Necroptosis activation in Alzheimer’s disease

Antonella Caccamo¹,⁷, Caterina Branca¹,⁷, Ignazio S Piras², Eric Ferreira¹, Matthew J Huentelman², Winnie S Liang², Ben Readhead³, Joel T Dudley³, Elizabeth E Spangenberg⁴, Kim N Green⁴, Ramona Belfiore¹,⁵, Wendy Winslow¹ & Salvatore Oddo¹,⁶✉

Alzheimer’s disease (AD) is characterized by severe neuronal loss; however, the mechanisms by which neurons die remain elusive. Necroptosis, a programmed form of necrosis, is executed by the mixed lineage kinase domain-like (MLKL) protein, which is triggered by receptor-interactive protein kinases (RIPK) 1 and 3. We found that necroptosis was activated in postmortem human AD brains, positively correlated with Braak stage, and inversely correlated with brain weight and cognitive scores. In addition, we found that the set of genes regulated by RIPK1 overlapped significantly with multiple independent AD transcriptomic signatures, indicating that RIPK1 activity could explain a substantial portion of transcriptomic changes in AD. Furthermore, we observed that lowering necroptosis activation reduced cell loss in a mouse model of AD. We anticipate that our findings will spur a new area of research in the AD field focused on developing new therapeutic strategies aimed at blocking its activation.

RESULTS
Necroptosis is activated in human AD brains
To determine whether necroptosis was activated in sporadic AD brains, we measured RIPK1, RIPK3 and MLKL in the temporal gyrus of AD (n = 12) and control (CTL) cases (n = 11) using buffers of increasing stringency (see Supplementary Table 1 for case information). RIPK1 was not detectable in the Tris-buffered saline (TBS) fraction and we found no statistically significant difference (P > 0.05) between CTL and AD brains in the Triton X-100 fraction (Supplementary Fig. 1a,b). Similarly, RIPK3 and MLKL levels in the TBS and Triton X-100 fractions were not changed between AD and CTL cases (Supplementary Fig. 1a,c,d). In contrast, in RIPA and urea fractions, RIPK1 and MLKL levels were significantly higher in AD than in CTL cases (Fig. 1a–c), whereas RIPK3 was not detectable (full blots are shown in Supplementary Fig. 2a,b). These observations are consistent with previous reports showing that necroptotic markers, once activated, form insoluble amyloid-like structures. The changes in solubility of the necrosome have also been confirmed in the cortex of people with multiple sclerosis.

1Arizona State University-Banner Neurodegenerative Disease Research Center at the Biodesign Institute, Arizona State University, Tempe, Arizona, USA. 2Translational Genomics Research Institute, Phoenix, Arizona, USA. 3Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, New York, USA. 4Department of Neurobiology and Behavior, University of California, Irvine, California, USA. 5Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy. 6School of Life Sciences, Arizona State University, Tempe, Arizona, USA. 7These authors contributed equally to this work. Correspondence should be addressed to S.O. (oddo@asu.edu).

Received 5 September 2016; accepted 21 June 2017; published online 24 July 2017; doi:10.1038/nn.4608
Necroptosis is executed by phosphorylated MLKL at Thr357/Ser358 (pMLKL). Once phosphorylated, MLKL aggregates to form homodimers, which induce membrane damage that leads to cell loss. Using confocal imaging, we found a significant increase in pMLKL levels in human AD brains compared with CTL brains (Fig. 2h,i). The number of MLKL dimers was also significantly increased in AD brains (Fig. 2j,k; full blots are shown in Supplementary Fig. 2d). Furthermore, we assessed the amount of pMLKL in AD brains that colocalized with the membrane marker cadherin and found that 42.31 ± 2.0% of pMLKL immunoreactivity was localized to the membrane (Fig. 2l). Statistical evaluation showed that the Pearson’s correlation coefficient was R = 0.630 with a Bonferroni correction P value of 0.980, which indicates that there was a 98% likelihood that the colocalization was not random. We confirmed the increase in pMLKL in AD brains and its colocalization with cadherin using a second, different pMLKL antibody (Supplementary Fig. 3). To determine whether neurons undergoing necroptosis also show markers of apoptosis, we double-stained sections with pMLKL and a caspase-3 antibody. We found that the probability of finding colocalization between pMLKL and caspase-3 was not statistically significant between AD and CTL cases (P > 0.05; Supplementary Fig. 4a,b). These data provide, to the best of our knowledge, the first direct evidence of necrosome formation in human AD brains. This is notable, as necrosome formation is sufficient and necessary for necroptosis activation and thus cell death.

To determine the cell type in which pMLKL is most active, we labeled human AD brains with pMLKL and neuronal, astrocytic or microglia markers. We found that 60.22 ± 3.3% of pMLKL immunoreactivity colocalized with NeuN, 11.14 ± 1.4% colocalized with GFAP and 28.00 ± 2.6% colocalized with Iba1 (Supplementary Fig. 5a–c). These data suggest that most of the pMLKL is found in neurons and microglia.

Necroptosis activation negatively correlates with brain weight and MMSE

We leveraged a genome-wide mRNA screening between AD (n = 97) and CTL cases (n = 98) using microarray technology (see Supplementary Tables 1 and 2 for cases information and variables distributions, and Supplementary Data Set 1). After filtering out low-expression probes, we obtained a final data set of 26,583 transcripts. We detected one probe mapping to RIPK1 (NM_003804), one probe mapping to RIPK3 (NM_006871) and three probes mapping to MLKL, corresponding to two different transcript variants (NM_152649 and XM_001126647). Although RIPK3 mRNA levels were not significantly different between the two groups (P > 0.05), RIPK1 and MLKL mRNA levels were significantly higher in AD brains than in CTL brains (Fig. 3a–c and Supplementary Table 3). Among the probes mapping to MLKL, we focused on the transcript NM_152649, as it showed a higher average expression and a bigger change in expression levels than XM_001126647. We validated these RNA-expression analyses using a publicly available, independent data set (GSE5281; n = 12 AD cases and n = 6 CTL cases) generated from laser-captured neurons from medial temporal gyrus. We found that RIPK1 and MLKL mRNA levels were increased in AD cases compared with controls (Supplementary Table 3). These changes were statistically significant.
Figure 2 Necrosome formation in AD. (a–f) Microphotographs and quantitative analyses of brain sections from AD and CTL patients immunostained with the indicated antibodies. Quantitative analysis revealed that the number of colocalized pixels was significantly higher in AD patients than in CTL patients (b: t(28) = 2.870, P = 0.003; d: t(28) = 2.659, P = 0.013; f: t(28) = 2.437, P = 0.022). (g) Proteins extracted from AD and CTL brains were immunoprecipitated with an antibody against RIPK1 and probed for MLKL. The IgG band is shown as a control. Full blots are shown in Supplementary Figure 2. (h) Microphotographs of brain sections immunostained with a pMLKL specific antibody. (i) Representative western blot probe with a MLKL antibody. The two bands represent MLKL monomers and dimers. Full blots are shown in Supplementary Figure 2. (k) Quantitative analyses of the MLKL blots were obtained by normalizing the intensity value of the dimers over the intensity value of the monomers (t(22) = 2.322, P = 0.030). (l) Microphotographs of brain sections from AD and CTL patients colabeled with the indicated antibodies. Statistical evaluation by Mander’s correlation, followed by Costes randomization test revealed that, in CTL cases, 36.47 ± 1.4% of pMLKL immunoreactivity was in the membrane (R = 0.3288 and Costes P = 0.96). In AD cases, 42.31 ± 1.5% of pMLKL immunoreactivity was in the membrane (R = 0.360 and Costes P = 0.98). (m) Quantitative analyses of the pMLKL/cadherin colocalization showed that the number of colocalized pixels was significantly higher in AD cases compared with CTL cases (t(28) = 4.504, P = 0.0001). Data in b, d, f, g, i, k and m are presented as box plots and were analyzed by unpaired t test. In the box plots, the center line represents the median value, the limits represent the 25th and 75th percentile, and the whiskers represent the minimum and maximum value of the distribution. n = 15 cases for CTL and n = 15 cases for AD for a–f, l; n = 11 cases for CTL and n = 13 cases for AD for h–k; n = 7 cases for CTL and n = 7 cases for AD for g.*P < 0.05.

at a genome-wide level (correction for 20,514 tests; adjusted P = 0.023 and 0.005 for RIPK1 and MLKL, respectively). In contrast, RIPK3 mRNA levels were similar between the two groups (Supplementary Table 3). These data confirm that necroptotic markers are upregulated in postmortem human AD cases, and are consistent with the data shown in Supplementary Figure 5a, indicating that most of the pMLKL in AD colocalizes with neurons.

To probe for a linear relationship between gene expression and brain weight, we used a Pearson’s correlation coefficient. Brain weight did not correlate with RIPK3 and MLKL mRNA levels (RIPK3, CTL: R(95) = 0.188, P = 0.066; AD: R(91) = 0.002, P = 0.986; MLKL, CTL: R(95) = −0.060, P = 0.561; AD, R(91) = −0.095, P = 0.362; n = 93 AD cases and 97 CTL cases). However, we found a significant negative correlation between brain weight and RIPK1 expression in AD (Fig. 3d), but not in CTL cases (R(95) = 0.049, P = 0.636). The correlation in AD was still significant (P = 0.046), with a coefficient of β = −123.93, when we corrected for height and gender (Supplementary Table 3).
The Mini-Mental State Examination (MMSE) is a cognitive test that is routinely used to assess cognitive function. To determine the relation between MMSE and necroptosis, we used a quintile regression given the bimodal distribution of the MMSE scores. We ran ten different models ranging from the 10th to the 100th percentiles for both CTL and AD. In CTL cases, RIPK1, RIPK3 and MLKL expression did not correlate with the MMSE (Supplementary Table 4). In AD cases, for RIPK1, we obtained a negative significant regression (adjusted $P < 0.05$) for percentiles ranging from 30th (MMSE = 5) to 40th (MMSE = 7). In contrast, RIPK3 did not correlate with the MMSE. For MLKL, we obtained a negative significant regression (adjusted $P < 0.05$) in AD patients for percentiles ranging from the 30th (MMSE = 5) to 50th (MMSE = 12; Fig. 4f–h and Supplementary Table 4). When we evaluated the RIPK1:MLKL interaction with MMSE, the regression was significant for a wider range of percentiles (20th to 50th percent; Fig. 4i and Supplementary Table 4), which suggests that RIPK1 and MLKL mRNA levels covariate in the same sample in relation to the MMSE.

RIPK1 levels predicts gene expression dysregulations in AD

We generated a causal gene regulatory network to model RIPK1 interactions in AD-relevant tissue (Supplementary Fig. 6). The network was inferred from DNA and transcriptomic data generated from post-mortem samples across two brain regions, which we used to build RIPK1 networks in the anterior prefrontal cortex (APFC, Brodmann area 10, total individuals: n = 174; CTL, n = 63; definite AD, n = 68; likely AD, n = 20; possible AD, n = 23) and the entorhinal cortex (ERC, Brodmann area 36, total individuals: n = 80; CTL, n = 33; definite AD, n = 28; likely AD, n = 8; possible AD, n = 11), as represented in the Mount Sinai NIH Brain and Tissue Repository. This network inference approach leverages paired genetic and transcriptomic data, using cis-eQTL markers linked to RIPK1 as a causal anchor to resolve directed interactions between RIPK1 and its correlated genes via a causal inference test. This method of inference captures multi-order regulatory relationships that include direct interactions between RIPK1 expression and its neighbors, as well as indirect effects that may be mediated through additional genes. Across both regions, we identified 819 genes whose expression covariate with RIPK1 expression (APFC: 230 genes, 134 positively correlated, 127 negatively correlated; ERC: 598 genes, 183 positively correlated, 423 negatively correlated). These genes significantly overlapped with multiple, independent AD gene expression profiles, comprising non-demented AD (which are characterized by moderate neuropathology, without cognitive impairment) and clinical AD, across multiple brain regions (Fig. 5, Supplementary Table 5, and Supplementary Data Sets 2 and 3). There was uniform consistency between the signed relationship linking RIPK1 expression with its downstream neighbors, and the direction of differential expression in the clinical AD profiles; specifically, genes negatively regulated by RIPK1 overlapped with genes downregulated in AD. This large, concordant overlap between genes regulated by RIPK1 and genes differentially expressed across multiple AD severity and regional contexts suggests that RIPK1 activity could explain a substantial portion of transcriptomic changes in AD. We also found that RIPK1 regulated multiple genes linked with risk-associated variants for AD and other brain diseases (Fig. 5, Supplementary Table 5, and Supplementary Data Sets 2 and 3).

To identify possible mechanisms underlying necroptosis activation in AD, we compared the set of genes regulated by RIPK1 with a set of genes associated with Z-VAD-FMK-induced necroptosis in L929 cells. There was no significant overlap ($P > 0.05$) in either brain region (Supplementary Data Set 3). In contrast, we found a strong

---

To determine the relationship between RIPK1, RIPK3 and MLKL mRNA levels and AD neuropathology, we performed an ordinal logistic regression with plaque density and Braak stage, which evaluates the amount of Aβ plaques and neurofibrillary tangles, respectively.

None of the three necroptotic marker levels were predictive of the amyloid load (Supplementary Table 3). In contrast, the increase of RIPK1 and MLKL mRNA levels, but not RIPK3, was significantly and positively correlated with the Braak stage ($P < 0.05$; Fig. 4a–c and Supplementary Table 3). This significance increased when we evaluated the effect of the interaction of RIPK1:MLKL ($P = 0.007$; Supplementary Table 3), which suggests that both markers covariate in the same samples together with the Braak stage. We also found that, in CTL cases, there was a significant negative correlation between RIPK3 levels and Braak stage ($P < 0.001$; Supplementary Table 3). To further investigate the link between necroptosis and tau, we double-stained sections from AD cases with the tau antibody AT8 and RIPK1 or pMLKL antibodies. We found that 55.48 ± 2.8% and 46.85 ± 4.0% of AT8 immunoreactivity colocalized with RIPK1 and pMLKL, respectively (Fig. 4d,e). We confirmed this colocalization using CP13, which recognizes tau phosphorylated at Ser202, and an independent pMLKL antibody (Supplementary Fig. 3). These data indicate a link between necroptosis and tau pathobiology but not between necroptosis and Aβ pathology, and are consistent with evidence indicating that tau is more proximal than Aβ to neuronal loss in AD.

---

**Figure 3** Necroptosis activation is linked to reduced brain weight. (a–c) Boxplot of log2 expression values in AD and CTL patients for genes RIPK1, RIPK3 and MLKL, respectively. The data indicate that RIPK1 and MLKL levels were significantly higher in AD compared with CTL cases ($t(193) = 6.890$, $P = 7.5 \times 10^{-11}$; $t(193) = 7.017$, $P = 3.7 \times 10^{-11}$, respectively). In contrast, RIPK3 levels were not different between the two groups ($t(193) = -0.247$, $P = 0.805$). (d) Scatterplot and regression line for the regression (without covariates) between RIPK1 and brain weight with 95% confidence intervals. Data in a–c are presented as box plots and were analyzed by moderated t-test ($n = 98$ cases for CTL and $n = 97$ cases for AD). Data in d were analyzed by linear regression ($n = 93$ cases for AD). In the box plots, the center line represents the median value, the limits represent the 25th and 75th percentile, and the whiskers represent the minimum and maximum value of the distribution. *$P < 0.05$.**

---

To determine the relationship between RIPK1, RIPK3 and MLKL mRNA levels and AD neuropathology, we performed an ordinal logistic regression with plaque density and Braak stage, which evaluates the amount of Aβ plaques and neurofibrillary tangles, respectively.

None of the three necroptotic marker levels were predictive of the amyloid load (Supplementary Table 3). In contrast, the increase of RIPK1 and MLKL mRNA levels, but not RIPK3, was significantly and positively correlated with the Braak stage ($P < 0.05$; Fig. 4a–c and Supplementary Table 3). This significance increased when we evaluated the effect of the interaction of RIPK1:MLKL ($P = 0.007$; Supplementary Table 3), which suggests that both markers covariate in the same samples together with the Braak stage. We also found that, in CTL cases, there was a significant negative correlation between RIPK3 levels and Braak stage ($P < 0.001$; Supplementary Table 3). To further investigate the link between necroptosis and tau, we double-stained sections from AD cases with the tau antibody AT8 and RIPK1 or pMLKL antibodies. We found that 55.48 ± 2.8% and 46.85 ± 4.0% of AT8 immunoreactivity colocalized with RIPK1 and pMLKL, respectively (Fig. 4d,e). We confirmed this colocalization using CP13, which recognizes tau phosphorylated at Ser202, and an independent pMLKL antibody (Supplementary Fig. 3). These data indicate a link between necroptosis and tau pathobiology but not between necroptosis and Aβ pathology, and are consistent with evidence indicating that tau is more proximal than Aβ to neuronal loss in AD.

---

To determine the relationship between RIPK1, RIPK3 and MLKL mRNA levels and AD neuropathology, we performed an ordinal logistic regression with plaque density and Braak stage, which evaluates the amount of Aβ plaques and neurofibrillary tangles, respectively.

None of the three necroptotic marker levels were predictive of the amyloid load (Supplementary Table 3). In contrast, the increase of RIPK1 and MLKL mRNA levels, but not RIPK3, was significantly and positively correlated with the Braak stage ($P < 0.05$; Fig. 4a–c and Supplementary Table 3). This significance increased when we evaluated the effect of the interaction of RIPK1:MLKL ($P = 0.007$; Supplementary Table 3), which suggests that both markers covariate in the same samples together with the Braak stage. We also found that, in CTL cases, there was a significant negative correlation between RIPK3 levels and Braak stage ($P < 0.001$; Supplementary Table 3). To further investigate the link between necroptosis and tau, we double-stained sections from AD cases with the tau antibody AT8 and RIPK1 or pMLKL antibodies. We found that 55.48 ± 2.8% and 46.85 ± 4.0% of AT8 immunoreactivity colocalized with RIPK1 and pMLKL, respectively (Fig. 4d,e). We confirmed this colocalization using CP13, which recognizes tau phosphorylated at Ser202, and an independent pMLKL antibody (Supplementary Fig. 3). These data indicate a link between necroptosis and tau pathobiology but not between necroptosis and Aβ pathology, and are consistent with evidence indicating that tau is more proximal than Aβ to neuronal loss in AD.
The activation of RIPK1 and MLKL correlates with Braak stage. (a–c) Boxplot of log2 expression values in AD patients for each Braak stage for RIPK1, RIPK3 and MLKL, respectively. RIPK1 and MLKL expression levels positively correlate with Braak stage (t(92) = 2.107, P = 0.035; t(92) = 2.488, P = 0.013, respectively). RIPK3 levels did not correlate with Braak stage (t(92) = 0.677, P = 0.499). Data were analyzed by ordinal logistical regression and presented as box plots. Data were analyzed by ordinal logistical regression and are presented as box plots. In the box plots, the center line represents the median value, the limits represent the 25th (Q1) and 75th (Q3) percentile, the whiskers represent the minimum and maximum value of the distribution, and the points represents the individual experimental value. The points outside the whiskers were defined by the interquartile range (IQR) rule, meaning they fall below Q1 – 1.5 IQR or above the Q3 + 1.5 IQR. (d–f) Results for the quantile regression between MMSE and the expression levels of the three necroptotic markers, RIPK1, RIPK3 and MLKL. The scatter plots show the regression coefficients as a function of percentiles and the standard errors. For RIPK1, after FDR correction there was a significant negative regression for percentiles ranging from 30th to the 40th percentiles. For RIPK3, no significant correlation was detected for any of the percentiles. For MLKL, after FDR correction there was a significant negative regression for all percentiles ranging from the 30th to the 50th percentiles. (g–i) Results for the quantile regression between MMSE and RIPK1:MLKL. After FDR correction, the interaction RIPK1:MLKL negatively correlated with MMSE for a wider range of percentiles (20th-50th). n = 94 AD cases for a–c, n = 15 for d–e and n = 62 for f–i.

Inhibiting necroptosis reduces neuronal death in 5xFAD mice

To further probe for a role of necroptosis in AD, we first measured necroptotic markers in two widely used animal models of AD: 5xFAD and APP/PS1 mice27,28. We found that RIPK1, MLKL and pMLKL levels were not significantly different between 12-month-old APP/PS1 and non-transgenic littermates (P < 0.05). In contrast, RIPK1, MLKL and pMLKL levels were significantly higher in the brains of 11-month-old 5xFAD mice compared with non-transgenic littermates.
Thus, in 5xFAD mice, which are characterized by marked cell loss (Supplementary Fig. 7e and ref. 27), necroptotic markers were considerably increased. In contrast, in APP/PS1 mice, which do not demonstrate overt cell loss (Supplementary Fig. 5f and ref. 28), necroptotic markers were not upregulated.

We then sought to determine the effect of inducing necroptosis on AD-like pathology in mice and generated an adeno-associated virus (AAV) expressing a constitutively active, GFP-tagged form of MLKL29 (caMLKL), under a neuronal-specific promoter. The virus was stereotaxically injected into the third ventricle of 3-month-old APP/PS1 mice (n = 14 mice, 6 females and 8 males) and nontransgenic (NonTg) littermates (n = 12 mice, 5 males and 7 females). Control age- and gender-matched APP/PS1 and NonTg mice (n = 14 mice per genotype) were injected with an AAV expressing GFP alone. The mice were tested on the spatial version of the Morris water maze 3 months later. We trained mice four times per day for five consecutive days.

(P < 0.05; Supplementary Fig. 7a–d). Thus, in 5xFAD mice, which are characterized by marked cell loss (Supplementary Fig. 7e and ref. 27), necroptotic markers were considerably increased. In contrast, in APP/PS1 mice, which do not demonstrate overt cell loss (Supplementary Fig. 5f and ref. 28), necroptotic markers were not upregulated.

We then sought to determine the effect of inducing necroptosis on AD-like pathology in mice and generated an adeno-associated virus (AAV) expressing a constitutively active, GFP-tagged form of MLKL29 (caMLKL), under a neuronal-specific promoter. The virus was stereotaxically injected into the third ventricle of 3-month-old APP/PS1 mice (n = 14 mice, 6 females and 8 males) and nontransgenic (NonTg) littermates (n = 12 mice, 5 males and 7 females). Control age- and gender-matched APP/PS1 and NonTg mice (n = 14 mice per genotype) were injected with an AAV expressing GFP alone. The mice were tested on the spatial version of the Morris water maze 3 months later. We trained mice four times per day for five consecutive days.
days to learn the location of a hidden platform using cues located outside of the maze. Using a two-way ANOVA, we found that, for the escape latency, there was a significant effect for days \((F = 31.26, P < 0.0001)\) and genotype \((F = 21.37, P < 0.0001)\), as well as a significant genotype \(\times\) day interaction \((F = 2.16, P < 0.05; \text{Fig. 6a})\). The effect of day indicated that all of the mice learned the task across

---

**Figure 6** Necrotosis activation exacerbates cognitive deficits in APP/PS1 mice. (a, b) Learning curves of mice trained in Morris water maze (NonTg-GFP, \(n = 14\) mice; NonTg-MLKL, \(n = 12\) mice; APP/PS1-GFP, \(n = 14\) mice; APP/PS1-MLKL, \(n = 14\) mice). For the escape latency, day effect \((F(4, 250) = 31.26; P < 0.0001)\), group effect \((F(3, 250) = 21.37; P < 0.001)\) and group \(\times\) day interaction \((F(12, 250) = 2.16; P = 0.014)\). For distance traveled, day effect \((F(4, 250) = 45.48; P < 0.0001)\), group effect \((F(3, 250) = 31.46; P < 0.001)\) and group \(\times\) day interaction \((F(12, 250) = 1.87; P = 0.040)\). Post hoc tests indicated that the escape latency for APP/PS1-MLKL mice was significantly different than that for NonTg-GFP mice at days 3, 4 and 5 (shown by ‡); it was significantly different than that for NonTg-MLKL and APP/PS1-GFP mice at day 5 (shown by ‡‡ and ‡‡‡, respectively). Post hoc tests indicated that the distance traveled for APP/PS1-MLKL mice was significantly different than NonTg-GFP mice at days 2–5 (shown by ‡); it was significantly different than NonTg-MLKL mice at days 3 and 4 (shown by ‡‡); it was significantly higher than APP/PS1-GFP mice at days 2, 4 and 5 (shown by ‡‡‡). (c) Number of platform location crosses during a single 60-s probe trial \((F(3, 50) = 11.59; P < 0.0001)\). Post hoc tests showed that NonTg-MLKP performed significantly better than the other three groups (shown by ‡). In addition, APP/PS1-MLKL performed significantly worse than the other three groups (shown by ‡‡). (d) Number of platform location crosses analyzed as a percentage change indicated that the slopes for the two groups were significantly different from each other \((F(1, 38) = 4.67; P = 0.037)\). (e) Swim speed measured during a single 60-s probe trial. The values were not statistically significant among the groups \((F(3, 50) = 1.164; P = 0.333)\). (f, g) Microphotographs of NonTg brain sections injected with the GFP or the MLKL virus, respectively. Sections were stained with an anti-GFP antibody to visualize viral diffusion. (h–j) Sections from APP/PS1-GFP mice \((n = 8)\) were stained with the indicated antibodies. Mander’s correlation analysis indicated a 66.84 ± 3.2% colocalization between GFP and the neuronal marker NeuN, 20.06 ± 3.0% colocalization between GFP and the astrocytic marker GFAP and 31.14 ± 2.4% colocalized between GFP and the microglial marker Iba1. (k) Quantitative analysis of the colocalized pixels indicated that most of the virus-infected neurons. One-way ANOVA indicated that these values were significantly different among each other \((F(2, 23) = 70.35; P < 0.001)\). Bonferroni’s multiple comparison test indicated that all three groups were statistically different from each other. Data in a, b were analyzed by two-way ANOVA and are presented as mean ± s.e.m.; data in c, e, k were analyzed by one-way ANOVA and are presented as box plots. In the box plots, the center line represents the median value, the limits represent the 25th and 75th percentile, and the whiskers represent the minimum and maximum value of the distribution. Bonferroni’s was used for post hoc tests. Data in d were analyzed by linear regression and are presented as mean ± s.e.m.
we also measured soluble and insoluble F-APP/PS1-MLKL. We found that the APP/PS1-MLKL group performed significantly worse than the genotype-matched mice injected with the GFP virus (P < 0.05; APP/PS1-GFP; NonTg-GFP) on days 4 and 5, respectively. Notably, APP/PS1-MLKL mice performed significantly worse than NonTg-MLKL mice on day 5 (P < 0.05). When we analyzed the distance traveled to find the platform, we found a significant effect for days (F = 45.48, P < 0.0001) and genotype (F = 31.46, P < 0.0001), as well as a significant genotype × day interaction (F = 1.872, P < 0.05; Fig. 6b). Bonferroni’s post hoc test revealed that NonTg-GFP performed significantly better than any other group on days 4 and 5 (P < 0.05). Notably, APP/PS1-MLKL performed significantly worse than APP/PS1-GFP on days 2, 4 and 5 (P < 0.05), and significantly worse than NonTg-MLKL on days 3 and 4 (P < 0.05).

We conducted probe trials to evaluate spatial reference memory 24 h after the last training trial. We measured the number of platform location crosses and the swim speed during a single 60-s trial. One-way ANOVA revealed a significant difference among the four groups (F = 11.59, P < 0.0001; Fig. 6c). Bonferroni’s post hoc analyses indicated that the APP/PS1-MLKL group performed significantly worse than the other three groups (P < 0.05). To assess the degree of exacerbation, we normalized the number of platform location crosses for the two genotypes in relation to mice that received the GFP virus. We found a steeper slope for the APP/PS1 mice compared with NonTg mice, indicating that the MLKL virus had more severe effects on spatial memory in transgenic mice than in NonTg mice (Fig. 6d). Notably, the swim speed of mice during the probe trials was not statistically different among the four genotypes (Fig. 6e), indicating that the effects on learning and memory were independent of physical performance. These findings indicate that inducing necroptosis exacerbates cognitive decline in APP/PS1 mice to a greater degree than in NonTg mice.

At the end of the behavioral tests, to assess the extent of viral diffusion, as well as the specific cell type infected by the virus, we stained sections from APP/PS1-GFP and NonTg-GFP mice with an anti-GFP antibody. We observed strong GFP immunoreactivity in the hippocampus and adjacent cortical areas (Fig. 6f–g). Confocal imaging of sections from APP/PS1-GFP mice revealed 66.84 ± 3.2% colocalization between GFP and the neuronal marker NeuN, 20.06 ± 3.0% colocalization between GFP and the astrocyte marker GFAP, and 31.14 ± 2.4% colocalization between GFP and the microglia marker Iba1 (Fig. 6h–k). One-way ANOVA indicated that these values were significantly different from each other (F = 70.35, P < 0.001). Bonferroni’s multiple comparison test revealed that all three groups were statistically significant from each other (P < 0.05). These data indicate that caMLKL is preferentially expressed in neurons.

To probe for the mechanisms underlying the caMLKL-mediated changes in cognitive function, we assessed Aβ pathology, endogenous tau levels and neurodegeneration. We stained hippocampal sections from APP/PS1-GFP and APP/PS1-MLKL mice with an Aβ42-specific antibody and found that Aβ load was similar between the two groups (Supplementary Fig. 8a). We also measured soluble and insoluble Aβ40 and Aβ42 by sandwich ELISA and found no changes in Aβ levels between the two groups for any of the measurements (Supplementary Fig. 8c–f). Similarly, total tau levels (measured by the tau-5 antibody) and tau phosphorylated at Ser202 (measured by the CP13 antibody) were not statistically different between APP/PS1-GFP and APP/PS1-MLKL mice (Supplementary Fig. 8g–j). The lack of changes in Aβ and tau suggest that inducing necroptosis does not contribute to Aβ and tau accumulation.

We then assessed the degree of neuronal loss by staining hippocampal sections with NeuN, a commonly used neuronal marker.
Quantitative analyses of the NeuN staining revealed a significant reduction in the number of neurons among the four groups \((F = 21.77, P < 0.0001; \text{Fig. 7a})\). Bonferroni’s post hoc analyses indicated that mice injected with the caMLK virus had significantly fewer CA1 neurons than genotype-matched mice injected with the GFP virus (\text{Fig. 7b}). Notably, the number of NeuN-positive neurons in the APP/PS1-MLKL group was significantly lower than the number of NeuN-positive neurons in the NonTg-MLKL group. This difference is further highlighted by analyzing the data as a percentage change in neuronal loss between the GFP- and the MLKL-injected mice. These data indicate that the degree of MLKL-induced cell loss was higher in APP/PS1 treated than in NonTg mice (\text{Fig. 7c}), suggesting that the APP/PS1 brains are more susceptible to activation of necroptosis.

To probe for a direct link between necroptosis and neuronal loss in AD, we plated primary cortical neurons from APP/PS1 and wild-type (WT) littermate mice. After 7 d in vitro (DIV), neurons were treated with 10 \(\mu\)M of the necroptosis inhibitor 7-CI’O-necrostatin (Nec1S) or vehicle. Treatment was repeated at DIV 11. We then stained neurons at DIV 11 and 15 with NeuN and counted the total number of neurons. Using a two-way ANOVA, we found that there was a significant effect for time \((F = 76.13, P < 0.0001)\) and groups \((F = 7.109, P = 0.0002)\), as well as a significant time \(\times\) group interaction \((F = 7.109, P = 0.0002; \text{Fig. 8a})\). A post hoc test with Bonferroni’s correction revealed that the number of neurons was similar among the four groups at DIV 11. In contrast, at DIV 15, the total number of neurons in the APP/PS1-vehicle group was significantly different than the other three groups. The APP/PS1-Nec1S group had more neurons than the APP/PS1-vehicle group, and as many neurons as both wild-type groups. Nec1S did not significantly affect the decrease in the number of wild-type neurons. We then isolated proteins from neurons of the same four groups and measured the levels of total and phosphorylated MLKL by western blot. We found that pMLKL was not detectable at DIV 11. In contrast, at DIV 15, the ratio of pMLKL/MLKL was significantly different among the four groups \((F = 11.30, P < 0.0005)\). Bonferroni’s post hoc analyses indicated that all the groups were significantly different from the APP/PS1-vehicle.

Representative brain sections from 5xFAD mice treated with Nec1S or vehicle stained with Fluoro-Jade. (\text{Fig. 8f}) The graph shows the quantitative analyses of the Fluoro-Jade staining revealed a significant difference between groups \((f(7) = 4.353; P = 0.0033)\). (\text{Fig. 8g}) The graph shows the quantitative analyses of pMLKL/MLKL measured by western blot \((5xFAD-vehicle, n = 4, 5xFAD-Nec1S, n = 4)\). Analyses revealed a significant difference between groups \((f(6) = 2.627; P = 0.0341)\). Data in \(\text{a and c} are presented as \text{mean ± s.e.m. and were analyzed by two-way ANOVA. Data in b and d are presented as box plots and were analyzed by one-way ANOVA. Data in f,g are presented as box plots and were analyzed by unpaired t test. In the box plots, the center line represents the median value, the limits represent the 25th and 75th percentile, and the whiskers represent the minimum and maximum value of the distribution.} *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 8 Necroptosis contributes to neuronal death in 5xFAD mice. (a) Primary neurons from APP/PS1 and wild-type (WT) mice were treated with Nec1S or vehicle (APP/PS1, \(n = 36\) wells from 9 mice per drug per time point; WT, \(n = 12\) wells from 3 mice per drug per time point). The graph shows the number of NeuN-positive neurons after 11 and 15 DIV. There was a significant effect for time \((F(1,184) = 76.13; P < 0.0001)\) and groups \((F(3,184) = 7.109; P = 0.0002)\) as well as a significant time \(\times\) group interaction \((F(3,184) = 7.109; P = 0.0002)\). (b) The graph shows the quantitative analyses of pMLKL/MLKL measured by western blot \((\text{APP/PS1-vehicle, } n = 5; \text{APP/PS1-Nec1S, } n = 5; \text{WT-vehicle, } n = 4; \text{WT-Nec1S, } n = 4)\). Analyses revealed a significant difference among the four groups \((F(3,14) = 10.20; P = 0.0008)\). Bonferroni’s post hoc analyses revealed that all of the APP/PS1-vehicle group was significantly different from the other three groups. (c) Primary neurons from APP/PS1 and wild-type mice were infected with an AAV-expressing GFP and then treated with Nec1S or vehicle. We then measured GFP fluorescence in live cells as well as a significant time \(\times\) group interaction \((P = 0.0002)\) as well as a significant time \(\times\) group interaction \((P = 7.109; P = 0.0002)\). Bonferroni’s post hoc analyses revealed that all of the APP/PS1-vehicle group was significantly different from the other three groups. (d) The graph shows the quantitative analyses of NeuN-positive neuron loss after 11 and 15 DIV. There was a significant effect for time \((F(3,344) = 78.43; P < 0.0001)\) as well as a significant time \(\times\) group interaction \((F(3,344) = 78.43; P < 0.0001)\). (e) The graph shows the quantitative analyses of NeuN-positive neuron loss after 11 and 15 DIV. There was a significant effect for time \((F(3,344) = 78.43; P < 0.0001)\) as well as a significant time \(\times\) group interaction \((F(3,344) = 78.43; P < 0.0001)\). (d) The graph shows the quantitative analyses of NeuN-positive neuron loss after 11 and 15 DIV. There was a significant effect for time \((F(3,344) = 78.43; P < 0.0001)\) as well as a significant time \(\times\) group interaction \((F(3,344) = 78.43; P < 0.0001)\).
In contrast, at DIV 15, the APP/PS1–vehicle group had lower GFP levels than the other three groups. pMLKL levels were significantly different among the four groups at DIV 15 ($F = 11.30, P < 0.0005$), but were not detectable at DIV 11. This difference was driven by the APP/PS1–vehicle group; indeed, the ratio of pMLKL/MLKL for this group was statistically different compared with the other three groups (Fig. 8d and Supplementary Fig. 9c,d). The results of these two independent experiments indicate that Nec-1S reduces in vitro cell death of APP/PS1 primary cortical neurons.

We then treated 8-month-old 5xFAD mice with Nec1S ($n = 5$) or vehicle ($n = 4$). Following a previously published protocol$^{13}$, mice received a single intraperitoneal injection of 10 mg per kg of body weight Nec1S, after which they were given 0.5 mg ml$^{-1}$ Nec1S in their drinking water for 21 d. Notably, Nec1S-treated 5xFAD mice had fewer Fluoro-Jade-positive neurons than vehicle-treated 5xFAD mice ($t = 2.627, P = 0.0341$; Fig. 8e,f). Western blot data confirmed that necroptosis was reduced in Nec1S-treated 5xFAD mice, as indicated by a lower pMLKL/MLKL ratio ($t = 4.35, P = 0.0033$; Fig. 8g and Supplementary Fig. 9e). These data provide compelling evidence that necroptosis contributes to neuronal loss in 5xFAD mice.

**DISCUSSION**

Neuronal loss is a cardinal feature of AD and invariably affects multiple brain regions. Notably, brain atrophy is evident in asymptomatic individuals 10 years before the onset of dementia.$^{30}$ Despite this indisputable evidence, the precise mechanism by which neurons die remains unknown.$^{31–33}$ Identifying the mechanisms leading to neuronal loss is essential for the development of an effective therapeutic strategy to treat or slow down the progression of AD. However, the vast majority of ongoing clinical trials are designed to reduce a toxic insult (for example, removal of Aβ from the brain). Growing evidence suggests that there are multiple causes for AD; thus, targeting the mechanisms of neurodegeneration is critical, as it may have beneficial effects independently of the trigger$^{34}$. Alternatively, it could be used in concomitance with other therapeutics that aim to block the neurotoxic insult. Here we provide, to the best of our knowledge, the first direct evidence that necroptosis is activated in human AD brains, as well as in a mouse model of AD that develops neuronal loss. Our results open new venues of research and interventions for this insidious disorder, which affects more than 40 million people worldwide$^{1}$. From a basic biology perspective, it will be essential to dissect the mechanisms underlying necroptosis induction in AD; such studies may reveal new insights into the pathogenesis of this disorder. From a therapeutic perspective, our data strongly suggest that reducing necroptosis may be a valid therapeutic target for AD.

Evidence suggests that there is a favorable environment for apoptosis in AD brains. For example, DNA fragmentation has been detected in postmortem human brains$^{35}$. In addition, activation of several caspases has also been reported. However, caspase activation and DNA fragmentation do not necessarily lead to apoptosis$^{36}$. For example, caspase-3 activation might be involved in synaptic function in the adult brain$^{37}$, whereas DNA fragmentation routinely occurs during DNA repair in response to various stressors$^{38}$. Apoptotic bodies and/or chromatin condensation, which are more proximal to death by apoptosis, have not been reported in AD$^{39}$. It is estimated that it takes up to 24 h for a neuron to undergo apoptosis. Thus, given the extensive number of neurons reported that have DNA fragmentation and other apoptotic features, if apoptosis were the only key factor involved in neuronal loss in AD, this disorder would be an acute one, rather than the chronic, slow progressive disease that it is$^{33}$. It is tempting to speculate that early activation of caspases and DNA fragmentation, in the absence of apoptotic bodies and chromatin condensation, might instead be evidence of necroptosis activation. Indeed, recent evidence highlights an extensive crosstalk between apoptosis and necroptosis$^{40}$.

Necroptosis contributes to the pathogenesis of amyotrophic lateral sclerosis and multiple sclerosis$^{13,14}$. Our data consistently show, across different cohorts, that RIPK1 levels were increased in human AD brains. Although the mechanisms regulating RIPK1 expression in AD remain elusive, the activation of this kinase can be regulated by different signaling pathways, including chronic tumor necrosis factor α (TNFα)-mediated inflammation, which is a feature of AD$^{41}$. Once activated, RIPK1 protects cells from caspase-8 mediated apoptosis$^{42,43}$. In contrast, chronic activation of RIPK1 leads to necroptosis activation. We generated a causal gene regulatory network to model RIPK1 regulatory activity in AD-relevant tissue. Our data clearly indicate that upregulation of RIPK1 alone may account for the gene expression changes reported in AD brains. These findings are consistent with previous reports suggesting that RIPK1 is involved in the formation and regulation of protein complexes involved in gene regulation$^{46}$.

Although RIPK3 levels were not statistically different between AD and CTL cases, we found that RIPK3 was associated with RIPK1 and MLKL to a greater degree in AD brains than in CTL brains. These findings are consistent with the overall mechanisms underlying necroptosis activation: RIPK1 binds to RIPK3, which in turn binds to MLKL to form the necosome. In other words, necrosome formation, which is an irreversible marker of necroptosis activation, is dependent on the physical interaction between RIPK1, RIPK3 and MLKL, and not on the relative levels of RIPK3. After the necosome forms, RIPK3 phosphorylates MLKL, which becomes the executioner of necroptosis. Once phosphorylated, MLKL aggregates to form homodimers, binds to phosphatidylinositol phosphates, permeabilizes membranes and induces cell death$^{3,10}$. Notably, phosphorylation and dimerization of MLKL are sufficient and necessary for necroptosis induction$^{44–46}$. Consistent with these data, we found that MLKL phosphorylation and aggregation was increased in AD brains, driving its localization to the membrane. Moreover, we found that RIPK1 and MLKL correlated with Braak stage only in AD patients, suggesting that tau accumulation could be a key trigger for necroptosis activation; it has been consistently shown that neurofibrillary tangles represent a proximal event to neuronal loss in AD and correlate well with cognitive function$^{47}$. To this end, we found that, for both Braak stage and MMSE, the significance of the correlation increased when we evaluated the interaction between RIPK1 and MLKL, leading to the hypothesis that it is not only the absolute expression of these proteins that may contribute to cell loss in AD, but also their coexpression.

In summary, we report, to the best of our knowledge, the first direct evidence of necroptosis activation in AD. These findings may serve as a springboard for future evaluation of triggers of necroptosis in AD. Moreover, these findings may open up a new phase of drug discovery for AD focused on identifying small molecule inhibitors of RIPK1, RIPK3 and/or MLKL.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

**ACKNOWLEDGMENTS**

We thank E. Reiman for discussion and assistance, P. Coleman for kindly providing access to his expressing data set. We thank A. Rodin and A. Tran for contributing to the editing of the manuscript. We thank D. Green for kindly providing the MLKL constructs. We are grateful to the Banner Sun Health Research Institute Brain and Body Donation Program of Sun City, Arizona for providing the human tissue.
Data for the RIPK1 causal regulatory gene network were generated from postmortem brain tissue collected through the Mount Sinai VA Medical Center Brain Bank and were provided by E. Schadt. The computational resources and expertise provided by the Department of Scientific Computing at the Icahn School of Medicine at Mount Sinai also contributed to this study. This work was supported by grants from the Arizona Alzheimer's Consortium and the US National Institutes of Health (R01 AG037637 to S.O., and R01 NS083801 and P50 AG16573 to K.N.G. The Brain and Body Donation Program is supported by the US National Institute of Neurological Disorders and Stroke (U24 NS072026 National Brain and Tissue Resource for Parkinson's Disease and Related Disorders), the National Institute on Aging (P03 AG19610 Arizona Alzheimer's Disease Core Center), the Arizona Department of Health Services (contract 211002, Arizona Alzheimer’s Research Center), the Arizona Biomedical Research Commission (contracts 4001, 0011, 05-091 and 1001 to the Arizona Parkinson’s Disease Consortium), and the Michael J. Fox Foundation for Parkinson’s Research.

AUTHOR CONTRIBUTIONS
A.C. and C.R. designed and performed the experiments and analyzed the data. I.S.P. and M.J.H. performed the statistical analyses. E.F. performed the confocal imaging and quantification. W.S.L. generated the expression data from the microarray analyses used to generate the RIPK1 causal regulatory network. B.R. and J.T.D. generated the RIPK1 causal regulatory gene network and performed the associated gene set analysis. E.E.S. and K.N.G. performed the experiments on SxEAD mice. B.R. performed the colocalization experiments described in Supplementary Figure 5. W.W. performed the immunoprecipitation experiments. S.O. conceptualized and designed the experiments, analyzed the data, and wrote the manuscript. All of the authors contributed to the preparation of the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

ONLINE METHODS

Human tissue and mice. The human samples were obtained from the Brain and Body Donation Program at the Banner Sun Health Research Institute, whose average postmortem interval (PMI) is 2.75 h. The cases were selected randomly among the tissue available by personnel of the Brain and Body Donation program. Groups were matched based on their clinical and neuropathological phenotype. The generation of APP/PS1 and 5xFAD has been described previously.[27,28] We backcrossed the APP/PS1 to a pure 129S6 background for 12 generations. The 5xFAD mice are on a C57BL/6 background. All mice were housed 4–5 per cage at 23°C, kept on a 12-h light/dark cycle and were given ad libitum access to food and water. Mice were randomly assigned to a specific group based on their genotype, and there were no other factors that determined groups’ selection. No mice were excluded from the statistical analyses. For the behavioral analyses (see below), the experimenters were blinded to genotype and treatment. For the other experiments, data collection and analysis were not performed blind to the conditions of the experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Arizona State University.

Immunohistochemistry. Coronal sections from hippocampus and temporal gyrus of CT1 and AD cases were pre-mounted on coated slides and washed in TBS. After blocking in TBSB (TBS, 2% BSA and 0.1% Triton X-100), tissue was incubated overnight at 4°C with the following primary antibodies: RIPK1 (1:200, BD Transduction Laboratories, catalog number 610459), MLKL (1:200, Novus Biologicals, catalog number NB51-56729), RIPK3 (1:200, RD Systems catalog number MAB7604), pMLKL Ser358/Thr357 (1:200, Abcam, catalog number ab187091), pMLKL Ser358 (1:200, SAB Signalway Antibody, catalog number 12837), NeuN (1:200, Cell Signaling, catalog number 24307P), Iba1 (1:200, Wako, catalog number 09-19741), GFAP (1:200, Cell Signaling, catalog number 36705), Caspase 3 (1:200, Millipore, catalog number AB3623), phospho-tau pSer202/Thr205 (AT8, 1:200, Thermo Fisher Scientific, catalog number MN1020), pSer202/Thr205 (1:200, Abcam, catalog number ab6528 and ab6529) with CP13 (1:200, Cell Signaling, catalog number 93957). Sections were washed to remove the excess of primary antibody and incubated with the appropriate secondary fluorescent antibodies for 1 h at 25°C (1:200, Thermo Fisher Scientific, catalog numbers A-11034, A-11034, A21429, and A-21424). The excess of secondary antibody was washed and the sections were coveredslipped with Prolong Diamond antifade mountant (Thermo Fisher Scientific). For Fluoro-Jade staining, tissue from APP/PS1 and 5xFAD mice was mounted on coated slides and rehydrated on decreasing concentrations of ethanol. After rehydration, the tissue was pre-treated for 5 min in potassium permanganate and then incubated for 30 min in the dark at 25°C in Fluoro-Jade (Millipore) and DAPI (Sigma-Aldrich). The tissue was then washed with water and let dry overnight. The next day the slides were cleared in xylene and coveredslipped with DPX mounting solution (Sigma-Aldrich). The immunohistochemistry on the brain sections from APP/PS1 was conducted following a similar protocol as we previously detailed.[49] In this case, sections were stained with the following antibodies: Aβ42 (1:200, Millipore, catalog number AB5087P), NeuN (1:200, Millipore, catalog number MAB377), Iba1 (Wako), GFAP (Cell Signaling), and GFP (1:200, Thermo Fisher Scientific, catalog number A-11122).

Confocal microscopy and image analysis. All images were acquired at a resolution size of x = 1.024, y = 1.024 and z = 1, using a 63× oil immersion objective and zoom of 1.5 using the Leica DM 2500 confocal microscope. Image fields within the x-y plane of the tissue section were randomly selected. For each image acquired, channels were split to allow separate pixel quantification in both the S61-nm and 635-nm channels. To quantify total protein in each image field, channels were split to allow separate pixel quantification in both the S61-nm and 635-nm channels. To quantify total protein in each image field, channels were split to allow separate pixel quantification in both the S61-nm and 635-nm channels. To quantify total protein in each image field, channels were split to allow separate pixel quantification in both the S61-nm and 635-nm channels.

Protein extraction. Human temporal gyrus tissue samples were extracted in sequential buffers, from lower to higher detergent strength. Briefly, the samples were homogenized in TBS (50 mM Tris-HCl, pH 7.4, 175 mM NaCl, 5 mM EDTA, 1 mM N-ethylmaleimide) containing protease inhibitors (Roche) and phosphatase inhibitors (Millipore). The protein extract was centrifuged at 120,000g for 30 min at 4°C. The resulting supernatant represented the TBS soluble fraction. Pellets were homogenized in TBS/1% Triton X-100 and spun at 1,000g for 10 min at 4°C. The supernatant represented the Triton soluble fraction. Pellets were washed in TBS/1% sucrose and then homogenized in RIPA buffer. The samples were centrifuged at 1,000g for 10 min at 4°C and the supernatant was collected and stored as RIPA fraction. The detergent-insoluble pellets were then re-suspended in 8 M urea/5% SDS and stored as urea fraction. The frozen mouse hemispheres were homogenized in 1 ml of cold T-PER (Thermo Fisher Scientific) protein extraction buffer containing complete protease inhibitor (Roche) and phosphatase inhibitor (Millipore). Brain homogenates were ultra-centrifuged at 100,000g for 1 h at 4°C. The supernatant was recovered and stored at −80°C until used for western blots.

Western blot and ELISA. The proteins were run using reducing conditions in precast gels (Thermo Fisher Scientific) and transferred to nitrocellulose membranes (iBlot, Thermo Fisher Scientific). The membranes were incubated for 60 min in 5% nonfat milk (Great Value) in Tris-buffered saline with Tween (TBST; 0.1 M Tris, 0.15 M NaCl, and 0.1% Tween 20). After blocking, membranes were incubated overnight at 4°C in 5% milk in TBS-T with the appropriate primary antibodies: RIPK1 (1:1,000, BD Biosciences, catalog number 610459), RIPK3 (1:1,000, Novus Biologicals, catalog number NB51-77299), MLKL (1:1,000, Novus Biologicals, catalog number NB51-56729), pMLKL (1:1,000, Abcam, catalog number ab196436), tau-5 (1:500, Thermo Fisher Scientific, catalog number MA5-12808), and β-actin (1:10,000, Cell Signaling Technology, catalog number 3700). The next day, the blots were washed three times with TBS-T for 10 min and incubated in the specific secondary antibodies (1:20,000, LI-COR Biosciences, catalog numbers 926-68020 and 926-32211) for 1 h at 25°C. The blots were then washed with TBS-T, and imaged/quantified using a LICOR Odyssey CLX (LI-COR Biosciences) attached to a Dell computer (OptiPlex 7010) running Windows 7 and Image Studio (version 10.11, LI-COR Biosciences). For the detection of MLKL monomers and dimers, proteins were run in 3–8% tris-acetate gel (Thermo Fisher Scientific) in non-reducing conditions. The ELISA experiments to measure Aβ levels were done following the protocol we detailed in previous publications.[49] Antigens validation: western blots. To validate the RIPK1 antibody, we loaded on a gel proteins extracted from wild type cells, RIPK1 knockout cells (Horizon Discovery, catalog number HZGHCH000060C015), RIPK1 knockout cells transfected with a RIPK1 expressing plasmid (Addgene, catalog number 78042), and AD human cases, non-transgenic and 5xFAD mice. Notably, the expected band of 73 kDa (arrow in Supplementary Fig. 1a) was not present in the knock-out cells. To validate the RIPK3 antibody, we loaded on a gel proteins extracted from wild type mice, RIPK3 knockout mice (obtained from Genentech), wild-type cells transfected with a RIPK3 expressing plasmid (Addgene, catalog number 41387), CTL and AD human cases, non-transgenic and 5xFAD mice. The expected band of 55 kDa (arrow in Supplementary Fig. 1b) was not present in the RIPK3 knockout mice and was present in the cells transfected with the RIPK3-expressing
plasmid. Notably, in this case the band ran a little slower, as the plasmid had a GFP tag to its C-terminal. To validate the MLKL antibody, we loaded on a gel proteins extracted from wild type cells, MLKL knockout cells (Horizon Discovery, catalog number HGCH000072c0013), MLKL knockout cells transfected with a MLKL-expressing plasmid pRetox-TRE3G MLKL-Flag (obtained from P. Fitzgerald, St. Jude Children’s Research Hospital), CTL and AD human cases, and non-transgenic and 5xFAD mice. Notably, the expected band of 51 kDa (arrow in Supplementary Fig. 10c) was not present in the knockout cells, but it was present when these cells were transfected with an MLKL plasmid. To validate the phospho-specific MLKL antibody, we loaded on a gel protein extracted from MLKL knockdown and wild-type cells. As a positive control, cells were treated with 1 ng/ml TNFα (PeproTech) and 50 μM pan caspase inhibitor Z-VAD-FMK (R&D Systems) for 4 h before protein extraction. This is a known protocol to induce necroptosis in vitro35. Proteins extracted from NonTg and 5xFAD mice were also added. As shown in Supplementary Figure 10d, we detected a strong band in the positive control but not in the MLKL knockdown cells.

Immunohistochemistry. To validate the RIPK1, RIPK3, and MLKL antibodies for immunohistochemistry, we used the knockout out cell lines for RIPK1 and MLKL listed above with their matched control (HAP-1 cell line), and primary fibroblasts isolated from RIPK3 knockout and wild-type mice. As shown in Supplementary Figure 10e, only background staining was evident in the knockout cells. To validate the pMLKL antibodies, we induced necroptosis in HAP-1 cells with 1 ng/ml TNFα and 50 μM caspase inhibitor Z-VAD-FMK for 4 h. Cells were then stained with the two pMLKL antibodies used in this work. A strong immunoreactivity for both pMLKL antibodies was only evident after inducing necroptosis (Supplementary Fig. 10f).

Immunoprecipitation. Samples from AD and CTL brains were homogenized in TBS/Triton-X100. Protein concentrations were determined using the Bradford assay (Thermo Fischer Scientific). 100 μg of proteins per each sample was incubated with 4 μg of anti-RIPK1 (BD Transduction Laboratories), for 48 h while rotating at 4 °C. After incubation, 100 μl of pre-washed Protein G magnetic beads (Biorad) were added to each sample. Samples and beads were incubated at 4 °C for 3 h. The beads were then washed three times with TBS-T and mixed with 15.5 μl of 4x loading buffer containing 1.5 μl reducing buffer (Thermo Fischer Scientific). The beads were boiled at 90 °C for 5 min and placed on ice. Samples were loaded onto a 10% Bis-Tris gel in MOPS running buffer (Thermo Fischer Scientific). Gels were transferred to a nitrocellulose membrane and incubated overnight at 4 °C with anti-MLKL (1:1,000, Novus Biologicals) followed by one hour incubation at 25 °C with anti-rabbit IgG-HRP (1:20,000, Thermo Fischer Scientific). Blots were then washed and developed with Super Signal West Dura (Thermo Fisher Scientific) and processed on film. The quantification of the bands was performed using ImageJ.

RNA extraction and microarray analysis. The total RNA extracted from frozen temporal gyrus tissue was isolated with RNEasy Mini Kit (Qiagen) starting with 20–50 mg of tissue. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to manufacturer’s protocol. The raw data were background corrected using Genome Studio data analysis software (Illumina), and then exported for the following analysis. For quality control purposes, hierarchical clustering, MDS, and density plots were inspected to detect outliers and low quality samples using the R package ArrayQualityMetrics36. The expression data set was annotated with the R-package lumiHumanAll.db, originally consisting of 42,179 informative probes. After filtering for low expressed and non-annotated probes, we obtained a final data set of 26,583 probes corresponding to 21,122 unique genes. The HumanHT-12v4 BeadChip array contains multiple probes matching a single gene; however, no summarization of the data was performed to avoid the loss of information because different probes can correspond to multiple transcript variants/isoforms for the same gene. Variation of raw data was stabilized using a variance-stabilizing transformation (VST), and the data were normalized using the quantile method by using the lumiExpression function with default parameters. The moderated t-statistics, as well as FDR correction method, were used for detection of differential expression and p-value correction, respectively33. Transcripts with an adjusted \( P < 0.05 \) were considered statistically significant. VST and differential expression analysis were conducted using the R-package Lumi32.

The normalized expression values for RIPK1 (NM_003804), MLKL (NM_152649) and RIPK3 (NM_006871) from the microarray experiments are available as Supplementary Data Set 1.

For validation purposes, we reanalyzed the publicly available data set GSE5281. Data were processed with the Affymetrix U133 Plus 2.0 array. In this data set, there were samples obtained from the tissue bank at the Banner Sun Health Research Institute and samples obtained from the tissue bank at Washington University. To obtain an independent cohort, we excluded the samples obtained from the Banner Sun Health Research Institute. The raw data (*.CEL files) were analyzed applying the Robust Multi-Array Average (RMA) normalization method35. Differential expression was computed using a linear model, and the significance was assessed using the moderated-t statistics as implemented in the limma R-package34. The \( P \) values were corrected using the FDR correction method.

Construction of RIPK1 causal gene regulatory network. We performed causal inference testing35 to build a causal gene regulatory network focused on RIPK1 in post-mortem brain tissue. This approach requires paired gene expression and genotype data for a large number of samples to establish the direction of regulation between RIPK1 and its correlated genes. RNA sequencing data was obtained from the Accelerating Medicines Partnership – Alzheimer’s disease (AMP-AD) Knowledge Portal (synapse ID: syn3157743). Post-mortem samples were collected from the superior temporal gyrus (STG, Brodmann Area 22), anterior prefrontal cortex (APFC, Brodmann Area 10), entorhinal cortex (ERC, Brodmann Area 36) and inferior frontal gyrus (IFG, Brodmann Area 44) by the Mount Sinai NIH Brain and Tissue Repository. Samples with RIN less than 6, were removed from the analysis. Single end reads were aligned reads to human genome reference (GRCh37 ensembl version 7035), using STAR-RNASeq (2.4.0g) read aligner36, and accepted mapped reads were summarized to gene level using the feature-Counts function of the subread software package37,38. Genes with at least 1 count per million mapped reads39 in at least half of the sample libraries were retained, and normalized using the voom function in the Limma package40-42.

Whole Exome Sequencing data used in this study can be obtained from the Accelerating Medicines Partnership–Alzheimer’s Disease (AMP-AD) Knowledge Portal (synapse ID: syn4645334). Reads were aligned to human genome hg19 using BWA aligner43. DNA sequence variants were called using the DNAseq Variant Analysis workflow of GATK Best Practices version 3 (ref. 62). Variants with a minor allele frequency <0.05, or with missing calls in >10 samples were removed from further analysis. Common variants were imputed using IMPUTE2 (refs. 63,64), using 1,000 Genomes Phase 3 reference genotype45.

We used the Matrix eQTL software66, to identify cis-eQTLs for RIPK1, classifying DNA markers within 1MB of RIPK1 gene boundaries that significantly associate with RIPK1 expression (controlling for age, sex, ethnicity, RNA-sequencing batch, RIN and PMI as covariates), assuming an additive linear model for associating genotype dosage with RIPK1 expression. We classified DNA markers with an association FDR < 0.25 as RIPK1 cis-eQTL, identifying multiple markers in two of the four brain regions, the ERC and APFC.

We then identified the subset of these RIPK1 cis-eQTLs that are conditionally independent in their association with RIPK1, and used them for causal inference testing. For each brain region, we ranked cis-eQTL according to ascending P-value, and recursively attempted cis-eQTL discovery while conditioning on the other quantitative trait (such as the expression of genes correlated with RIPK1).
Causal relationships can be inferred from a chain of mathematical conditions, requiring that for a given trio of loci (L), a potential causal mediator, i.e., RIPK1 (G) and a quantitative trait (T), the following conditions must be satisfied to establish that G is a causal mediator of the association between L and T:

(a) L and G are associated
(b) L and T are associated
(c) L is associated with G, given T
(d) L is independent of T, given G

Although CIT includes tests for linkage (conditions a and b), to control the number of candidate L / G / T trios that are submitted to the CIT function, we performed multiple pre-filtering steps, which are aimed at establishing association between L and G, and L and T, before we submit a particular trio for CIT. Association between L and G is established in the course of the RIPK1 cis-eQTL analysis (described above). Nominal association between L and T is established using matrix eQTL testing results (for that specific cis-eQTL) with an association P < 0.05.

While CIT outputs a P value, it is actually the highest P value of the four constituent hypothesis tests, reflecting each of the conditions required to establish causal mediation. This results in a non-uniform CIT P value distribution under null conditions, which can make appropriate multiple testing correction unreliable. To overcome this, we employed a permutation based approach to assess the significance of candidate causal relationships, where candidate traits (T) are randomly shuffled, separately within each genotype dosage group (0, 1 or 2) for each permutation. The false discovery rate was estimated by counting the proportion of permutations (1000 per trio) with a CIT P value lower than the test CIT P-value.

To minimize the number of false positive inferences, we performed two separate tests for each candidate trio. We tested models that include RIPK1 expression (G) as causal for transgene expression (T) (causal model) and separately, the G being regulated by the T (reactive model). We thus classified candidate genes (T) as regulated by RIPK1, if they achieved a causal model FDR < 0.05 and a reactive model FDR > 0.05. (see Supplementary Table 5 for full causal inference testing results).

Gene set enrichment testing. We then separated the downstream neighbors of RIPK1 into directed sets, subdividing according to whether they are positively or negatively regulated by RIPK1 and calculated gene set enrichments using Fisher's exact text, and one-sided P values (to identify over-representation of gene sets) were adjusted using the Benjamini-Hochberg method13. Gene sets used throughout the enrichment analysis were derived from the molecular signatures database26, as well as multiple expression profiles of AD brains obtained from the literature2,24.

Genetic disease associations shown in Figure 5 are based on a combination of data from the Human Gene Mutation Database26, Online Mendelian Inheritance in Man70, ClinVar71, GWAS Catalog72 and Harmonizome73. The RIPK1 network was visualized using Cytoscape24. Network generation and gene set enrichments were performed using the R project for statistical computing version 3.2.5.

Morris water maze. To assess spatial learning and memory, we performed the Morris water maze test, which was conducted during the light cycle in a circular tank of 1.5 m in diameter located in a room with extra maze cues. The tank was filled with water maintained at 23 °C throughout the duration of the testing. Non-toxic white paint was added to the water to make it opaque. A 14-cm-wide tank of 1.5 m in diameter located in a room with extra maze cues. The tank was filled with water to make it opaque. A 14-cm-wide platform was kept 1.5 cm beneath the surface of the water, and thus was not visible to mice during the test. Mice received four trial per day for 5 consecutive days. If a mouse was not able to find the platform within 60 s, it was gently placed on the platform by the researcher and kept it there for 10 s. At the end of each trial, the mice were placed in a worm cage for 25 s before starting the next trial. The probe trials were performed 24 h after the last training trial. For the probe trials, the platform was removed and the mice were allowed to swim for 60 s. A video camera placed on the ceiling recorded the training and probe trials for each mouse. The results were analyzed using the EthoVisionXT tracking system. The experimenters were blinded to genotype and treatment.

Viral constructs generation and injections. AAVs were generated by Vector BioLabs with the following constructs: AAV1-CamKIIr-caMLKL-2A-eGFP-WPRE and AAV1-CamKIIr-eGFP-WPRE for the experimental and control viruses, respectively. The final titer for each virus was 3.8 × 10^{13} GC/ml.

Using a 5-µl Hamilton syringe, 5 µl of viral suspension were injected into the third ventricle of anesthetized mice. Mice were anesthetized with isoflurane. Using bregma as a reference point, the stereotaxic coordinates of the injections were: −2.46 mm antero-posterior; 0 mm lateral; 2 mm dorso-ventral from the skull. The virus was injected at 0.5 µl/min, after which the needle is left in place for 3 additional min before it was slowly removed. The experimenter was blinded to the genotype of the mice.

Primary neuronal cultures. For the GFP experiments. Primary neurons were isolated from APP/PS1 and NonTg P0 pups. The tissue was then dissociated in Neurobasal media (Thermo Fisher Scientific) and spun down for 3 min at 5,000 g at 4 °C. The supernatant was discarded and the pellet was re-suspended in 1 ml of Neurobasal media supplemented with B27 (Thermo Fisher Scientific). An equal volume per each sample was seeded into a Poly-D-Lysine-coated (10 µg/ml, Sigma-Aldrich) black 96-well tissue culture plate. After 1 day in vitro (DIV), neurons were infected with 1µl/ml CamKIIa-GFP AAV (virus titer 1 × 10^{13} GC/ml) and at day 7 post infections the neurons were treated either with 10 µM 7-CI-O-necrostatin 15 (Nec1S, Millipore) or DMSO (Sigma-Aldrich). Neurons were treated until 15 DIV and the GFP fluorescence was measured at 11 and 15 DIV. For every measurement, fresh media with or without Nec1S was added to the wells. All fluorescent measurements (excitation 488 nm, emission 509 nm) were performed using the Synergy HT multi-mode microplate reader with the Gen5 software (BioTek). The reader was pre-heated to 37 °C to maintain optimal conditions for the neurons during the measurements. The experimenter was blinded to group allocation.

For the NeuN experiments. Neurons were extracted using the same protocol we used for the GFP experiments, plated on round coverslips of 13 mm in diameter coated with Poly-D-Lysine-coated (10 µg/ml, Sigma-Aldrich), and placed into 24-well plates. At DIV 7, neurons were treated with 10µM Nec-1s(Millipore) or DMSO (Sigma-Aldrich). At 11 and 15 DIV, plates were fixed with 4% paraformaldehyde and stored at 4 °C. At the end of the experiment neurons per each time point were stained using a NeuN antibody (1:200, Millipore) and mounted on slides using a mounting media with DAPI (Thermo Fisher Scientific). Four fields per coverslip were imaged using a 40x objective. Lasers at 405 nm and 488 nm were respectively used to image DAPI and NeuN. Image fields were selected in an unbiased manner.

Nec1S in vivo treatment. To reduce necroptosis in vivo, we used the same treatment paradigm used by25. Briefly, 8-month-old 5xFAD and NonTg mice were given a single intraperitoneal injection of 10 mg/kg Nec1S or vehicle (Focus Biomolecules). Mice were then given 0.5 mg/ml Nec1S or vehicle in their drinking water, which contained 2% sucrose, for 21 d.

Statistical analyses. For WB and IF data, the statistical analyses were conducted using GraphPad Prism 5. Sample description and regression statistics were conducted using the R software.

Samples description. The age distribution in AD and CTL cases was assessed with mean, s.d., and quartiles. The comparison was conducted using a t-test for equal variance, after assessing the normal distribution with the Shapiro-Wilk test and the variance homoscedasticity with the Levene's test. The distribution of gender and ApoE genotypes in AD cases and controls was assessed with Fisher's exact test with 20,000 simulated P values.

Variables description. The normality of distribution of continuous variables (Height, Weight, BMI, Brain Weight) and MMSE, considered a continuous interval variable, was assessed with the Shapiro-Wilk test and by visual inspection of the distribution plots. MMSE showed a strong departure from normality, thus the bimodal distribution confirmed by Hartigan test. The distribution between AD cases and controls was assessed using the t-test with equal variance, after assessing homoscedasticity with the Levene's test (normal distributed variables) and non-parametric Mann-Whitney U test (non-normal distributed variables). The distribution of ordinal variables (Braak stage and plaque density) between AD cases and controls was assessed using the Fisher's exact test with 20,000 simulated P values.

Regression analysis. For continuous variables (brain weight), the linear relationship between gene expression and variables was first assessed using a Pearson's correlation. For variables showing a significant correlation (P < 0.05), we conducted a linear regression analysis. For ordinal variables (Braak stage and
plaque density), we conducted an ordinal logistic regression analysis. Finally, for MMSE we conducted a quantile regression analysis due to the bimodal distribution, running 10 models from 10th to the 100th percentiles and correcting P-values with the FDR method. For western blot and IHC, a two-tailed unpaired t-test was used to analyze select pairwise comparisons as specified in the results section. Pearson correlation coefficients corresponding to colocalization in double labeled IHC samples. Costes randomized P-values were obtained using ImageJ (NIH). Where representative images are shown, statistical analyses were done on all the samples. No statistical methods were used to pre-determine sample sizes, but our sample sizes were similar to those reported in previous publications. For the human analyses, no data points were excluded. In the analyses for brain weight, Braak stage, and MMSE the sample size was smaller than the total number of cases, due to unavailability of these variables from the Banner Sun Health Research Institute Brain and Body Donation Program. Unless otherwise specified, in all box-plots the center line represents the median value, the limits represent the 25th and 75th percentile, the whiskers represent the minimum and maximum value of the distribution, and the points represent the single experimental value.

Code availability. We used functions implemented in the R packages: ggplot2, stats, car, limma, annotate, lumiHumanAll.db, lumiHumanIDMapping, AnnotationDbi, MASS, quantreg, multtest (https://cran.r-project.org; https://bioconductor.org). For the construction of the RIPK1 causal gene regulatory network, we used the Matrix eQTL software and the causal inference testing algorithm for assigning sequence reads to genomic features. Bioinformatics 30, 923–930 (2014).

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.


A Supplementary Methods Checklist is available.

Reporting Checklist for Nature Neuroscience

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. For more information, please read Reporting Life Sciences Research.

Please note that in the event of publication, it is mandatory that authors include all relevant methodological and statistical information in the manuscript.

### Statistics reporting, by figure

- Please specify the following information for each panel reporting quantitative data, and where each item is reported (section, e.g. Results, & paragraph number).
- Each figure legend should ideally contain an exact sample size (n) for each experimental group/condition, where n is an exact number and not a range, a clear definition of how n is defined (for example x cells from x slices from x animals from x litters, collected over x days), a description of the statistical test used, the results of the tests, any descriptive statistics and clearly defined error bars if applicable.
- For any experiments using custom statistics, please indicate the test used and stats obtained for each experiment.
- Each figure legend should include a statement of how many times the experiment shown was replicated in the lab; the details of sample collection should be sufficiently clear so that the replicability of the experiment is obvious to the reader.
- For experiments reported in the text but not in the figures, please use the paragraph number instead of the figure number.

Note: Mean and standard deviation are not appropriate on small samples, and plotting independent data points is usually more informative. When technical replicates are reported, error and significance measures reflect the experimental variability and not the variability of the biological process; it is misleading not to state this clearly.

<table>
<thead>
<tr>
<th>TEST USED</th>
<th>n</th>
<th>DESCRIPTIVE STATS (AVERAGE, VARIANCE)</th>
<th>P VALUE</th>
<th>DEGREES OF FREEDOM &amp; F/T/Z/R/ETC VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>one-way ANOVA</td>
<td>9, 9, 10, 15</td>
<td>mice from at least 3 litters/group</td>
<td>error bars are mean +/- SEM</td>
<td>p = 0.044</td>
</tr>
<tr>
<td>unpaired t-test</td>
<td>15</td>
<td>slices from 10 mice</td>
<td>error bars are mean +/- SEM</td>
<td>p = 0.0006</td>
</tr>
<tr>
<td>RIPPA</td>
<td>11, 12</td>
<td>number of human cases</td>
<td>box plot</td>
<td>p = 0.002</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>----------------------</td>
<td>---</td>
<td>-----------</td>
</tr>
<tr>
<td>1b</td>
<td>unpaired t-test</td>
<td>Fig. legend</td>
<td>11, 12</td>
<td>number of human cases</td>
</tr>
<tr>
<td>1c</td>
<td>RIPA</td>
<td>Fig. legend</td>
<td>11, 12</td>
<td>number of human cases</td>
</tr>
<tr>
<td>1c</td>
<td>UREA</td>
<td>Fig. legend</td>
<td>11, 12</td>
<td>number of human cases</td>
</tr>
<tr>
<td>1e</td>
<td>unpaired t-test</td>
<td>Fig. legend</td>
<td>12, 14</td>
<td>number of human cases</td>
</tr>
<tr>
<td>1f</td>
<td>unpaired t-test</td>
<td>Fig. legend</td>
<td>12, 14</td>
<td>number of human cases</td>
</tr>
<tr>
<td>1g</td>
<td>unpaired t-test</td>
<td>Fig. legend</td>
<td>12, 14</td>
<td>number of human cases</td>
</tr>
<tr>
<td>2b</td>
<td>unpaired t-test</td>
<td>Fig. legend</td>
<td>15, 15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>2d</td>
<td>unpaired t-test</td>
<td>Fig. legend</td>
<td>15, 15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>2f</td>
<td>unpaired t-test</td>
<td>Fig. legend</td>
<td>15, 15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>2g</td>
<td>unpaired t-test</td>
<td>Fig. legend</td>
<td>7, 7</td>
<td>number of human cases</td>
</tr>
<tr>
<td>2i</td>
<td>unpaired t-test</td>
<td>Fig. legend</td>
<td>11, 13</td>
<td>number of human cases</td>
</tr>
<tr>
<td>2k</td>
<td>unpaired t-test</td>
<td>Fig. legend</td>
<td>11, 13</td>
<td>number of human cases</td>
</tr>
<tr>
<td>2l</td>
<td>Mander’s correlation</td>
<td>Fig. legend</td>
<td>15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>2l</td>
<td>AD</td>
<td>Fig. legend</td>
<td>15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>2m</td>
<td>unpaired t-test</td>
<td>Fig. legend</td>
<td>15, 15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>3a</td>
<td>moderated t-test</td>
<td>Fig. legend</td>
<td>97, 98</td>
<td>number of human cases</td>
</tr>
<tr>
<td>3b</td>
<td>moderated t-test</td>
<td>Fig. legend</td>
<td>97, 98</td>
<td>number of human cases</td>
</tr>
<tr>
<td>3c</td>
<td>moderated t-test</td>
<td>Fig. legend</td>
<td>97, 98</td>
<td>number of human cases</td>
</tr>
<tr>
<td>3d</td>
<td>linear regression</td>
<td>Fig. legend</td>
<td>93</td>
<td>number of human cases</td>
</tr>
<tr>
<td>4a</td>
<td>ordinal logistical regression</td>
<td>Fig. legend</td>
<td>94</td>
<td>number of human cases</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>ordinal logistical regression</td>
<td>Fig. legend</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>4c</td>
<td>ordinal logistical regression</td>
<td>Fig. legend</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>4d</td>
<td>Mander's correlation</td>
<td>Fig. legend</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>4e</td>
<td>Mander's correlation</td>
<td>Fig. legend</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>4f</td>
<td>quantile regression</td>
<td>Fig. legend</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>4g</td>
<td>quantile regression</td>
<td>Fig. legend</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>4h</td>
<td>quantile regression</td>
<td>Fig. legend</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>4i</td>
<td>quantile regression</td>
<td>Fig. legend</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>6a</td>
<td>two-way ANOVA followed by Bonferroni post hoc</td>
<td>Fig. legend</td>
<td>14, 12, 14, 14</td>
</tr>
<tr>
<td></td>
<td>6b</td>
<td>two-way ANOVA followed by Bonferroni post hoc</td>
<td>Fig. legend</td>
<td>14, 12, 14, 14</td>
</tr>
<tr>
<td></td>
<td>6c</td>
<td>one-way ANOVA followed by Bonferroni post hoc</td>
<td>Fig. legend</td>
<td>14, 12, 14, 14</td>
</tr>
<tr>
<td></td>
<td>6d</td>
<td>linear regression</td>
<td>Fig. legend</td>
<td>14, 12, 14, 14</td>
</tr>
<tr>
<td></td>
<td>6e</td>
<td>one-way ANOVA followed by Bonferroni post hoc</td>
<td>Fig. legend</td>
<td>14, 12, 14, 14</td>
</tr>
<tr>
<td></td>
<td>6h</td>
<td>Mander's correlation</td>
<td>Fig. legend</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>6i</td>
<td>Mander's correlation</td>
<td>Fig. legend</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>6j</td>
<td>Mander's correlation</td>
<td>Fig. legend</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>6k</td>
<td>one-way ANOVA followed by Bonferroni post hoc</td>
<td>Fig. legend</td>
<td>8, 8, 8</td>
</tr>
<tr>
<td></td>
<td>7b</td>
<td>one-way ANOVA followed by Bonferroni post hoc</td>
<td>Fig. legend</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>7c</td>
<td>linear regression</td>
<td>Fig. legend</td>
<td>18</td>
</tr>
<tr>
<td>Page</td>
<td>Figure</td>
<td>Description</td>
<td>N</td>
<td>Details</td>
</tr>
<tr>
<td>------</td>
<td>--------</td>
<td>-------------</td>
<td>---</td>
<td>---------</td>
</tr>
<tr>
<td>8a</td>
<td></td>
<td>two-way ANOVA followed by Bonferroni post hoc</td>
<td>12, 36</td>
<td>4 wells per mouse/treatment/time point from 3 WT and 9 APP/PS1</td>
</tr>
<tr>
<td>8b</td>
<td></td>
<td>two-way ANOVA followed by Bonferroni post hoc</td>
<td>4, 4, 5, 5</td>
<td>1 well per mouse</td>
</tr>
<tr>
<td>8c</td>
<td></td>
<td>two-way ANOVA followed by Bonferroni post hoc</td>
<td>16, 72</td>
<td>8 wells per mouse/treatment/time point from 2 WT and 9 APP/PS1</td>
</tr>
<tr>
<td>8d</td>
<td></td>
<td>one-way ANOVA followed by Bonferroni post hoc</td>
<td>4, 4, 5, 5</td>
<td>1 well per mouse</td>
</tr>
<tr>
<td>8f</td>
<td></td>
<td>unpaired t-test</td>
<td>4, 5</td>
<td>mice</td>
</tr>
<tr>
<td>8g</td>
<td></td>
<td>unpaired t-test</td>
<td>4, 4</td>
<td>mice</td>
</tr>
<tr>
<td>8h</td>
<td></td>
<td>unpaired t-test</td>
<td>11, 12</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8i</td>
<td></td>
<td>unpaired t-test</td>
<td>11, 12</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8j</td>
<td></td>
<td>unpaired t-test</td>
<td>11, 12</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8k</td>
<td></td>
<td>unpaired t-test</td>
<td>11, 12</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8l</td>
<td></td>
<td>unpaired t-test</td>
<td>11, 12</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8m</td>
<td></td>
<td>unpaired t-test</td>
<td>15, 15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8n</td>
<td></td>
<td>Mander’s correlation</td>
<td>15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8o</td>
<td></td>
<td>Mander’s correlation</td>
<td>15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8p</td>
<td></td>
<td>unpaired t-test</td>
<td>15, 15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8q</td>
<td></td>
<td>Mander’s correlation</td>
<td>15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8r</td>
<td></td>
<td>Mander’s correlation</td>
<td>15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8s</td>
<td></td>
<td>Mander’s correlation</td>
<td>15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8t</td>
<td></td>
<td>unpaired t-test</td>
<td>15, 15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8u</td>
<td></td>
<td>Mander’s correlation</td>
<td>15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8v</td>
<td></td>
<td>Mander’s correlation</td>
<td>15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8w</td>
<td></td>
<td>Mander’s correlation</td>
<td>15</td>
<td>number of human cases</td>
</tr>
<tr>
<td>8x</td>
<td></td>
<td>unpaired t-test</td>
<td>7, 5</td>
<td>mice</td>
</tr>
<tr>
<td>8y</td>
<td></td>
<td>unpaired t-test</td>
<td>8, 8</td>
<td>mice</td>
</tr>
</tbody>
</table>
## Representative figures

1. Are any representative images shown (including Western blots and immunohistochemistry/staining) in the paper?
   
   If so, what figure(s)?

   Fig. 1a, d: western blots and immunofluorescent staining
   Fig. 2a, c, e, g, h, j, and l: western blots and immunofluorescent staining
   Fig. 4d, e: immunofluorescent staining
   Fig. 6f-j: immunofluorescent staining
   Fig. 7a: immunofluorescent staining
   Fig. 8e: immunofluorescent staining
   Suppl. Fig. 1a: western blots
   Suppl. Fig. 3a, c, e: immunofluorescent staining
   Suppl. Fig. 4a: immunofluorescent staining
   Suppl. Fig. 5a-c: immunofluorescent staining
   Suppl. Fig. 7e-f: immunofluorescent staining
   Suppl. Fig. 8a-b: immunohistochemistry
   Suppl. Fig. 10e-f: immunofluorescent staining
   
   While representative images are shown, the statistical analyses were performed on the entire sample as indicated in each figure legend.

   We reported this statement in the Statistical Analyses section, within the Methods.

## Statistics and general methods

1. Is there a justification of the sample size?

   If so, how was it justified?

   Where (section, paragraph #)?

   Even if no sample size calculation was performed, authors should report why the sample size is adequate to measure their effect size.

   An a priori power analysis was not performed, our sample sizes are similar to those reported in previously published papers, in which this sample size has been demonstrated to be appropriate to detect significant effects.

   We reported this statement in the Statistical Analyses section, within the Methods.
2. Are statistical tests justified as appropriate for every figure?
   Where (section, paragraph #)?

   a. If there is a section summarizing the statistical methods in the methods, is the statistical test for each experiment clearly defined?
   Yes, there is a "Statistical Analyses Section" within the Methods. However, the statistical test used for each experiment is clearly defined in the figure legends.

   b. Do the data meet the assumptions of the specific statistical test you chose (e.g. normality for a parametric test)?
   Where is this described (section, paragraph #)?
   Yes.
   This statement is reported in the Statistical Analyses section, within the Methods.

   c. Is there any estimate of variance within each group of data?
   Is the variance similar between groups that are being statistically compared?
   Where is this described (section, paragraph #)?
   The estimate of variance is reported in graphical form in each graph.
   Yes, the variance is similar between groups.
   This statement is reported in the Statistical Analyses section, within the Methods.

   d. Are tests specified as one- or two-sided?
   The tests were two sided

   e. Are there adjustments for multiple comparisons?
   Yes, there are. The results showed in Fig. 3a-c and Fig. 4f-i were corrected for multiple comparisons. The results showed in Fig. 6c, Fig. 6e, Fig. 6k, Fig. 7b, Fig. 8b, and Fig. 8d were analyzed by one-way ANOVA, followed by Bonferroni's post hoc analysis. The results showed in Fig. 6a-b, Fig. 8a, Fig. 8c were analyzed by two-way ANOVA, followed by Bonferroni's post hoc analysis.

3. To promote transparency, *Nature Neuroscience* has stopped allowing bar graphs to report statistics in the papers it publishes. If you have bar graphs in your paper, please make sure to switch them to dot plots (with central and dispersion statistics displayed) or to box-and-whisker plots to show data distributions.

4. Are criteria for excluding data points reported?
   Was this criterion established prior to data collection?
   Where is this described (section, paragraph #)?
   No data points were excluded. In the analyses for brain weight (Fig. 3d), Braak stage (Fig. 4a-c), and MMSE (Fig. 4d-g) the sample size was smaller than the total number of cases, due to unavailability of these variables from the Banner Sun Health Research Institute Brain and Body Donation Program.
   All the sample sizes were reported in Supplementary Tables 1 and 2.

5. Define the method of randomization used to assign subjects (or samples) to the experimental groups and to collect and process data.
   If no randomization was used, state so.
   Where does this appear (section, paragraph #)?
   Human cases were selected randomly among the tissue available. Mice were assigned to a specific group based on their genotype after birth and there were no other factors that determined group selection. No mice were excluded.
   This statement is reported in the Human tissue and mice section, within the Methods.
6. Is a statement of the extent to which investigator knew the group allocation during the experiment and in assessing outcome included?

If no blinding was done, state so.

Where (section, paragraph #)?

For the behavioral analyses shown in Fig. 6, the experimenters were blinded to genotype and treatment. No blinding was done for the other experiments.

This statement is reported in the Morris water maze and Human tissue and mice sections, within the Methods.

7. For experiments in live vertebrates, is a statement of compliance with ethical guidelines/regulations included?

Where (section, paragraph #)?

Yes.

Human tissue and mice section within the Methods

8. Is the species of the animals used reported?

Where (section, paragraph #)?

Yes.

Human tissue and mice section within the Methods

9. Is the strain of the animals (including background strains of KO/transgenic animals used) reported?

Where (section, paragraph #)?

Yes.

Human tissue and mice section within the Methods

10. Is the sex of the animals/subjects used reported?

Where (section, paragraph #)?

Yes.

Human tissue and mice section within the Methods

11. Is the age of the animals/subjects reported?

Where (section, paragraph #)?

Yes, the age for animals/subject is reported. For human cases, see Supplementary Tables 1. For mice, see first and second paragraphs in the "Brains with AD-like pathology are more prone to necroptosis" section within the Results.

12. For animals housed in a vivarium, is the light/dark cycle reported?

Where (section, paragraph #)?

Yes.

Human tissue and mice section within the Methods.

13. For animals housed in a vivarium, is the housing group (i.e. number of animals per cage) reported?

Where (section, paragraph #)?

Yes.

Human tissue and mice section within the Methods.

14. For behavioral experiments, is the time of day reported (e.g. light or dark cycle)?

Where (section, paragraph #)?

Yes.

Morris water maze section within the Methods

15. Is the previous history of the animals/subjects (e.g. prior drug administration, surgery, behavioral testing) reported?

Where (section, paragraph #)?

For human subjects, we do not have access to their previous history.

For the behavioral studies in mice, we used mice that underwent stereotaxic surgeries for viral injections as reported in the second and third paragraphs of the "Brains with AD-like pathology are more prone to necroptosis" section within the Results.

a. If multiple behavioral tests were conducted in the same group of animals, is this reported?

Where (section, paragraph #)?

N/A
16. If any animals/subjects were excluded from analysis, is this reported?

Where (section, paragraph #)?

No animals/subjects were excluded from the analyses.

a. How were the criteria for exclusion defined?
Where is this described (section, paragraph #)?

N/A

b. Specify reasons for any discrepancy between the number of animals at the beginning and end of the study.
Where is this described (section, paragraph #)?

N/A

Reagents

1. Have antibodies been validated for use in the system under study (assay and species)?

Yes

a. Is antibody catalog number given?
Where does this appear (section, paragraph #)?

Yes.

"Immunohistochemistry" and "Western blot and ELISA" sections within the Methods.

b. Where were the validation data reported (citation, supplementary information, Antibodypedia)?
Where does this appear (section, paragraph #)?

Supplementary Figures 10 and "Antibody Validation" section within the Methods.

2. Cell line identity

N/A

a. Are any cell lines used in this paper listed in the database of commonly misidentified cell lines maintained by ICLAC and NCBI Biosample?
Where (section, paragraph #)?

N/A

b. If yes, include in the Methods section a scientific justification of their use--indicate here in which section and paragraph the justification can be found.

N/A

c. For each cell line, include in the Methods section a statement that specifies:
   - the source of the cell lines
   - have the cell lines been authenticated? If so, by which method?
   - have the cell lines been tested for mycoplasma contamination?
Where (section, paragraph #)?

N/A
Data availability

Provide a Data availability statement in the Methods section under "Data availability", which should include, where applicable:

- Accession codes for deposited data
- Other unique identifiers (such as DOIs and hyperlinks for any other datasets)
- At a minimum, a statement confirming that all relevant data are available from the authors
- Formal citations of datasets that are assigned DOIs
- A statement regarding data available in the manuscript as source data
- A statement regarding data available with restrictions

See our data availability and data citations policy page for more information.

Data deposition in a public repository is mandatory for:

a. Protein, DNA and RNA sequences
b. Macromolecular structures
c. Crystallographic data for small molecules
d. Microarray data

Deposition is strongly recommended for many other datasets for which structured public repositories exist; more details on our data policy are available here. We encourage the provision of other source data in supplementary information or in unstructured repositories such as Figshare and Dryad.

We encourage publication of Data Descriptors (see Scientific Data) to maximize data reuse.

Where is the Data Availability statement provided (section, paragraph #)?

- The statement "The data that support the findings of this study are available from the corresponding author upon reasonable request" is reported in the "Data availability" section at the end of the Methods.
- All the datasets used in this study, with ID and name, are reported in the "Accession codes" section. Specifically:
  - The microarray data are available in the Gene Expression File 1. This sentence is reported in the "Necroptosis activation negatively correlates with brain weight and MMSE" section within the Results.
  - The external dataset used for validation was already deposited in the database Gene Expression Omnibus (GEO). The ID, GSE5281, is reported in the "Necroptosis activation negatively correlates with brain weight and MMSE" section within the Results.
  - The external datasets used for generating the RIPK1 causal gene regulatory network were already deposited on www.synapse.org: syn3157743: MSBB AD RNA-seq Gene Expression Data; and syn4645334: MSBB AD Whole Exome Sequencing (WES) Data. The IDs are reported in the "Construction of RIPK1 causal gene regulatory network" section within the Methods.

Computer code/software

Any custom algorithm/software that is central to the methods must be supplied by the authors in a usable and readable form for readers at the time of publication. However, referees may ask for this information at any time during the review process.

1. Identify all custom software or scripts that were required to conduct the study and where in the procedures each was used.

   N/A

2. If computer code was used to generate results that are central to the paper’s conclusions, include a statement in the Methods section under "Code availability" to indicate whether and how the code can be accessed. Include version information as necessary and any restrictions on availability.

   We used functions implemented in the R packages: ggplot2, stats, car, lumi, limma, annotate, lumiHumanAll.db, lumiHumanIDMapping, AnnotationDbi, MASS, quantreg, multtest (https://cran.r-project.org; https://bioconductor.org). For the construction of the RIPK1 causal gene regulatory network, we used the Matrix eQTL software and the causal inference testing (CIT), available in Shabalin, Bioinformatics 2012, and Millstein et al., BMC Genet 2009.

Human subjects
1. Which IRB approved the protocol?  
   Where is this stated (section, paragraph #)?  

   Only post mortem human tissue was used and thus no IRB approval was needed

2. Is demographic information on all subjects provided?  
   Where (section, paragraph #)?  

   Yes, Supplementary Tables 1-2.

3. Is the number of human subjects, their age and sex clearly defined?  
   Where (section, paragraph #)?  

   Yes, Supplementary Table 1.

4. Are the inclusion and exclusion criteria (if any) clearly specified?  
   Where (section, paragraph #)?  

   N/A

5. How well were the groups matched?  
   Where is this information described (section, paragraph #)?  

   The groups were matched for age and sex. This information is reported in Supplementary Table 1.

6. Is a statement included confirming that informed consent was obtained from all subjects?  
   Where (section, paragraph #)?  

   N/A

7. For publication of patient photos, is a statement included confirming that consent to publish was obtained?  
   Where (section, paragraph #)?  

   N/A

### fMRI studies

For papers reporting functional imaging (fMRI) results please ensure that these minimal reporting guidelines are met and that all this information is clearly provided in the methods:

1. Were any subjects scanned but then rejected for the analysis after the data was collected?  
   
   N/A

   a. If yes, is the number rejected and reasons for rejection described?  
      Where (section, paragraph #)?  

      N/A

2. Is the number of blocks, trials or experimental units per session and/or subjects specified?  
   Where (section, paragraph #)?  

   N/A

3. Is the length of each trial and interval between trials specified?  
   N/A

4. Is a blocked, event-related, or mixed design being used? If applicable, please specify the block length or how the event-related or mixed design was optimized.  
   N/A
5. Is the task design clearly described?
   Where (section, paragraph #)?
   N/A

6. How was behavioral performance measured?
   N/A

7. Is an ANOVA or factorial design being used?
   N/A

8. For data acquisition, is a whole brain scan used?
   If not, state area of acquisition.
   N/A

9. How was this region determined?
   N/A

10. Is the field strength (in Tesla) of the MRI system stated?
    a. Is the pulse sequence type (gradient/spin echo, EPI/spiral) stated?
       N/A
    b. Are the field-of-view, matrix size, slice thickness, and TE/TR/flip angle clearly stated?
       N/A

11. Are the software and specific parameters (model/functions, smoothing kernel size if applicable, etc.) used for data processing and pre-processing clearly stated?
    N/A

12. Is the coordinate space for the anatomical/functional imaging data clearly defined as subject/native space or standardized stereotaxic space, e.g., original Talairach, MNI305, ICBM152, etc? Where (section, paragraph #)?
    N/A

13. If there was data normalization/standardization to a specific space template, are the type of transformation (linear vs. nonlinear) used and image types being transformed clearly described? Where (section, paragraph #)?
    N/A

14. How were anatomical locations determined, e.g., via an automated labeling algorithm (AAL), standardized coordinate database (Talairach daemon), probabilistic atlases, etc.?
    N/A

15. Were any additional regressors (behavioral covariates, motion etc) used?
    N/A

16. Is the contrast construction clearly defined?
    N/A

17. Were repeated measures used (multiple measurements per subject)?
    N/A
<table>
<thead>
<tr>
<th>Question</th>
<th>Answer</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. If so, are the method to account for within subject correlation and the assumptions made about variance clearly stated?</td>
<td>N/A</td>
</tr>
<tr>
<td>18. If the threshold used for inference and visualization in figures varies, is this clearly stated?</td>
<td>N/A</td>
</tr>
<tr>
<td>19. Are statistical inferences corrected for multiple comparisons?</td>
<td>N/A</td>
</tr>
<tr>
<td>a. If not, is this labeled as uncorrected?</td>
<td>N/A</td>
</tr>
<tr>
<td>20. Are the results based on an ROI (region of interest) analysis?</td>
<td>N/A</td>
</tr>
<tr>
<td>a. If so, is the rationale clearly described?</td>
<td>N/A</td>
</tr>
<tr>
<td>b. How were the ROI’s defined (functional vs anatomical localization)?</td>
<td>N/A</td>
</tr>
<tr>
<td>21. Is there correction for multiple comparisons within each voxel?</td>
<td>N/A</td>
</tr>
<tr>
<td>22. For cluster-wise significance, is the cluster-defining threshold and the corrected significance level defined?</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Additional comments

Additional Comments