 2*	GAB2	0.251	0.0018	0.077	0.6138	Transmembrane receptor protein tyrosine kinase adaptor activity
Inositol polyphosphate-5- phosphatase D	INPP5D	0.911	1.76E-07	0.524	0.0031	Inositol phosphatase activity
IQ motif containing GTPase activating protein 2	IQGAP2	-0.337	0.0798	-0.681	0.0159	Actin binding
Myelin protein zero like 1 ^{**}	MPZL1	0.188	0.1824	0.300	0.0347	Structural molecule activity
Ras and Rab interactor 3 [*]	RIN3	0.529	0.0098	0.237	0.5371	GTPase activator activity
Rho GTPase activating protein 20 [*]	ARHGAP20	-0.338	0.0006	-0.044	0.8427	GTPase activator activity
Serine/threonine kinase 32B [*]	STK32B	-0.412	0.0383	-0.069	0.7908	Protein serine/threonine kinase activity
Cytoskeleton organization						
Bridging integrator 1 [*]	BIN1	0.115	0.0115	-0.128	0.4945	Actin filament binding
Cordon-bleu WH2 repeat protein**	COBL	-0.002	0.9956	-0.286	0.0663	Actin monomer binding
CUB and Sushi multiple domains 1^*	CSMD1	-0.315	0.0146	-0.224	0.3286	Protein binding
DLC1 Rho GTPase activating protein ^{**}	DLC1	-0.018	0.8286	-0.426	0.0880	GTPase activator activity
Electron transport						
Cytochrome c, somatic [*]	CYCS	-0.129	0.0797	-0.108	0.4746	Electron transfer activity
Extracellular matrix organization						
ADAM metallopeptidase with thrombospondin type 1 motif 20**	ADAMTS20	-0.040	0.8181	0.344	0.0244	Metalloendopeptidase activity
Thrombospondin type 1 domain containing 4**	THSD4	-0.261	0.3363	-0.933	0.0400	Hydrolase activity
Immune response, inflammation						
Clusterin	CLU	0.685	0.0015	0.477	0.0059	$A\beta$ and misfolded protein binding
Protein tyrosine kinase 2 beta *	РТК2В	-0.274	0.0687	-0.110	0.3080	ATP binding
SLP adaptor and CSK interacting membrane protein [*]	SCIMP	2.519	0.0067	2.527	NA	Molecular adaptor activity
Triggering receptor expressed on myeloid cells 2	TREM2	1.849	4.88E-08	1.176	0.0025	$A\beta$, lipid, and protein binding
Triggering receptor expressed on myeloid cells like 2*	TREML2	1.424	7.83E-05	0.389	0.4646	Signaling receptor activity
Lipid metabolism						
Aldehyde oxidase 1	AOX1	0.605	5.12E-05	0.616	0.0011	Aldehyde oxidase activity
Apolipoprotein C1	APOC1	1.640	0.0002	1.093	0.0461	Lipid binding
Apolipoprotein E	APOE	0.744	0.0140	0.556	0.0049	Lipid binding
	ECHDC3	0.914	0.0048	1.207	0.0027	Lyase activity

Table 1. continued

Biological pathway/gene name	Gene symbol	HPC		BLA		Biological function	
		Log₂ FC	padj	Log₂ FC	padj		
Enoyl-CoA hydratase domain containing 3							
Phospholipase C gamma 2	PLCG2	0.509	0.0124	0.389	0.0982	Phosphatidylinositol and phospholipase activity	
Sortilin related receptor 1 [*]	SORL1	-0.200	4.69E-05	-0.157	0.1056	Aβ, low-density lipoprotein particle, neuropeptide and GTPase binding	
Thromboxane A synthase 1	TBXAS1	1.116	0.0010	1.040	3.08E-06	Oxidoreductase activity	
UDP glucuronosyltransferase family 1 member A10	UGT1A10	1.562	1.19E-08	1.005	0.0001	Glucuronosyltransferase activity	
UDP glucuronosyltransferase family 1 member A8	UGT1A8	1.569	1.15E-08	0.990	9.72E-05	Glucuronosyltransferase activity	
Protein transport and homeostasis							
F-box and leucine-rich repeat protein 7 [*]	FBXL7	-0.489	0.0437	0.066	0.8875	Protein binding and ubiquitination	
HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1 [*]	HECW1	-0.211	0.0285	-0.289	0.1478	Ubiquitin protein ligase activity	
Heparan sulfate-glucosamine 3- sulfotransferase 1	HS3ST1	0.259	0.0014	0.382	0.0363	Sulfotransferase activity	
Rhomboid 5 homolog 1 [*]	RHBDF1	0.364	0.0018	0.069	0.7152	Growth factor binding	
Sequestosome 1 [*]	SQSTM1	0.108	0.0355	-0.024	0.8687	Protein kinase and ubiquitin binding	
SEC24 homolog B, COPII coat complex component	SEC24B	-0.111	0.0150	-0.120	0.0843	SNARE and zinc ion binding	
Transglutaminase 6 [*]	TGM6	-1.436	0.0501	-0.852	NA	Protein-glutamine gamma- glutamyltransferase activity	
USP6 N-terminal like	USP6NL	0.181	0.0521	-0.366	0.0726	GTPase activator activity	
Dmx like 1 [*]	DMXL1	-0.186	0.0085	-0.087	0.4498	Vacuolar acidification	
Synaptic function, ion transport							
Neuronal calcium sensor 1 [*]	NCS1	-0.197	0.0298	-0.051	0.4838	Calcium binding	
Na+/K+ transporting ATPase interacting 2 [*]	NKAIN2	-0.145	0.0029	-0.123	0.2962	Sodium/potassium-transporting ATPase activity	
Phosphodiesterase 78 [®]	PDE7B	-0.352	0.0042	0.442	0.4354	c-AMP phosphodiesterase activity	
Phosphatidylinositol binding clathrin assembly protein [*]	PICALM	0.104	0.0952	0.022	0.8404	A β , SNARE, clathrin, and GTPase binding	
Solute carrier family 24 member 4 [*]	SLC24A4	-0.257	0.0121	-0.086	0.7272	Calcium/potassium/sodium transporter activity	
Solute carrier family 4 member 8 [*]	SLC4A8	-0.152	0.0227	-0.132	0.3902	Sodium/bicarbonate/chloride transporter activity	
Tetraspanin 13 [*]	TSPAN13	-0.211	0.0375	-0.128	0.3426	Calcium channel regulator activity	
Transcriptional regulation							
AHNAK nucleoprotein*	AHNAK	0.793	0.0072	0.234	0.3389	RNA and protein binding	
BCAS3 microtubule associated cell migration factor*	BCAS3	-0.122	0.0286	2.299	NA	Acetyltransferase activator activity	
BCL3 transcription coactivator	BCL3	2.499	8.48E-06	1.203	0.1014	DNA-binding transcription factor binding	
CUGBP Elav-like family member 1 [*]	CELF1	-0.134	0.0681	-0.053	0.4822	RNA binding	
General transcription factor IIH subunit 3**	GTF2H3	-0.089	0.2043	0.198	0.0294	Metal ion binding	
Histone deacetylase 9	HDAC9	-0.206	0.0724	-0.386	0.0449	DNA-binding transcription factor binding	
ST18 C2H2C-type zinc finger transcription factor	ST18	-0.229	0.0346	0.389	0.0315	DNA-binding transcription factor activity, RNA polymerase II-specific	
Unclassified							
Ataxin 7 like 1 [*]	ATXN7L1	-0.141	0.0086	0.022	0.9034	Protein binding	
Leucine zipper protein 2	LUZP2	-0.187	0.0565	-0.251	0.0840	Extracellular region	

Table 1. continued							
Biological pathway/gene name		Gene symbol	НРС		BLA		Biological function
			Log₂ FC	padj	Log ₂ FC	padj	
N A	lembrane spanning 4-domains 4A	MS4A4A	0.705	0.0989	1.178	0.0588	Protein binding
P si	rotein phosphatase 1 regulatory ubunit 37 [*]	PPP1R37	-0.096	0.0642	-0.040	0.6481	Protein phosphatase inhibitor activity
T	ransmembrane and coiled-coil	TMCO4	0.708	0.0002	0.467	0.0063	Protein binding

Differential expression of GWAS-identified AD-associated genes in the hippocampus (HPC) and/or basolateral amygdala (BLA) of APP/Tau mice compared with non-transgenic (WT) mice. Genes are grouped according to their biological function sorted alphabetically with Log₂ fold changes (Log₂ FC) indicating downregulated (<0) or upregulated (>0) mRNA levels and significantly deregulated (adjusted *p*-value (padj) <0.1) only in the hippocampus (marked with*) or BLA (marked with**) or in both hippocampus and BLA (not marked).

analysis lacks cell-type resolution, which restricts the identification of the specific cellular population(s) responsible for the transcriptomic alterations underlying the dysfunction of hippocampal and amygdalar neural circuits. By contrast, single-cell transcriptomics showed deregulation of synaptic, signaling, myelination, immune response, metabolism and protein homeostasis pathways in neurons, astrocytes, microglia and oligodendrocytes in AD brain ([81], for a review). In this regard, integrative gene co-expression analyses of murine and human bulk and single-cell transcriptomics may help to identify key cell-type-specific genes affected in specific neural circuits during AD pathology. The deregulation of multiple AD-associated genes related to inflammation, including microglial and astrocytic genes, synapse function and ion transport, suggest their direct relationship with synapse dysfunction and hippocampal-dependent cognitive deficits in AD. Considering that APP/Tau mice exhibit transcriptional changes closely linked to molecular drivers of AD, it may be a useful model for investigating how AD-risk genes contribute to pathological changes and cellular vulnerability in AD.

In summary, our study reveals complex interactions between Aβ and tau, including synergistic and region-specific effects on neuropathology, synapse integrity, immune response, and, ultimately, on cognitive and emotional function. The occurrence of sex-specific differences in pathology, the faithful reproduction of pathological and clinical AD features, and the identification of transcriptional profiles linked to AD risk genes in APP/Tau mice suggest that this may be a useful model for studying sex-specific pathophysiological mechanisms and evaluating novel therapeutic targets. Finally, since both pathologies can alter brain function by affecting different biological processes, we reinforce the need to develop therapeutic approaches in AD that simultaneously target multiple pathophysiological mechanisms.

DATA AVAILABILITY

Raw bulk RNA-seq data were deposited to the Gene Expression Omnibus (GEO) under accession numbers GSE280199 and GSE279050. Data and materials, including specific experimental protocol information, are available under request.

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ACKNOWLEDGEMENTS

We thank Drs. E Sanz and A Quintana for providing the RiboTag mice, A Delprat and VA Díaz for excellent technical support, and M Puigdellívol for critical reading of the manuscript. We thank the Servei d'Estabulari, Servei de Genòmica i Bioinformàtica and Institut de Neurociències Histology and Microscope facilities of UAB, and Genomics Unit of Centre for Genomic Regulation (CRG) for excellent technical support. This work was supported by research projects funded by Agencia Estatal de Investigación, Ministerio de Ciencia, Innovación y Universidades of Spain, with FEDER funds (PID2019-106615RB-I00, PID2022-137668OB-I00 and PDC2021-121350-I00 to CAS; PID2019-107677GB-I00 and PID2022-136597NB-I00 to ARM; SAF2017-89271-R, PID2020-11751ORB-I00 and PID2023-151925OB-I00 to JRA), Junta de Andalucía (P20-0881 to ARM), Instituto de Salud Carlos III (CIBERNED CB06/05/0042 to JRA), and Generalitat de Catalunya (2021 SGR00142) and BrightFocus Foundation (A20220475 to CAS). APD was supported by Juan de la Cierva-Incorporación (IJC2019-042468-I) and Ramón y Cajal (RYC2022-037843-I) Programs, and YAT by a Marie Skłodowska-Curie Postdoctoral Fellowship under the Horizon Europe programme (GA. 101061171). MDCL (BES-2017-082072), PS (PREP2022-000069), AD (FPU 18/02486) and IMG (FPU 17/04283) were supported, respectively, by Formación Personal Investigador and Formación de Profesorado Universitario predoctoral fellowships from Ministerio de Ciencia, Innovación y Universidades of Spain.

AUTHOR CONTRIBUTIONS

MDCL, APD and CAS designed the experiments and coordinated the study. MDCL designed and performed pathological, immunofluorescence and behavioral analyses. MDCL, AD and APD performed the biochemical and expansion microscopy synaptic characterization and transcriptomic analyses. PS performed biochemical analysis. YAT, IMG, HCC and ARM designed and performed the electrophysiological experiments. MDCL, AD, APD, ARM, JRA and CAS discussed and interpreted the data. MDCL, APD and CAS wrote the manuscript with input of all authors. The authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41380-025-02901-9.

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