Neuronal deficiency of p38α-MAPK ameliorates symptoms and pathology of APP or Tau-transgenic Alzheimer’s mouse models

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Funding information
Deutsche Forschungsgemeinschaft (DFG), Grant/Award Number: LI1725/2-1; German federal ministry of research and education, Grant/Award Number: 031L0101D; HOMFORexcell program

Abstract
Alzheimer’s disease (AD) is the leading cause of dementia with very limited therapeutic options. Amyloid β (Aβ) and phosphorylated Tau (p-Tau) are key pathogenic molecules in AD. P38α-MAPK is specifically activated in AD lesion sites. However, its effects on AD pathogenesis, especially on p-Tau-associated brain pathology, and the underlying molecular mechanisms remain unclear. We mated human APP-transgenic mice and human P301S Tau-transgenic mice with mapk14-floxed and neuron-specific Cre-knock-in mice. We observed that deletion of p38α-MAPK specifically in neurons improves the cognitive function of both 9-month-old APP and Tau-transgenic AD mice, which is associated with decreased Aβ and p-Tau load in the brain. We further used next-generation sequencing to analyze the gene transcription in brains of p38α-MAPK deficient and wild-type APP-transgenic mice, which indicated that deletion of p38α-MAPK regulates the transcription of calcium homeostasis-related genes, especially downregulates the expression of grin2a, a gene encoding NMDAR subunit NR2A. Cell culture experiments further verified that deletion of p38α-MAPK inhibits NMDA-triggered calcium influx and neuronal apoptosis. Our systemic studies of AD pathogenic mechanisms using both APP- and Tau-transgenic mice suggested that deletion of neuronal p38α-MAPK attenuates AD-associated brain pathology and protects neurons in AD pathogenesis. This study supports p38α-MAPK as a novel target for AD therapy.

Abbreviations: AD, Alzheimer’s disease; adcy, adenylate cyclase; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; APP, Alzheimer’s amyloid precursor protein; APPβ, human APP-transgenic; APPΔ9, non-APP-transgenic; Aβ, amyloid β peptide; BACE1, β-secretase 1; BDNF, brain-derived neurotrophic factor; C99, C-terminal 99-aminoacid APP fragment; CCL-2, chemokine (C–C motif) ligand 2; Chi3l3, chitinase-like 3; ER, endoplasmic reticulum; ERK, like extracellular signal-related kinases; FA, formic acid; gapdh, glyceraldehyde 3-phosphate dehydrogenase; gnas, guanine nucleotide-binding protein; GO, gene ontology; gria1, glutamate ionotropic receptor AMPA type subunit 1; grin2a, glutamate ionotropic receptor NMDA type subunit 2A; Iba-1, ionized calcium-binding adapter molecule 1; IGF -1, insulin growth factor 1; iNOS, inducible nitric oxide synthase; JNK, C-Jun N-terminal kinase; LTD, long term depression; LTP, long term potentiation; Mrc1, mannose receptor, C type 1; Munc18-1, Munc18-1 protein mammalian homolog; p38α-MAPK, p38 mitogen-activated protein kinase type α; NeuN, neuronal nuclei; NMDAR, N-methyl-d-aspartate receptors; PSD-95, postsynaptic density protein 95; p-Tau, hyper-phosphorylated Tau; RAB, high-salt reassembly buffer; RIPA, radioimmuno precipitation assay; RT, room temperature; sec, seconds; sigmar1, sigma non-opioid intracellular receptor1; slc8a1, solute carrier family 8 (sodium/calcium exchanger) member; SNAP-25, synaptosome-associated protein 25; Tau6, human Tau-transgenic; Tau9, non-Tau-transgenic; TNF-α, tumor necrosis factor α.

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1 | INTRODUCTION

Alzheimer’s disease (AD) is pathologically characterized by extracellular deposits of amyloid β peptide (Aβ) and intracellular neurofibrillary tangles primarily composed of hyper phosphorylated Tau (p-Tau).\(^1\) Growing evidence shows that Aβ initiates AD pathogenesis: (a) Aβ aggregates directly injure synaptic junctions and neurons in the neocortex and limbic system,\(^2\) (b) aggregated Aβ triggers microglia-dominated neurotoxic inflammatory activation,\(^3\) and (c) both Aβ and neuroinflammation induce phosphorylation of Tau,\(^4,5\) and drive Tau pathology to expand along axonal projections to the entire neocortex.\(^5,7\) Therefore, the reduction of cerebral Aβ is desirable in AD therapy.

The β-secretase (BACE1) is a speed-limiting enzyme in Aβ generation from Alzheimer’s amyloid precursor protein (APP).\(^8\) Many approaches to inhibit BACE1 have been attempted; unfortunately, these studies have not yet led to efficacious therapy for AD patients.\(^9\) In contrast, the intervention of BACE1 potentially brought severe side effects and even safety concerns for AD patients.\(^9\) In the intervention of BACE1, we recently observed that reduction of p38α-MAPK, a stress-associated kinase which is highly activated at AD lesion sites in early disease stages,\(^11,12\) facilitated lysosomal degradation of BACE1 in neurons.\(^13\) We argue that inhibition of p38α-MAPK might be an alternative method to block BACE1-mediated Aβ production. Moreover, deficiency of p38α-MAPK reduces Aβ-triggered inflammatory activation in cultured microglia.\(^14\) p38-MAPK has been shown to phosphorylate recombinant Tau protein,\(^15\) and has been observed to bind p-Tau in the AD brain.\(^12,16\) There is also evidence that p38-MAPK might mediate Aβ-induced synaptic impairment.\(^17,18\) An exploratory clinical study on p38α-MAPK inhibitor did show a promising result that p38α-MAPK inhibition might improve episodic memory and impact amyloid deposits in AD patients.\(^19\) Thus, it is worthwhile to extensively investigate the pathophysiological role of p38α-MAPK in AD.

Administration of p38α-MAPK inhibitor in both APP- and Tau-transgenic AD mice suppresses inflammatory activation and attenuates neuronal deficits in the brain.\(^20,22\) However, pharmacological experiments are not able to distinguish the effect from neuronal and microglial p38α-MAPK. Deletion of p38α-MAPK specifically in neurons reduced cerebral Aβ and BACE1 proteins in 5xFAD mice,\(^23\) which corroborates our previous finding.\(^13\) However, this study did not further decode the underlying mechanisms mediating neuronal protection. Moreover, most preclinical studies on p38α-MAPK have used APP-transgenic mice as AD models and neglected the effects of p38α-MAPK on p-Tau-associated pathology, which prevent comprehensively understanding the pathogenic role of p38α-MAPK in AD.

To address these questions, we deleted p38α-MAPK in neurons of both APP- and Tau-transgenic mice and observed that deletion of neuronal p38α-MAPK attenuated cognitive dysfunctions in association with decreased loads of Aβ and p-Tau, and inhibited inflammatory activation in the brain. We further compared the transcriptome in brains from neuronal p38α-MAPK-deficient and wild-type APP-transgenic mice, and showed that deletion of p38α-MAPK might regulate neuronal calcium homeostasis and protect neurons in AD.

2 | MATERIALS AND METHODS

2.1 | Animal models and cross-breeding

Our APP-transgenic (APP\(^\text{tg}\)) mice over-expressing human mutated APP (KM670/671NL) and presenilin-1 (L166P) under Thy-1 promoters\(^24\) were kindly provided by M. Jucker, Hertie Institute for Clinical Brain Research, Tübingen; p38\(^\text{fl/fl}\) mice carrying loxP site-flanked mapk14 gene were provided by K. Otsu, Osaka University;\(^25\) and K. Nave, Max-Planck-Institute for Medicine, Göttingen, kindly provided Nex-Cre mice, expressing Cre recombinase from the endogenous locus of nex gene.\(^26\) APP\(^\text{tg}\), p38\(^\text{fl/fl}\), and Nex-Cre mice, all on a C57BL6 genetic background, had been cross-bred in our previous study to build AD animal models with (APP\(^\text{tg}\)p38\(^\text{fl/fl}\)Cre\(^+/−\)) and without (APP\(^\text{tg}\)p38\(^\text{fl/fl}\)Cre\(^−/−\)) deletion of p38α-MAPK in neurons.\(^13\) In order to investigate physiological function of p38α-MAPK in neurons, we also examined non-APP-transgenic (APP\(^\text{wt}\)) mice with (APP\(^\text{wt}\)p38\(^\text{fl/fl}\)Cre\(^+/−\)) and without (APP\(^\text{wt}\)p38\(^\text{fl/fl}\)Cre\(^−/−\)) deletion of neuronal p38α-MAPK. To evaluate the effect of neuronal p38α-MAPK on p-Tau-induced phenotype, we cross-bred p38\(^\text{fl/fl}\) and Nex-Cre mice, with P301S Tau-transgenic (Tau\(^\text{tg}\)) mice (imported from the Jackson Laboratory, Bar Harbor, MA, USA; Stock: #008169), which over-express the human Tau mutant (P301S) under the direction of mouse prion protein promoter.\(^27\) For this study, 9-month-old male and female mice were used. All animal experiments were performed in accordance with relevant national rules and authorized by the local research ethical committee (permission numbers: 40/2014 and 49/2016).
2.2 | Morris water maze

The Morris water maze test, consisting of a 6-day training phase and a 1-day probe trial, was used to assess the cognitive function of APP<sup>tg</sup> or Tau<sup>tg</sup> mice and their APP<sup>wt</sup> littermates, as previously described.28

2.3 | Tissue collection for histological and biochemical analysis

Animals were euthanized by inhalation of isoflurane. The brain was removed and divided. The left hemisphere was immediately fixed in 4% paraformaldehyde (Sigma-Aldrich GmbH, Taukirchen, Germany) and embedded in paraffin. A 0.5-μm-thick piece of tissue was sagittal cut from the right hemisphere. The cortex and hippocampus were separated and homogenized in TRIzol (Thermo Fisher Scientific, Darmstadt, Germany) for RNA isolation. The remainder of the right hemisphere was snap-frozen in liquid nitrogen and stored at −80°C until biochemical analysis. To evaluate the activity of p38-MAPK, we also collected heart, left lobe of liver, and skeletal muscles of quadriceps.

2.4 | Western blot analysis

Frozen mouse brains were homogenized on ice in radioimmunoprecipitation assay buffer (RIPA buffer; 50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% SDS, 0.5% sodiumdeoxycholate, 1% NP-40, and 5 mM EDTA) supplemented with protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) and phosphatase inhibitors (50 nM okadaic acid, 5 mM sodium pyrophosphate, and 50 mM NaF; Sigma-Aldrich). To quantify p-Tau and total Tau (t-Tau) proteins, the brain tissue was sequentially homogenized in ice-cold high-salt reassembly buffer (RAB; 0.1 M MES, 1 mM EGTA, 0.5 mM MgSO4, 0.75 M NaCl, 20 mM NaF, and 1 mM PMSF), RIPA buffer, and 70% formic acid (FA).28 Human Aβ, Tau, and other brain proteins were quantified with previously established Western blot using antibodies listed in Table 1.

2.5 | Golgi - Cox staining

Serial 100-μm-thick sagittal sections were cut from mouse brains using a vibratome (VT1000S, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) and stained with Rapid Golgi Staining Kit (FD NeuroTechnologies, Columbia, MA, USA). Images of neurons at hippocampal CA1 and CA3 and at cortex (layer II/III) were obtained on a Zeiss Axiolmager.Z2 microscope with a 63× oil-objective and a z-stack distance of 0.5 μm (Zeiss Microscopy, Göttingen, Germany). The number of spines per micrometer of dendritic length was determined in second- or third-order dendritic branches of apical dendrites. At minimum, three serial sections per animals with 500 μm of interval were analyzed. Moreover, the morphology of dendritic spines were analyzed and spines were grouped into mature (mushroom), immature (thin and stubby), and filopodia-like spines according to the published classification.29 The experimenter was blinded to the genotypes of mice during the entire experiment.

### Table 1

| Antibodies              | Supplier                     | Species       | Type          | Reference            |
|-------------------------|------------------------------|---------------|---------------|----------------------|
| p38-MAPK                | Cell Signaling Technology    | Rabbit        | Polyclonal    | Catalog no. 9212     |
| phosphorylated p38-MAPK | Cell Signaling Technology    | Rabbit        | Polyclonal    | Catalog no. 9211     |
| PSD-95                  | Cell Signaling Technology    | Rabbit        | Polyclonal    | Catalog no. 2507     |
| SNAP-25                 | Cell Signaling Technology    | Rabbit        | Polyclonal    | Catalog no. 3926     |
| Munc18-1                | Cell Signaling Technology    | Rabbit        | Polyclonal    | Catalog no. 13414    |
| synaptophysin           | Abcam                        | Mouse         | Monoclonal    | Clone SY38           |
| Aβ                      | Merck Chemicals GmbH        | Mouse         | Monoclonal    | Clone W0-2           |
| Tau                     | Thermo Fisher Scientific     | Mouse         | Monoclonal    | Clone HT7            |
| Phosphorylated Tau      | Thermo Fisher Scientific     | Mouse         | Monoclonal    | Clone AT8            |
| Grin2a                  | Cell Signaling Technology    | Rabbit        | Polyclonal    | Catalog no. 4205     |
| β-actin                 | Cell Signaling Technology    | Rabbit        | Monoclonal    | Clone 13E5           |
| α-tubulin               | Abcam                        | Mouse         | Monoclonal    | Clone DM1A           |
| LC3B                    | Cell Signaling Technology    | Rabbit        | Monoclonal    | Clone D11            |
| Cleaved Caspase-3       | Cell Signaling Technology    | Rabbit        | Monoclonal    | Clone 5A1E           |
2.6 | Immunohistological analysis

Serial 50-μm-thick sagittal sections were cut from the paraffin-embedded hemisphere. Human Aβ in APP<sup>Fl</sup> mouse brains was stained with mouse anti-human Aβ antibody (clone 6F3/3D; Dako Deutschland GmbH, Hamburg, Germany) and microglia labeled with rabbit anti-ionized calcium-binding adapter molecule (Iba)-1 antibody (Wako Chemicals, Neuss, Germany). For each animal we labeled four sections with an interval of 10 layers between neighbouring sagittal sections. In the whole hippocampus and cortex, volumes of Aβ were estimated with the Cavalieri method, and Iba-1-positive cells were counted with the Optical Fractionator as described previously<sup>30</sup> on a Zeiss AxioImager.Z2 microscope equipped with a Stereo Investigator system (MBF Bioscience, Williston, VT, USA).

To evaluate Tau pathology in Tau<sup>Fl</sup> mice, four serial 50-μm-thick sections were chosen as for Aβ analysis. Brain tissues were stained according to the published protocols<sup>31</sup> with a mouse monoclonal antibody against human phospho-Tau (Ser202, Thr205) (clone: AT8; Thermo Fisher Scientific) or with thioflavine S (Sigma-Aldrich GmbH). Thioflavine S staining was used to identify neurofibrillary tangles. Because of low numbers of p-Tau or thioflavine S staining-positive cells in the cortex and hippocampus, we did not use stereological analysis, but counted labeled cells in the regions under 40× objective. In the channels for Alexa Fluor 488 and p38α-MAPK (clone: 9F12; Novus Biologicals, VT, USA), we imaged sections in the channel for CY3 to count p38α-MAPK-staining positive cells. More than 800 NeuN-positive cells and >400 NeuN-negative cells were counted and the percentages of p38α-MAPK-positive cells within these two groups of cells were calculated.

2.7 | Quantitative PCR for analysis of gene transcripts

Total RNA was isolated from mouse brains and reverse transcribed. Gene transcripts of pro-and anti-inflammatory markers were quantified with our established protocol<sup>30</sup> using Taqman gene expression assays of mouse tnf-α, il-1β, chemokine (C–C motif) ligand 2 (ccl-2), inducible nitric oxide synthase (inos), il-10, arginase 1 (arg1), mannose receptor C type 1 (mrc1), chitinase-like 3 (chil3), brain-derived neurotrophic factor (bdnf), insulin growth factor (igf)-1, and glyceraldehyde 3-phosphate dehydrogenase (gapdh) (Thermo Fisher Scientific). After next generation sequencing analysis, the transcription of following target genes encoding: adenylate cyclase 3 and 7 (adcy3, adcy7), ATPase plasma membrane Ca<sup>2+</sup> transporting 4 (atp2b4), guanine nucleotide-binding protein (gnas), grin2a, 5-hydroxytryptamine receptor 7 (htr7), solute carrier family 8 (sodium/calcium exchanger), member 1 (slc8a1), sphingosine kinase 1 (spk1), transmembrane protein 63C (mem63c), phosphoinositide-specific phospholipase C (plch1), and nuclear factor of activated T cells 2 (nfatc2), was determined using SYBR green binding technique with primers shown in Table 2. For nfatc2 detection, two different sets of primers for transcript variants were used.

2.8 | RNA purification for next generation sequencing

RNA from 9-month-old p38α-MAPK-deficient and wild-type APP<sup>Fl</sup> littermate mice was purified using the RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The concentration and integrity of RNA were tested using the Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). RNA samples with RNA integrity number > 6 were used to build RNA-sequencing libraries.

2.9 | Preparation of RNA-sequencing library

The RNA-seq library was built with 600 ng total RNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (Ipswich), NEBNext Ultra Directional RNA Library Prep Kit for Illumina (E7420) and NEBNext Multiplex Oligos for Illumina (Index-Set2) (New England Biolabs GmbH, Frankfurt am Main, Germany). Briefly, mRNA was enriched using AMPure XP Beads (Beckman Coulter GmbH, Krefeld, Germany), fragmented using enzymes, and then, reverse transcribed into cDNA with random primers. Thereafter, double stranded cDNA was adapter-ligated and amplified by PCR for 10 cycles. The quality of libraries was evaluated using the High Sensitivity DNA Chip in the Agilent 2001 Bioanalyzer and quantified by qPCR with the PerfeCTa NGS quantification kit from QuantaBio (Beverly, MA, USA).
Illumina Sequencing and Sequence alignment

The libraries were sequenced on the Illumina HiSeq2500 platform (San Diego, CA, USA) with 101-bp single-end reads. After QC with FastQC Version 0.11.2 (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/), reads were adaptor-trimmed (Q < 20) with Cutadapt (Version 1.4.132) with a wrapper Trim Galore! (Version 0.3.3) (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Reads were aligned with the grape-nf pipeline (https://github.com/guigolab/grape-nf) wrapping STAR (Version 2.4.0j 33) and RSEM (Version 1.2.21 34). Read counts were imported into R with tximport (Version 1.6.0 35). Differentially expressed genes were identified with edgeR (Version 3.20.936). Transcripts with an absolute log2-fold change greater than 2 and a FDR value below 0.01 were considered differentially expressed.

Gene ontology (GO) and pathway analysis

The biological function of individual genes was identified by GO analysis (http://cbl-gorila.cs.technion.ac.il/). KEGG pathways with high gene transcript clustering were processed and downloaded using the online software Pathview.37,39

Culture of primary neurons

Cortex was collected from embryos (E14 ± 0.5) from the breeding of APPWTp38fl/flNex-Cre−/− (or APPWTp38fl/flNex-Cre−/−) male and APPWTp38fl/flNex-Cre−/− female mice. After digestion with trypsin for 15 minutes at 37°C, the single cell suspension (2×10^5 cells) was seeded on a poly-L-lysine-coated coverslip with 15 mm of diameter. Primary neurons were cultured for 14 ± 2 days at 37°C and 10% CO2 in neurobasal medium supplemented with 2% B27, 0.25% L-Glutamine, and 0.1% Glutamax (all reagents were bought from Thermo Fisher Scientific).

Calcium imaging of primary neurons

The functional analysis of altered calcium homeostasis was evaluated by calcium imaging. We incubated the neurons with 5 μM Fluo-4 (Thermo Fisher Scientific) for 30 minutes at RT. After washing, we aligned the coverslip in a perfusion chamber RC-20 (Warner Instruments, Hamden, CT, USA) and applied a constant perfusion (0.5 mL/min) with imaging buffer (148 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Glucose, 10 mM HEPES [pH 7.38]). Calcium images were taken with an inverse Olympus microscope (IX-70; Olympus Deutschland GmbH, Hamburg, Germany), equipped with a 20× objective (Olympus) and a CCD camera (Orca-II-ER; Hamamatsu Photonics Deutschland GmbH, Herrsching am Ammersee, Germany). The basal activity of neurons was detected for 90 seconds. Then, the neurons of both genotypes were stimulated with either 100 μM L-glutamate or 50 μM NMDA (both from Sigma-Aldrich) in imaging buffer. When the calcium response reached a plateau, 500 μM L-glutamate (at 400 seconds) or 10 mM NMDA (at 300 seconds) was further administered to neurons. As a control, 75 mM KCl was used to stimulate neurons. Images were processed and analyzed with the software CellObserver Z1 and Axiovision (Zeiss Microscopy, Göttingen, Germany). Region of interests were set around the fluorescent neuronal cell bodies. The ratio F/F0 was calculated (F: fluorescent intensity at different time points, F0: basal fluorescent intensity.

| Gene     | Sense                         | Antisense                      |
|----------|-------------------------------|--------------------------------|
| Adcy3    | AGCTTGTTGGGCTTCTCTATCTT       | CCATCCAGTGCTGTGTGTTG           |
| Adyc7    | ACATGCCAATGCGTTAAGAT          | TCTAGCTAGGCTATCCGG             |
| Atp2b4   | CCCTCGAGATTTGGAGAAGC          | GAGTCGATGAGGAGGACAGG           |
| GAPDH    | ACAACTTTGGCATGGTGGAA          | GATGCAAGGATGGTGTTCG            |
| Gnas     | GACCTGTCGACGTACTCTCCTT        | TCCACGGAGACATTGTGCC            |
| Grin2a   | CCATCTGGGCGGATCCTAGGG         | AGATGGTTGGTGACACAGGAG          |
| HTR7     | GCAAGGCCCCTTTATCTTGGA         | GCACTGGAGTAGGGCTAGCAT          |
| Nfatc2_1207 | CTGGGCAGAATTTCCGTGTG         | GCCATTTGCCTCAAGGAG             |
| Nfatc2_4293 | CGGCCACACTTACCCCTACTG        | ATAGGACCCCGACTAATTG            |
| Plch1    | GCAAAGTGAGGGCTTTCAAGAA        | GCATAGAGACAGAGGCTAGCT          |
| Slc8a1   | TAGGCCCTTTTCATCACAGC          | AAGCAAATTCAGCCTGCAA            |
| Sphk1    | GAGCCAGTGCCCTTCATGTC          | GCATAACACAGCCCTACAG            |
| Tmem63c  | TCAGCTCTCGTTCCAACTTT          | ATGGGTTGGAGACACTTGT            |

| TABLE 2  | List of primer sequences used for SYBR green based quantitative RT-PCR

Gene Sense Antisense
Adcy3 AGCTTGTTGGGCTTCTCTATCTT CCATCCAGTGCTGTGTGTTG
Adyc7 ACATGCCAATGCGTTAAGAT TCTAGCTAGGCTATCCGG
Atp2b4 CCCTCGAGATTTGGAGAAGC GAGTCGATGAGGAGGACAGG
GAPDH ACAACTTTGGCATGGTGGAA GATGCAAGGATGGTGTTCG
Gnas GACCTGTCGACGTACTCTCCTT TCCACGGAGACATTGTGCC
Grin2a CCATCTGGGCGGATCCTAGGG AGATGGTTGGTGACACAGGAG
HTR7 GCAAGGCCCCTTTATCTTGGA GCACTGGAGTAGGGCTAGCAT
Nfatc2_1207 CTGGGCAGAATTTCCGTGTG GCCATTTGCCTCAAGGAG
Nfatc2_4293 CGGCCACACTTACCCCTACTG ATAGGACCCCGACTAATTG
Plch1 GCAAAGTGAGGGCTTTCAAGAA GCATAGAGACAGAGGCTAGCT
Slc8a1 TAGGCCCTTTTCATCACAGC AAGCAAATTCAGCCTGCAA
Sphk1 GAGCCAGTGCCCTTCATGTC GCATAACACAGCCCTACAG
Tmem63c TCAGCTCTCGTTCCAACTTT ATGGGTTGGAGACACTTGT
at the time point $0$). Calcium influx ($\Delta \text{Ca}^{2+}$) = $(F-F0)/F0$. Four independent experiments were performed for each stimulator. The mean value of $\Delta \text{Ca}^{2+}$ from all analyzed cells on the same coverslip was calculated for each animal.

To evaluate intracellular calcium buffering capacity, we stimulated p38α-MAPK-deficient and wild-type neurons with 100 µM l-glutamate for 20 seconds after 60 seconds of baseline acquisition. Thereafter, a constant perfusion (0.5 mL/min) with imaging buffer was applied to the chamber to wash out l-glutamate for around 180 seconds (until the fluorescence intensity returned to the baseline level). The time during which the intensity of Fluo-4 fluorescence went back to the baseline after wash-out was calculated as decay time (see Figure 6F).

To investigate the effects of p38α-MAPK deletion on calcium influx after blocking NMDAR, we continued to perfuse p38α-MAPK-deficient and wild-type neurons from the wash-out experiment (described above) constantly with imaging buffer containing 100 µM NMDAR antagonist, 2-APV (Tocris Bioscience, Wiesbaden-Nordenstadt, Germany; Cat. No.: 0106) at 0.5 mL/min for 300 seconds. Thereafter, neurons were again stimulated with 100 µM l-glutamate for 20 seconds in presence of 100 µM 2-APV. After a further 180-sec treatment with 100 µM 2-APV, neurons were washed with pure imaging buffer (see Figure 6F).

In all calcium imaging experiments, the experimenter was blinded to the genotypes of cells.

### 2.14 Apoptosis assay of p38α-MAPK-deficient and wild-type primary neurons

To evaluate the effects of p38α-MAPK deficiency on calcium influx-induced neuronal death, we treated cultured p38α-MAPK-deficient and wild-type neurons with 100 µM l-glutamate or 50 µM NMDA for 16 hours. Thereafter, neurons were harvested and lysed in RIPA buffer. Quantitative Western blot was used to detect caspase-3 activity in the cell lysate with antibodies specifically against cleaved caspase-3 (Clone 5A1E; Cell Signaling Technology). This antibody detects cleaved (active) caspase-3 without showing pro-caspase-3.

### 2.15 Statistics

Data were presented as mean ± SEM. For multiple comparisons, one-way or two-way ANOVA followed by Bonferroni, Tukey, or Dunnett T3 post hoc test (dependent on the result of Levene's test to determine the equality of variances) was used. Two independent-samples Students $t$ test was used to compare means for two groups of cases. All statistical analyses were performed with SPSS version 19.0 for Windows (IBM, New York, NY, USA). Statistical significance was set at $P < .05$.

### 3 RESULTS

#### 3.1 Determination of lesion sites-associated activation of p38-MAPK in APP-transgenic mice and establishment of neuronal p38α-MAPK-deficient AD mouse models

Based on previous reports showing that p38-MAPK is activated in association with AD pathological changes, we hypothesized that phosphorylation of p38-MAPK would be greater in APP$^{tg}$ mice compared to non-APP-transgenic (APP$^{wt}$) animals. We measured p38-MAPK activities in brain, heart, muscles, and liver of 9-month-old APP$^{tg}$ and APP$^{wt}$ littermates. The results showed that phosphorylation of p38-MAPK was increased in the brain of the APP$^{tg}$ mice, compared to the APP$^{wt}$ littermates, but not in the other organs tested (Figure 1A,B; $t$ test, $P = .025$), confirming that p38-MAPK is activated in association with AD lesions.

To verify the deletion of p38α-MAPK in the brain, we measured the cerebral protein levels of p38-MAPK in 9-month-old APP$^{tg}$/p38α$^{fl/fl}$Cre$^{-/-}$ and APP$^{tg}$/p38α$^{fl/fl}$Cre$^{-/-}$ littermate mice and found the levels of p38-MAPK to be 75% lower in Cre-positive mice compared to Cre-negative littermates (Figure 1C,D; p38-MAPK/α-tubulin: 0.245 ± 0.036 vs 1.005 ± 0.162, respectively; $t$ test, $P = .003$).

We then co-stained p38α-MAPK and NeuN, a neuronal marker, in brains of 9-month-old APP$^{tg}$/p38α$^{fl/fl}$Cre$^{-/-}$ and APP$^{tg}$/p38α$^{fl/fl}$Cre$^{-/-}$ littermates. In the cortex of APP$^{tg}$/p38α$^{fl/fl}$Cre$^{-/-}$ mice, p38α-MAPK protein was stained and shown in puncta around the nucleus in 48.67 ± 6.95% NeuN antibody-stained cells (Figure 1E), whereas, only 12.81 ± 3.86% NeuN-positive cells in APP$^{tg}$/p38α$^{fl/fl}$Cre$^{-/-}$ mice showed p38α-MAPK signals (Figure 1F; $t$ test, $P < .001$). In NeuN-negative brain cells, the staining of p38α-MAPK was not different between these two groups of AD mice (Figure 1F; $t$ test, $P > .05$). The cellular specificity of Cre-mediated p38α-MAPK deletion in neurons of APP$^{tg}$ mice corroborates our previous observation.

Interestingly, in the additional experiments, we observed that deletion of p38α-MAPK was able to further inhibit phosphorylation of the rest of p38-MAPK protein in p38α-MAPK-deficient APP$^{tg}$ mouse brain but not in p38α-MAPK-deficient APP$^{wt}$ mice (Figure 1G,H; one-way ANOVA, $P < .05$).

In our study, we produced a total of 361 new-born mice from the following two breeding pairs: (a) APP$^{tg}$/p38α$^{fl/fl}$Cre$^{-/-}$ [male] and APP$^{wt}$/p38α$^{fl/fl}$Cre$^{-/-}$ [female], and (b) APP$^{tg}$/p38α$^{fl/fl}$Cre$^{-/-}$ [male] and APP$^{wt}$/p38α$^{fl/fl}$
The genotypes (percentages) of the newborn mice were APP\textsuperscript{wt}\,p38\textsuperscript{flo/flo}\,Cre\textsuperscript{−/−} (19.11%), APP\textsuperscript{wt}\,p38\textsuperscript{flo/flo}\,Cre\textsuperscript{+/−} (30.75%), APP\textsuperscript{tgp}\,p38\textsuperscript{flo/flo}\,Cre\textsuperscript{−/−} (22.44%), and APP\textsuperscript{tgp}\,p38\textsuperscript{flo/flo}\,Cre\textsuperscript{+/−} (27.70%), as shown in Table 3 (χ\textsuperscript{2} test between different genotypes, \(P = .984\)). Mice with deletion of p38α-MAPK appeared to be generally healthy, fertile, and behaviorally normal, demonstrating that deletion of neuronal p38α-MAPK is not toxic to the survival of mice.
TABLE 3 Genotype distribution of newborns from the following two breeding pairs: (i) app<sup>wt</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> [male] and app<sup>wt</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> [female]; and (ii) app<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> [male] and app<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> [female]

| Genotype          | Male  | Female | Total |
|-------------------|-------|--------|-------|
| app<sup>wt</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> | 34 (33.8) | 35 (35.2) | 69 (69.0) |
| app<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> | 53 (54.4) | 58 (56.6) | 111 (111.0) |
| app<sup>wt</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> | 41 (39.7) | 40 (41.3) | 81 (81.0) |
| app<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> | 49 (49.0) | 51 (51.0) | 100 (100.0) |
| Total             | 177 (177.0) | 184 (184.0) | 361 (361.0) |

Note: Values in brackets indicate the expected counts from <sup>χ</sup><sub>2</sub> test. 

3.2 | Deletion of neuronal p38α-MAPK attenuates cognitive deficits and synaptic impairments in APP-transgenic mice

In order to test the pathogenic effects of neuronal p38α-MAPK on AD, we used the Morris water maze test to examine the cognitive function of 9-month-old APP<sup>wt</sup> and APP<sup>p</sup> littermate mice with and without deletion of p38α-MAPK in neurons. As shown in Figure 2A-C, the swimming time and distance to reach the platform for all tested mice significantly decreased when the training time increased (two-way ANOVA, <sup>P</sup> < .05); however, the swimming velocity did not differ between various groups of mice or for the same mice on different training dates (two-way ANOVA, <sup>P</sup> > .05).

We compared different genotypes of mice with respect to their behavior during the acquisition phase of the water maze test. APP<sup>wt</sup> littermate mice with or without deficiency of p38α-MAPK in neurons (APP<sup>wt</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> and APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup>) showed no significant differences in either swimming time or swimming distance before climbing onto the escape platform (Figure 2A,B; two-way ANOVA, <sup>P</sup> > .05). Compared to APP<sup>wt</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> or APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> littersmates, 9-month-old APP<sup>wt</sup> mice with wild-type p38α-MAPK expression in neurons (APP<sup>wt</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup>) spent significantly more time (Figure 2A; two-way ANOVA, <sup>P</sup> < .05) and traveled longer distances (Figure 2B; two-way ANOVA, <sup>P</sup> < .05) in the water maze before reaching the escape platform. Interestingly, APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> mice with the ablation of p38α-MAPK specifically in neurons performed significantly better than their APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> littersmates in searching for and finding the platform after 3 days of training (Figure 2A,B; two-way ANOVA followed by post hoc test showing APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> vs APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> mice: <sup>P</sup> < .05).

Twenty-four hours after the end of the training phase, the escape platform was removed and a 5-minute probe trial was performed to test the memory of the mice. We evaluated three parameters: duration of time the mouse needed to find the original platform region, total number of times the mouse crossed the original platform region, and the total time the mouse spent in the original platform region. Compared to APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> and APP<sup>wt</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> littersmates, APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> mice took significantly longer time for their first visit to the region where the platform had been located, crossed the platform region with significantly less frequency, and spent significantly less time in the platform region during the total 5-minute probe trial (Figure 2D-F; one-way ANOVA followed by post hoc test, <sup>P</sup> < .01). Interestingly, when compared to APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> mice, APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> mice were able to reach the original platform region in significantly less time and crossed the region more frequently (Figure 2D,E; one-way ANOVA followed by post hoc test, <sup>P</sup> = .03 and 0.05, respectively). We observed no significant differences between the two APP<sup>wt</sup> control groups (APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> and APP<sup>wt</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> littermate mice) in any of the three parameters analyzed in the probe trial (Figure 2D-F; one-way ANOVA followed by post hoc test, <sup>P</sup> > .05).

We also used Western blot analysis to quantify the levels of four synaptic-structure proteins: Munc18-1, synaptophysin, SNAP-25, and PSD-95 in the brain homogenate of 9-month-old APP<sup>wt</sup> and APP<sup>p</sup> littermate mice. As shown in Figure 2 (panels G, H and J), protein levels of Munc18-1 and synaptophysin in APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> mice were significantly lower than levels of these proteins derived from APP<sup>wt</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> and APP<sup>wt</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> mice (one-way ANOVA followed by post hoc test, <sup>P</sup> < .05). Interestingly, the reduction in Munc18-1 and synaptophysin proteins due to APP-transgenic expression was rescued by the deletion of p38α-MAPK in neurons (one-way ANOVA followed by post hoc test, <sup>P</sup> < .05). SNAP-25 protein levels were significantly higher in brains from APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> mice than in brains from APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> control mice (Figure 2G,I; one-way ANOVA followed by post hoc test, <sup>P</sup> < .05). Comparison of APP<sup>wt</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> and APP<sup>wt</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> littermate mice showed no significant differences in protein levels of these four tested synaptic proteins (Figure 2G-K; one-way ANOVA followed by post hoc test, <sup>P</sup> > .05).

To further clarify whether deletion of neuronal p38α-MAPK protected synaptic integrity in APP<sup>p</sup> mice, we evaluated the spine density and analyzed the morphology of spines of neurons in dendritic branches of apical dendrites in hippocampal CA1 and CA3 areas, and in the cortex layer II/III. Consistent with the Western blot results, Golgi staining revealed that the spine density of neurons in all three tested brain regions was significantly higher in APP<sup>p</sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> mice than in...
Moreover, dendritic spines were grouped into mature, immature and filopodia-like spines based on their morphologies. Deficiency of p38α-MAPK appeared to preserve more mature (mushroom-shaped) spines than the spines of other types of morphology (Figure S1C-G; t test, P < .05).

### 3.3 Deletion of neuronal p38α-MAPK reduces cerebral Aβ burden and neuroinflammation in APP-transgenic mice

After observing that deletion of p38α-MAPK improved the cognitive performance in APPtg mice but not in APPwt mice, we hypothesized that this effect on cognitive function may be linked to reductions in cerebral Aβ burden and neuroinflammation. APPp38αfl/flCre−/− littermates (Figure S1A,B; t test, P < .05). Moreover, dendritic spines were grouped into mature, immature and filopodia-like spines based on their morphologies. Deficiency of p38α-MAPK appeared to preserve more mature (mushroom-shaped) spines than the spines of other types of morphology (Figure S1C-G; t test, P < .05).
littermates, we analyzed Aβ pathology in the AD mice, as Aβ is the key molecule leading to neurodegeneration in AD. We used the stereological Cavalieri method to measure Aβ volume, adjusted relative to the volume of analyzed tissues, in 9-month-old APPtgp38αfl/flCre−/− and APPtgp38αfl/flCre+/− mice. The volume of immunoreactive Aβ load in the APPtgp38αfl/flCre−/− mice (1.364% ± 0.140% in the hippocampus and 1.344% ± 0.096% in the cortex) was significantly higher than in the APPtgp38αfl/flCre+/− mice (0.876% ± 0.086% in the hippocampus and 0.847% ± 0.079% in the cortex; Figure 3A,B; t test, P = .008 and 0.001, respectively), suggesting that deletion of p38α-MAPK in neurons reduces the cerebral Aβ burden.

Western blot analysis using human Aβ-specific antibody was performed to determine the levels of Aβ monomer, dimers and trimers and of C99 in the homogenate of cortex and hippocampus derived from 9-month-old APPtgp38αfl/flCre−/− and APPtgp38αfl/flCre−/− littermate mice. As shown in Figure 3C-E, deletion of p38α-MAPK in neurons reduced all four detected peptides by 35% when

**FIGURE 3** Deletion of p38α-MAPK in neurons reduces cerebral Aβ in APP-transgenic mice. Nine-month-old APP-transgenic (tg) littermate mice with (ko) and without (wt) deletion of neuronal p38α-MAPK were analyzed for cerebral Aβ load after immunohistochemical staining of human Aβ (A). The Aβ volume was estimated with Cavalieri method and adjusted by the relevant brain volume. Deletion of p38α-MAPK in neurons significantly reduced the cerebral Aβ volume (B; t test; n ≥ 10 per group). The cerebral Aβ in APPtgp38αfl/flCre−/− mice was also evaluated by detecting Aβ in the brain homogenate with quantitative Western blot (C). Both, normalization of Aβ against β-actin (D) and against APP (E), show reduced Aβ load after deletion of p38α-MAPK in neurons (C-E; t test; n ≥ 4 per group). The protein levels of APP were evaluated by quantitative Western blot and not different between APPtgp38αfl/flCre−/− and APPtgp38αfl/flCre+/− littermate mice (F and G; t test; n ≥ 8 per group). Deletion of p38α-MAPK in neurons significantly reduced BACE1 protein in the brain of 9-month-old APPtgp38αfl/flCre−/− mice (H and I; t test; n ≥ 5 per group)
measured with β-actin as an internal control, or by 25% with APP as an internal control (t test, P < .05). Moreover, we observed that APP proteins did not differ between APP<sup>p38α<sup>fl/fl</sup>Cre<sup>+/−</sup> and APP<sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> mice (F and G; t test, P > .05); and BACE1 protein was significantly reduced in APP<sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> mice (Figure 3H,I; t test P = .002). Thus, the marked reduction of C99 fragments and Aβ oligomers in the brain corroborated our recent findings that p38α-MAPK deficiency inhibits the protein level and the activity of BACE1 in neurons and decreases cerebral Aβ load.13

Microglial inflammatory activation as another important pathogenic mechanism in AD had been analyzed with our established protocols.30 We observed that deletion of p38α-MAPK significantly reduced the number of Iba1-positive cells (Figure S2A,B; t test, P < .05), downregulated the transcription of pro-inflammatory genes (inf-α, il-1β, and inos) and upregulated transcription of anti-inflammatory genes (il-10 and mrc1) in the brain of APP<sup>+</sup> mice but not in APP<sup>−/−</sup> mice (Figure S2C-J; one-way ANOVA followed by post hoc test, P < .05). Deletion of neuronal p38α-MAPK in APP<sup>+</sup> mice also led to significantly upregulated transcription of bdnf and igf-1 in the brain, both of which encode inflammation-related growth factors and promote neuronal protection and regeneration (Figure S2K,L; one-way ANOVA followed by post hoc test, P < .05). The transcription of other tested genes, including ccl-2, arg1, and chi3l3, in APP<sup>+</sup> mouse brains was not changed by neuronal deficiency of p38α-MAPK (Figure S2E,HJ; one-way ANOVA followed by post hoc test, P > .05).

3.4 Deletion of neuronal p38α-MAPK alters gene expression profile in APP-transgenic mice

After observing that deletion of neuronal p38α-MAPK improved cognitive function in APP<sup>+</sup> mice, we performed transcriptome analysis to identify potential mechanisms that mediate neuronal protection. Next-generation sequencing of mRNA isolated from cortex and hippocampus of APP<sup>p38α<sup>fl/fl</sup>Cre<sup>+/−</sup> and APP<sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> littermate mice resulted in 36 437 sequenced transcript identifications for 15 011 individual genes with different transcript variants. Among identified gene transcripts, 253 gene transcripts were altered by log2 fold change < −2 or >2 and with a FDR corrected P < .01. From these altered gene transcripts 107 were downregulated and 146 were upregulated in APP<sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> mice as compared with APP<sup>p38α<sup>fl/fl</sup>Cre<sup>−/−</sup> littermate controls (see Figure 4A,B). All significantly altered transcripts, also with P value of <.05, are shown by their gene name and with their corresponding log FC and FDR values in Table S1.

By applying the Kyoto Encyclopedia of Genes and Genomes pathway analysis tool of the Pathview annotation software, we performed pathway enrichment analysis of all 253 significantly altered gene transcripts. Pathways such as MAPK signaling, spliceosome formation and calcium signaling were found to be enriched for genes differentially regulated by neuronal deletion of p38α-MAPK. Heat maps showed the differences in transcript variants of gene expression (Figure 4C-E). As expected, the MAPK signaling pathway was altered in p38α-MAPK-deficient mouse brain. In this pathway, the gene transcription of mapk9, also known as C-Jun N-terminal kinase (jnk2) was strongly upregulated in p38α-MAPK-deficient mouse brain, which might indicate a compensatory mechanism. JNK2 acts in a parallel MAPK signaling cascade and shares some common substrates with p38α-MAPK. Interestingly, the transcription of genes associated with the spliceosome was significantly modified by deletion of neuronal p38α-MAPK, which might represent an important mechanism through which p38α-MAPK regulates gene transcription, as the spliceosome removes introns from a transcribed precursor messenger RNA (pre-mRNA). Indeed disturbance in spliceosome function with reduced maturation of pre-mRNA has been reported in Aβ-challenged neuronal cells.32 In addition to these genes that were included into the enrichment analysis (see Figure 4E), there were 11 calcium signaling-related genes (adarb1, ano4, dmb, map2, nfatc2, plch1, s100a5, sox5, tec, tfap2b, and tmem63c), whose transcription was also significantly changed by deletion of p38α-MAPK in neurons (see Table S1). Using the online database, PanglaoDB (https://panglao-db.se/), we analyzed the cellular origin of transcriptionally altered genes. We observed that these spliceosome pathway and calcium signaling-related genes whose transcription was changed by p38α-MAPK deficiency were enriched in neuronal populations instead of in microglia and other glial cells.43

To validate the results derived from sequencing experiments, we performed quantitative RT-PCR to measure transcripts of the following genes, which are involved in calcium signaling and/or synaptic plasticity: adcy3, adcy7, aptb4, gnas, grin2a, htr7, slc8a1, spik1, tmem63c, plch1, and nfatc2. As shown in Figure 5, the alteration of gene transcription screened by next-generation sequencing could be confirmed by PCR (t test, P < .05).

3.5 Deletion of neuronal p38α-MAPK differently regulates glutamate and NMDA-induced calcium influx and cell death in primary cultured neurons

After observing that p38α-MAPK deficiency altered the transcription of calcium homeostasis-related genes in brain tissues, we asked whether p38α-MAPK regulates
the calcium influx in neurons. We cultured p38α-deficient and wild-type cortical neurons from the breeding between APP\textsuperscript{p38α\textsuperscript{fl/fl}}Nex-Cre\textsuperscript{+/−} (or \textsc{APP\textsuperscript{wt}p38α\textsuperscript{fl/fl}}Nex-Cre\textsuperscript{+/−}) male mice and APP\textsuperscript{wt}p38α\textsuperscript{fl/fl}Nex-Cre\textsuperscript{−/−} female mice. We first validated the neuronal reduction of p38α-MAPK by quantitative Western blot. The p38α-MAPK-deficient

| FDR  | Total | Regulation of Transcripts |  |
|------|-------|----------------------------|---|
| <0.05 | 422   | 175, 247                  |   |
| <0.01 | 253   | 107, 146                  |   |

(A) Graph showing fold change log\textsubscript{2} < -2; >2 of regulated transcripts. (B) Scatter plot showing log\textsubscript{10} [FDR] for fold change log\textsubscript{2} values. (C) Heatmap showing MAPK Signaling genes akt1, daxx, gng12, map2k3, mapk9, mknk1, rps6ka6 in ko and wt conditions. (D) Heatmap showing Spliceosome genes acin1, ddx5, elf4a3, prpf38b, prpf40b, snrpa, thoc2 in ko and wt conditions. (E) Heatmap showing Calcium Signaling genes adcy3, adcy7, atp2b4, gnas, grina2a, htr7, slc8a1, sphk1 in ko and wt conditions.
neurons (APP<sup>tg</sup>p38<sup>fl/fl</sup>Nex-Cre<sup>−/−</sup>) showed a reduced protein level of p38-MAPK by nearly 80% compared to the wild-type neurons (APP<sup>tg</sup>p38<sup>fl/fl</sup>Nex-Cre<sup>−/−</sup>) (Figure 6A; *t* test, *P* = .026). Interestingly, we observed that the protein level of Grin2a, a subunit of NMDAR, was lower in p38α-MAPK-deficient neurons than in wild-type cells (Figure 6B; *t* test, *P* = .010).

Then, we examined effects of APP/PS1-overexpression on calcium influx. We challenged APP<sup>tg</sup>p38<sup>fl/fl</sup>Nex-Cre<sup>−/−</sup> and APP<sup>wt</sup>p38<sup>fl/fl</sup>Nex-Cre<sup>−/−</sup> neurons with l-glutamate (at 100 and 500 µM) or NMDA (at 50 µM and 10mM) followed by 75 mM KCl. The calcium influx was not different between these two groups of neurons (Figure S3A,B; *n* ≥ 3 animals per group). In the culture medium of APP/PS1-transgenic neurons, we could not detect Aβ with Western blot (data not shown). The concentration of Aβ in calcium imaging chamber should be even lower after cells were further perfused with Aβ-free imaging buffer. We supposed that Aβ at such a low level was not able to trigger calcium influx as previously reported. Moreover, overexpression of PS1 appeared not to alter glutamate receptors-mediated calcium influx in our cultured neurons, although mutant PS1 potentially regulates calcium release from endoplasmic reticulum and affects store-operated calcium channels. Thus, we used both APP-transgenic (APP<sup>tg</sup>) and wild-type (APP<sup>wt</sup>) neurons for the following experiments.
We treated APP^{p38α/−}Nex-Cre^{−/−} and APP^{p38α/−}Nex-Cre^{−/−} neurons with 75 mM KCl to induce membrane depolarization. Substantial calcium influx was clearly induced in both neurons and the responses were higher in p38α-MAPK-deficient neurons than in p38α-MAPK-wild-type cells (Figure 6C; two-way ANOVA, P < .001). When
cells were activated with 100 µM and 500 µM L-glutamate, deletion of p38α-MAPK significantly enhanced calcium influx as compared with p38α-MAPK-wild-type cells (Figure 6D; two-way ANOVA, P < .001). The calcium influx could be further induced by adding 75 mM KCl after glutamate treatments. Similarly, we treated neurons with NMDAR-specific agonist, NMDA. Interestingly, p38α-MAPK-deficient neurons showed little calcium influx (F/F0 from 0.990 to 1.159) even after application of 10 mM NMDA, whereas, p38α-MAPK-wild-type neurons demonstrated pronounced calcium influx after treatment with 50 µM NMDA (F/F0 from 1.00 to 1.286) (Figure 6E; two-way ANOVA, P < .001). After NMDA treatments, p38α-MAPK-deficient and wild-type cells were further stimulated with 75 mM KCl and displayed a comparable increase of calcium signals, which indicated that the mild elevation of calcium signal in p38α-MAPK-deficient neurons was not due to the neurotoxicity of NMDA.

We also treated APP<sup>gfp</sup>p38<sup>fli/fli</sup>Nex-Cre<sup>+/−</sup> and APP<sup>gfp</sup>p38<sup>fli/fli</sup>Nex-Cre<sup>−/−</sup> neurons with 100 µM L-glutamate. The peak level of calcium influx in p38α-MAPK-deficient neurons was significantly higher than in p38α-MAPK-wild-type neurons (Figure 6F; F/F0: 1.67 ± 0.04 vs 1.52 ± 0.04 in APP<sup>gfp</sup>p38<sup>fli/fli</sup>Nex-Cre<sup>+/−</sup> and APP<sup>gfp</sup>p38<sup>fli/fli</sup>Nex-Cre<sup>−/−</sup> neurons, respectively; t test, P = .37). Interestingly, when L-glutamate was washed out, the intensity of calcium-associated Fluor-4 fluorescence dropped significantly faster in p38α-MAPK-deficient neurons than in p38α-MAPK-wild-type neurons (Figure 6G; two-way ANOVA, P < .001). The decay time for calcium fluorescence was 32.00 ± 5.34 seconds vs 123.00 ± 26.47 seconds in APP<sup>gfp</sup>p38<sup>fli/fli</sup>Nex-Cre<sup>+/−</sup> and APP<sup>gfp</sup>p38<sup>fli/fli</sup>Nex-Cre<sup>−/−</sup> neurons, respectively (Figure 6G; t test, P = .013).

To investigate how p38α-MAPK deficiency enhanced calcium influx, we continued to co-treat APP<sup>gfp</sup>p38<sup>fli/fli</sup>Nex-Cre<sup>+/−</sup> and APP<sup>gfp</sup>p38<sup>fli/fli</sup>Nex-Cre<sup>−/−</sup> neurons from the wash-out experiment with 100 µM L-glutamate and a NMDAR antagonist, APV-2. Calcium flux was again induced although the intra-neuronal levels of calcium were lower than in cells treated with glutamate alone (Figure 6F). The peak level of calcium influx in p38α-MAPK-deficient neurons was still higher than that in p38α-MAPK-wild-type neurons (Figure 6F; F/F0: 1.34 ± 0.03 vs 1.09 ± 0.03 in APP<sup>gfp</sup>p38<sup>fli/fli</sup>Nex-Cre<sup>+/−</sup> and APP<sup>gfp</sup>p38<sup>fli/fli</sup>Nex-Cre<sup>−/−</sup> cells, respectively; t test, P = .001). When p38α-MAPK deficiency-associated enhancement of calcium influx was compared in neurons that were challenged with L-glutamate in presence and absence of APV-2, we observed that blocking NMDAR potentially amplified the effect of p38α-MAPK deficiency on calcium influx. When peak levels of calcium influx in p38α-MAPK-wild-type neurons were set at 100% in both experimental settings, p38α-MAPK deletion elevated maximal calcium influx to 122.93 ± 3.11% in the presence of APV-2 and to 109.99 ± 2.61% in the absence of APV-2 (Figure 6F; H; t test, P = .019).

In the end, we analyzed the neurotoxic effects of calcium influx upon challenges of L-glutamate and NMDA. Deletion of p38α-MAPK significantly inhibited activation of caspase-3 in cultured neurons after treatments with both L-glutamate at 100 µM (Figure 7A) and NMDA at 50 µM (Figure 7B) (t test between p38α-MAPK deficient and wild-type neurons, P = .032 for L-glutamate and 0.039 for NMDA).

3.6 Deletion of p38α-MAPK in neurons attenuates cognitive deficits and synaptic impairments in Tau-transgenic mice

APP<sup>gfp</sup> mice cannot model all pathological changes of AD, such as those associated with p-Tau. To investigate the effects of neuronal p38α-MAPK on the Tau-associated pathological changes in AD, we cross-bred p38α<sup>fli/fli</sup> mice,<sup>25</sup> Nex-Cre mice,<sup>26</sup> and P301S Tau-transgenic (Taul<sup>gfp</sup>) mice<sup>27</sup> to create neuronal p38α-MAPK-deficient Tau<sup>gfp</sup> AD mice. The lower level of p38α-MAPK protein in the brain homogenate derived from Tau<sup>gfp</sup>p38α<sup>fli/fli</sup>Nex-Cre<sup>+/−</sup> mice compared to Tau<sup>gfp</sup>p38α<sup>fli/fli</sup>Nex-Cre<sup>−/−</sup> mice confirmed the deletion of p38α-MAPK (Figure 8A; t test, P < .001). We also measured p38-MAPK activities in brains of 9-month-old Tau<sup>gfp</sup>p38α<sup>fli/fli</sup>Nex-Cre<sup>+/−</sup> and Tau<sup>gfp</sup>p38α<sup>fli/fli</sup>Nex-Cre<sup>−/−</sup> mice,
FIGURE 7 Deletion of p38α-MAPK attenuates both l-glutamate and NMDA-induced apoptosis in primary neurons. Primary neurons were cultured from APP-transgenic embryos with (ko) and without (wt) deletion of p38α-MAPK in neurons. Western blot detected significant reduction of cleaved caspase-3 (Casp-3) in p38α-MAPK-knockout cells compared to p38α-MAPK-wt controls after treatments with 100 μM l-glutamate (Glu) (A; t test; n ≥ 4 per group) and 50 μM NMDA (B; t test; n ≥ 4 per group) for 16 h.

and their non-Tau-transgenic (Tauwt) littermate controls (Tautwtp38αfl/flCre+/− and Tautwtp38αfl/flCre−/−). The phosphorylation of p38-MAPK was increased in the brain of Tautg mice compared to Tauwt mice. Deletion of p38α-MAPK inhibited the phosphorylation of p38α-MAPK in Tautg mouse brain but not in Tauwt brain (Figure 8C,D; one-way ANOVA, P < .05).

We have recently observed that the cognitive function of Tauwt mice is impaired in the Morris water maze test.28 Compared to Tautwtp38αfl/flCre+/− litters, 9-month-old Tautwtp38αfl/flCre+/− mice traveled in significantly less time and over less distance to reach the escaping platform in the training phase, but the swimming velocity did not differ between these two groups of mice (Figure 8E-G; two-way ANOVA, P < .05). Similarly, in the probe trial, Tautwtp38αfl/flCre+/− mice found the original region for platform within a shorter time, crossed the target position more frequently and explored in the target region for longer duration than Tautwtp38αfl/flCre−/− litters (Figure 8H-J; t test, P < .05).

Levels of the synaptic structure proteins Munc18-1, PSD-95, and synaptophysin are reduced in the brains of Tautg mice compared to wild-type (Tauwt) controls.28 Interestingly, the protein levels of Munc18-1 and synaptophysin in brain homogenates of 9-month-old Tautwtp38αfl/flCre+/− mice were significantly elevated by the deletion of p38α-MAPK in neurons compared to Tautwtp38αfl/flCre−/− mice (Figure 8K,M; t test, P < .05). Deletion of neuronal p38α-MAPK in Tautg mice tended to restore the cerebral protein level of PSD-95, but the change was not significant (Figure 8N; t test, P = .05).

3.7 | Deletion of p38α-MAPK in neurons reduces cerebral p-Tau protein in Tau-transgenic mice

As p-Tau mediates toxic effects of Aβ in AD pathogenesis,46,47 we investigated effects of neuronal deficiency of p38α-MAPK on cerebral p-Tau levels. We counted AT8-positive cells in cortex and hippocampus of 9-month-old Tauwtp38αfl/flCre+/− and Tauwtp38αfl/flCre−/− mice. The total number of AT8-immunoreactive cells adjusted to the investigated area in Tautwtp38αfl/flCre−/− mice (16.51 ± 1.94/mm² in cortex and 35.40 ± 4.75/mm² in hippocampus) was significantly higher than that in Tautwtp38αfl/flCre+/− mice (7.50 ± 2.53/mm² in cortex, and 18.71 ± 4.34/mm² in hippocampus; Figure 9A,B; t test for Tautwtp38αfl/flCre+/− vs Tautwtp38αfl/flCre−/− mice, P = .013 and 0.021, respectively). We also stained brain tissues with thioflavine S to identify neurofibrillary tangles using a published method.31 We observed very few thioflavine-staining-positive cells in the whole brain (Figure 9C). However, deletion of p38α-MAPK did reduce the number of staining-positive cells in both cortex and hippocampus (Figure 9D; t test, P = .039 and 0.039, respectively).

We extracted Tau proteins from 9-month-old Tauwtp38αfl/flCre+/− and Tauwtp38αfl/flCre−/− mice with RAB, RIPA, and FA buffers. Western blots revealed that both the protein levels of p-Tau seen with direct densitometry and ratios of p-Tau/t-Tau were significantly lower in RIPA and FA fractions derived from Tauwtp38αfl/flCre+/− mice, compared to Tauwtp38αfl/flCre−/− mice (Figure 9F,G; t test, P < .05). In the RAB fraction, deletion of neuronal p38α-MAPK tended to decrease the cerebral p-Tau level in Tauwt mice, although the difference was not significant (Figure 9E; t test, P > .05). In all three fractions of brain homogenate, Tauwtp38αfl/flCre+/− and Tauwtp38αfl/flCre−/− litters did not differ in t-Tau protein levels (data not shown).

We have recently observed that activated autophagy is able to degrade p-Tau in the tau-transgenic mouse brain.28 We further detected autophagy markers, LC3B-I and LC3B-II, in RIPA-soluble brain homogenate. As shown in Figure 9H, the ratio of LC3B-II/I was significantly higher in Tautwtp38αfl/flCre+/− mouse brain than that in Tautwtp38αfl/flCre−/− mice (t test, P < .05).

4 | DISCUSSION

Aβ and p-Tau are two major pathogenic molecules in AD. In our study, we demonstrated that deletion of neuronal p38α-MAPK improves cognitive function of both 9-month-old APP and tau-transgenic AD mice, which is associated with reduced Aβ and p-Tau load, and shifting from pro- to anti-inflammatory activation in the brain. We
FIGURE 8 Deletion of p38α-MAPK in neurons improves cognitive function and attenuates synaptic impairments in Tau-transgenic mice. The deletion of p38α-MAPK in Tau-transgenic (tg) mice was confirmed by detecting p38-MAPK in the brain homogenate with quantitative Western blot (A and B; t test; n ≥ 8 per group). The homogenates of brain derived from 9-month-old Tau⁹ and non-Tau-transgenic (wt) littermate mice with (ko) without (wt) deletion of p38α-MAPK were analyzed for phosphorylated (p-p38) and total (t-p38) p38-MAPK with Western blot. In this experiment, the loading protein for each lane was adapted to have similar amount of t-p38. The protein level of p-p38 in Tau-tg mice with normal expression of p38α-MAPK was higher than in Tau-wt littermates. Deletion of p38α-MAPK reduced phosphorylation of p38-MAPK in Tau-tg mice but not in Tau-wt mice (C and D, one-way ANOVA followed by Bonferroni post hoc test, n ≥ 4 per group). Morris water maze was performed to evaluate the cognitive function of 9-month-old Tau⁹ mice with and without deletion of p38α-MAPK in neurons. During the training phase, p38α-MAPK wt mice spent significantly more time and traveled longer distances to reach the hidden platform than did their p38α-MAPK ko littermates. The swimming speed was not different between both genotypes (E-G; two-way ANOVA showing the effect of genotype; n ≥ 8 per group). In the probe trial, Tau-tg p38α-MAPK ko mice spent significantly shorter time in the first visit of the region where the platform was previously located, crossed the platform region with significantly more frequency and spent significantly longer time in the platform region during the total 5-minute experiment than their Tau-tg p38α-MAPK wt littermates (H-J; t test; n ≥ 8 per group). After water maze, the brain was collected for further biochemical analysis. The amount of synaptic structure proteins, such as Munc18-1, SNAP25, synaptophysin (SYN), and PSD95, was quantified with Western blot (K-N). Neuronal deficiency of p38α-MAPK was associated with a higher level of Munc18-1, synaptophysin and PSD95 in Tau⁹ mice (K, M and N; t test; n ≥ 5 per group).
Deletion of p38α-MAPK in neurons reduces p-Tau load in Tau-transgenic mice. Nine-month-old Tau-transgenic (tg) littermate mice with (ko) and without (wt) deletion of neuronal p38α-MAPK were analyzed for cerebral p-Tau load after immunofluorescent labeling with AT8 antibody (A) as well as for neurofibrillary tangles with thioflavine S (ThioS) staining (C). The p-Tau- or ThioS staining-positive cells were counted and adjusted by the relevant brain area. Deletion of p38α-MAPK in neurons significantly reduced the cerebral p-Tau-positive (B; t test; n = 8 per group) and ThioS staining-positive cells (D; t test; n ≥ 5 per group). Tau proteins were extracted from 9-month-old Tau tg mice with RAB, RIPA, and FA buffers and detected with Western blots for both phosphorylated and total Tau (p-Tau and t-Tau, respectively) (E-G). The protein levels of p-Tau are presented as both density (in arbitrary unit [AU]) and the ratio of p-/t-Tau. Deficiency of p38α-MAPK in neurons significantly reduces p-Tau proteins in RIPA and FA fractions, but not in RAB fraction (F and G; t test; n ≥ 4 per group). The RIPA fraction was further used to detect the autophagy marker LC3B-I and LC3B-II. Deletion of p38α-MAPK increased the ratio of LC3B-II/I (H; t test; n ≥ 6 per group).
further showed that p38α-MAPK regulates calcium homeostasis. Especially the NMDA receptor-mediated calcium influx in neurons was affected, which might regulate neuronal plasticity and death in AD and in other neurodegenerative diseases.

Our previous study has shown that inhibition of p38α-MAPK enhances autophagy in neurons, which promotes lysosomal degradation of BACE1. As APP and C-terminal fragment of APP (C99) are also substrates of autophagy, it is likely that p38α-MAPK inhibition reduces Aβ generation. Although we have not investigated p38α-MAPK-deficient microglia, other studies have shown that inhibition of p38α-MAPK potentially activates autophagy in macrophages. Since autophagy both promotes the recycling of Aβ-phagocytosing receptors CD36 and TREM2 and facilitates Aβ degradation in microglia, perhaps treatment with p38α-MAPK inhibitor has the potential to degrade Aβ in AD patients. We observed that deletion of neuronal p38α-MAPK inhibits pro-inflammatory, but enhances anti-inflammatory activation in the brain of APP-transgenic mice. The inflammatory changes are likely secondary to the cerebral Aβ reduction; however, provide a further protection for neurons. Moreover, autophagy is an efficient mechanism to degrade p-Tau in the brain, inhibition of p38α-MAPK not only inhibits Tau phosphorylation but should also increase p-Tau clearance in the AD brain. In Tau-transgenic mouse brain, we for the first time showed that deletion of p38α-MAPK in neurons attenuates p-Tau protein. It was correlated with enhancement of autophagy. However, as t-Tau protein levels in the brain are not affected by neuronal p38α-MAPK deficiency, the decrease of cerebral p-Tau is more likely due to the reduced phosphorylation of Tau proteins.

Furthermore, our study shows that phosphorylation of p38α-MAPK is only increased in the brain but not in heart, liver and muscles in APP-transgenic mice, which corroborates the observation that p38α-MAPK is locally activated at AD lesion sites in human brains. Our careful evaluation indicates that deficiency of neuronal p38α-MAPK does not affect the survival and cognitive function of APP-wild-type mice. Thus, inhibition of p38α-MAPK might serve preventive and therapeutic effects against AD progress by targeting several key pathogenic mechanisms and limit its potential off-target actions.

Apart from its effects on autophagy, we observed that p38α-MAPK regulates transcription of many calcium homeostasis-related genes in APP-transgenic mouse brain. Especially transcription and protein levels of NMDAR subunit NR2A (encoded by grl2a gene) are both downregulated in p38α-deficient mouse brains. NMDAR mediates effects of the primary excitatory neurotransmitter glutamate. Under physiological condition, activation of NMDAR is essential for neuronal plasticity or long-term potentiation (LTP); however, under pathological conditions, such as in AD, NMDAR might increase calcium influx, which leads to excitotoxicity. Aβ oligomers were reported to directly activate NMDARs, particularly NR2A-contained receptors. Aβ oligomers also block re-uptake of released glutamate from astrocytes and neurons, which leads to perisynaptic glutamate accumulation. In APP-transgenic mouse brain, the level of resting calcium increases in neurites surrounding Aβ deposits. Aβ oligomers-induced calcium overload in dendritic spines can be prevented by blocking NMDAR. Memantine, as a NMDAR antagonist, has been proved to improve cognitive function and daily living of AD patients. In our cultured neurons, deletion of p38α-MAPK suppresses NMDA-induced calcium influx and neuronal apoptosis, which supports that inhibition of p38α-MAPK protects neurons against calcium overload-induced neurotoxicity in AD.

Deletion of p38α-MAPK also controls the neuronal excitotoxicity by regulating calcium buffering. In this study, we observed that p38α-MAPK deficiency increased the gene expression of sodium/calcium exchanger 1 (Slc8a1) in APP mouse brain. Slc8a1 is located at plasma membranes, mitochondria, and endoplasmic reticulum (ER) of excitable cells, pumping cytoplasmic calcium out of cells or into calcium storage organelles. Moreover, we detected reduced gene expression of sigma non-opioid intracellular receptor 1 (Sigmar1) in p38α-MAPK-deficient mice. Sigmar1 resides at mitochondria-associated ER membrane, inhibiting store-operated calcium entry. Inhibition of Sigmar1 was reported to attenuate cognitive deficits in an AD mouse model with intraventricle Aβ injection. In our cultured neurons, deletion of p38α-MAPK enhances both glutamate-triggered calcium influx and the clearance of cytoplasmic calcium after glutamate removal. The rapid calcium clearance might compensate for the neurotoxic effects of elevated calcium level in p38α-MAPK-deficient neurons. Thus, inhibition of p38α-MAPK potentially protects neurons in AD brain by enhancing intracellular clearance of calcium, although the underlying mechanisms need to be further identified.

It should be noted that deletion of p38α-MAPK increased i-glutamate-induced calcium influx, but decreased cytoplasmic calcium when cells were activated by NMDA. The increase of glutamate-induced calcium influx in p38α-MAPK-deficient neurons is obviously mediated by non-NMDARs glutamate receptors, such as AMPA receptor, kainate receptors, and metabotropic-type glutamate receptors (mGluRs). In group 1 mGluRs activation-induced long-term depression (LTD), p38-MAPK activation initiates internalization of AMPA receptors at synapses. Thus, p38α-MAPK deficiency has the potential to preserve more AMPA receptors on the cell surface to facilitate the calcium entry. It is important to evaluate this possibility not only in cultured neurons but also in AD brain, as AMPA receptor-mediated calcium influx leads to synaptic dysfunction and neurodegeneration.

Excitatory synapses contain AMPA and NMDA receptors, and mGluRs on dendritic spines. AMPA receptors
mediate most neuronal basal transmission. Aβ treatments share the mechanisms with LTD to drive the endocytosis of synaptic AMPA receptors, which results in synaptic depression and dendritic spine loss.\(^8\) Inhibition of neuronal p38α-MAPK has the potential to inhibit Aβ-induced LTD by blocking internalization of AMPA receptors.\(^6\) Soluble Aβ also impairs LTP by increasing the activity of extrasyaptic NMDARs. Inhibition of p38-MAPK has been observed to abolish Aβ-caused inhibition of LTP in hippocampal slices.\(^17\) We did not analyze electrophysiological activity of neurons in our AD mice; however, we observed that deficiency of neuronal p38α-MAPK improves the cognitive function of APP-transgenic mice and prevents the loss of synaptic proteins. The preservation of maturated mushroom spines that are enriched with AMPA receptors\(^29\) further suggests protective effects of p38α-MAPK deficiency on AMPA-mediated neuronal transmission in APP-transgenic mice. Moreover, our transcriptome analysis shows that the transcription of gliA, which encodes AMPA receptor subunit 1, is upregulated in the brain of neuronal p38α-MAPK-deficient APP-transgenic mice (see Table S1). Thus, inhibition of neuronal p38-MAPK potentially improves synaptic plasticity in AD brain.

Our study shows that p38α-MAPK deficiency protects neuron in AD pathogenesis. However, physiological functions of p38-MAPK still need to be noted; for example, p38α-MAPK is essential in erythropoiesis.\(^64\) In our AD mice, neuronal p38α-MAPK was deleted from the birth. Our results indicate more preventive than therapeutic effects of p38α-MAPK inhibition in AD.

In the last decades, many clinical interventions, especially Aβ immunization,\(^65\) have been attempted to reduce cerebral Aβ. Unfortunately, none of them resulted in efficacious therapies for AD patients. Our study demonstrated that deficiency of neuronal p38α-MAPK ameliorates AD-associated brain pathology in both APP and Tau-transgenic animal models. As potential mechanisms, inhibition of neuronal p38α-MAPK not only reduces generation of Aβ and p-Tau, but also protects neurons by preventing calcium overload. Our studies support p38α-MAPK as a novel target for AD therapy, although further studies are required.

ACKNOWLEDGMENTS

We thank Dr M. Jucker (Hertie Institute for Clinical Brain Research, Tübingen) for providing APP-transgenic mice; and Dr K. Nave (Max-Planck-Institute for Medicine, Göttingen) for Nex-Cre mice. The floxed-p38α-MAPK (p38αf/f) mice were kindly provided by Dr K. Otsu (Osaka University) through the RIKEN Bioresource Center. We appreciate Mirjam Göttel for her excellent technical assistance. This work was supported by ‘Deutsche Forschungsgemeinschaft (LI1725/2-1; to YL), HOMFOSRExcell program (to LS), and German Federal Ministry of Research and Education grant for de.NBI (031L0101D; to KN).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

L. Schnöder and Y. Liu designed research; L. Schnöder, G. Gasparoni, A. Schottek, I. Tomic, and A. Christmann performed experiments; L. Schnöder, G. Gasparoni, and K. Nordström analyzed data; L. Schnöder and Y. Liu wrote the paper; K. Fassbender, J. Walter, and K.H. Schäfer contributed analytic tools; M.D. Menger provided animal facility. Y. Liu coordinated the whole study. All authors read and approved the final manuscript.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Schnöder L, Gasparoni G, Nordström K, et al. Neuronal deficiency of p38α-MAPK ameliorates symptoms and pathology of APP or Tau-transgenic Alzheimer’s mouse models. The FASEB Journal. 2020;34:9628–9649. https://doi.org/10.1096/fj.201902731RR