Neurons burdened by DNA double-strand breaks incite microglia activation through antiviral-like signaling in neurodegeneration

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DNA double-strand breaks (DSBs) are linked to neurodegeneration and senescence. However, it is not clear how DSB-bearing neurons influence neuroinflammation associated with neurodegeneration. Here, we characterize DSB-bearing neurons from the CK-p25 mouse model of neurodegeneration using single-nucleus, bulk, and spatial transcriptomic techniques. DSB-bearing neurons enter a late-stage DNA damage response marked by nuclear factor κB (NFκB)–activated senescent and antiviral immune pathways. In humans, Alzheimer’s disease pathology is closely associated with immune activation in excitatory neurons. Spatial transcriptomics reveal that regions of CK-p25 brain tissue dense with DSB-bearing neurons harbor signatures of inflammatory microglia, which is ameliorated by NFκB knockdown in neurons. Inhibition of NFκB in DSB-bearing neurons also reduces microglia activation in organotypic mouse brain slice culture. In conclusion, DSBs activate immune pathways in neurons, which in turn adopt a senescence-associated secretory phenotype to elicit microglia activation. These findings highlight a previously unidentified role for neurons in the mechanism of disease-associated neuroinflammation.

INTRODUCTION

Loss of genomic integrity is linked to aging and neurodegeneration (1, 2). DNA damage repair pathways are transcriptionally prominent in the aging brain, and many age-associated neurodegenerative diseases exhibit both accumulation of DNA lesions and reduced DNA repair efficiency (3–5). The most toxic of these lesions, the DNA double-strand break (DSB), can drive many phenotypes of aging including senescence, mutation, and cell death. Postmitotic neurons are particularly susceptible to these threats due to their long life span, high metabolic activity, and limited DSB repair capacity. While DSB accumulation in neurons is a well-documented feature of aging and neurodegeneration, the transcriptional profile adopted by such population of neurons remains largely unknown.

The accumulation of DSBs is an early feature of Alzheimer’s disease (AD), suggesting that they may act as an initiating lesion of toxicity (6). Multiple mouse models of neurodegeneration phenotype increased DSBs at early pathological stages, including the Tau P301S and P301L tauopathy models, the inducible CK-p25 model, and the hAPP-J20 amyloid pathology model (7–9). Recent studies characterizing DNA strand breaks in postmitotic neurons reveal that break location may underlie the functional decline of the brain in aging and neurodegenerative disease (10, 11). However, regardless of location, the downstream biological effects of DSB burden in neurons are unclear.

RESULTS

Identification of DSB-bearing neurons at early stages of disease in a mouse model of neurodegeneration

We used the CK-p25 mouse model of inducible neurodegeneration to understand how DSB-bearing neurons contribute to disease development. In these mice, the CamkII promoter drives the expression of the neurotoxic protein fragment p25 through a doxycycline (dox)–off system (13). P25 is the calpain-cleaved product of p35, an activator of cyclin-dependent kinase 5 (CDK5). Previously, we determined that the first pathologies observed in these mice are increased DSBs in neurons (7) and activation of microglia (16). These
that all \( \gamma H2AX \) hi nuclei were also labeled by CamKIIa (CamKIIa hi), and NeuN. In support of our immunostaining analyses, we found specific gene (fig. S1, G and H). \( H2AX^+ \) cells (89.15 ± 3.55%) with \( \gamma \) with green fluorescent protein (GFP), which is fused to the p25 transcription factor (fig. S1, E and F). \( H2AX^+ \) nuclei were detectable as early as 1 week after induction (1.038 ± 0.2627% population), peaked at 2 weeks (4.614 ± 0.9416% population), and gradually decreased thereafter (Fig. 1B). The significant reduction in \( H2AX^{hi} \) nuclei at 6 weeks corresponds with previous observations of neuronal loss in this model, suggesting that \( H2AX^{hi} \) cells degenerate by 6 weeks (15, 18). We were able to observe similar \( H2AX^{hi} \) population dynamics using immunofluorescent microscopy (fig. S1, A and B). We did not detect \( H2AX^{hi} \) nuclei after only 4 days off drug, suggesting that 1 week is the earliest time point at which this population appears.

We next sought to identify the cell type composition of \( H2AX^{hi} \) nuclei. Through immunofluorescent microscopy, we found that while most \( H2AX^{hi} \) nuclei were immunoreactive for NeuN, a general neuron marker, 21.51 ± 2.36% did not have strong NeuN immunoreactivity (fig. S1, C and D). Furthermore, none of the \( H2AX^+ \) nuclei overlapped with markers of microglia (Iba1), astrocytes [glial fibrillary acidic protein (GFAP)], or oligodendrocytes and oligodendrocyte precursor cells (Olig2), indicating that the 21.51% with low NeuN immunoreactivity were not likely of glial origin (fig. S1, C and D). We found that 74.17 ± 1.27% of \( H2AX^+ \) cells with low NeuN immunoreactivity were labeled with Neurol1, a neuronal transcription factor (fig. S1, E and F). \( H2AX^+ \) cells (88.98 ± 2.16%) with high NeuN immunoreactivity were labeled with Neurol1 as well (fig. S1, E and F). In addition, we observed that 85.22 ± 4.52% of \( H2AX^+ \) cells with low NeuN immunoreactivity were labeled with green fluorescent protein (GFP), which is fused to the p25 transgene and driven by the CamKII promoter, an excitatory neuron-specific gene (fig. S1, G and H). \( H2AX^+ \) cells (89.15 ± 3.55%) with high NeuN expression were also labeled with GFP (fig. S1, G and H). These immunostaining analyses indicated that \( H2AX^+ \) nuclei with low NeuN immunoreactivity were likely excitatory neurons with some level of cell identity degradation. Degradation of neuronal identity is observed within aging and AD patients (19), and reduced NeuN expression has been proposed as an indicator of declining neuronal health (20). Reduced NeuN expression or reduced NeuN immunoreactivity in neurons is an established feature of neuronal damage due to irradiation, ischemia, and axotomy (21-23).

To follow up on this observation, we performed additional flow cytometry analyses of \( H2AX^{mu} \) nuclei, staining for both CamKIIa and NeuN. In support of our immunostaining analyses, we found that all \( H2AX^{hi} \) nuclei were also labeled by CamKIIa (CamKIIa hi), even those with low NeuN immunoreactivity (NeuN lo) (fig. S1, I and J). Last, to validate that DNA damage response pathways were active in \( H2AX^{hi} \) nuclei, we confirmed colabeling with the active form of ATM kinase (p-ATM S1981) (fig. S1, K and L).

**DSB-bearing neurons express antiviral and senescence genes**

To formally analyze the population of \( H2AX^{hi} \) cells, we classified nuclei into four distinct populations: \( H2AX^{lo} \) NeuN hi (“baseline” neurons), \( H2AX^{hi} \) NeuN hi (stage 1 neurons), \( H2AX^{hi} \) NeuN lo (stage 2 neurons), and \( H2AX^{lo} \) NeuN lo (putative nonneuronal cells, which we refer to as “other”) (Fig. 1C). We used FANS followed by bulk RNA-seq to transcriptionally profile each population (Fig. 1D). All nuclei were collected for sequencing at the 2-week time point, which was when we observed the peak density of \( H2AX^{hi} \) nuclei.

We performed three differential expression analyses to characterize gene expression changes in these populations: stage 1 versus CK-p25 baseline, stage 2 versus CK-p25 baseline, and stage 2 versus stage 1. Large transcriptional changes were observed in the first two comparisons. A total of 3031 up-regulated and 717 down-regulated transcripts were identified in stage 1, and 5055 up-regulated and 3792 down-regulated transcripts were identified in stage 2 (log2 fold change ≥ [1.0], adjusted \( P < 0.05 \)) (fig. S2, A to C). Comparatively, few transcripts were found differentially expressed between stage 2 and stage 1 (186 up-regulated and 42 down-regulated transcripts in stage 2). Gene set enrichment analysis (GSEA) (24) revealed that both stage 1 and stage 2 neurons displayed a significant enrichment for genes implicated in DSB repair, apoptotic signaling, and cell cycle reentry, and a significant reduction in synaptic processes (Fig. 1E). These pathways were previously found to be dysregulated in both the CK-p25 mouse and AD human brain tissue (7, 25).

We also found that a number of innate immune pathways were enriched in stage 1 and stage 2 neurons. This included Gene Ontology terms “Senescence-associated secretory phenotype (SASP),” “Cytosolic sensors of pathogen-associated DNA,” and “Positive regulation of innate immune response” (Fig. 1E). Upon closer inspection, we observed an enrichment of genes linked to DSB-mediated activation of SASP signaling, particularly in stage 2 neurons. This included nucleic acid sensors such as Cgas and Zbp1; NfkB subcomponents Rela and Relb; SASP factors Il6, Il15, Cc12, and Cxcl10; and interferon (IFN)–stimulated genes Isg15 and Ifitm3 (Fig. 1F). Notably, a number of these genes are expressed in neurons following viral infection (14, 26). These genes are also classic markers of senescence, suggesting that DSB accumulation elicits senescent and antiviral-like pathways in neurons.

Because the CK-p25 model is characterized by the development of type 1 IFN–responsive microglia specifically by 2-week induction (16), we wanted to determine whether DSB-bearing neurons express cytokines before microglia. To do this, we performed multiplexed fluorescent RNAscope in situ hybridization in the CK-p25 cortex after 1 and 2 weeks of induction. We focused our profiling on Cxcl10 and Cc12 because these pro-inflammatory chemotactic molecules were highly expressed in stage 2 neurons and are known to be secreted by neurons upon viral infection, downstream of type 1 IFN (14, 27, 28). First, we performed RNAscope for the excitatory neuron marker Camk2a to again confirm whether \( H2AX^+ \) nuclei were of neuronal origin. Consistent with our RNA-seq and flow cytometry data, 98.81 ± 1.19% of \( H2AX^+ \) nuclei also expressed Camk2a (fig. S2D). This high positivity rate confirms that \( H2AX^+ \)...
Fig. 1. Neurons marked by DNA DSBs activate inflammatory signaling at early stages of neurodegeneration. 

(A) Flow cytometry dot plots of γH2AX\textsuperscript{hi} nuclei (turquoise) from CK and CK-p25 cortex. (B) Quantification of percent γH2AX\textsuperscript{hi} for 1 to 6 weeks. Each data point represents percent γH2AX\textsuperscript{hi} nuclei from one mouse. (C) Representative dot plots of γH2AX and NeuN immunoreactivity in 2-week CK-p25 cortex. (D) RNA-seq workflow. Bulk RNA-seq: n = 2 per genotype for bulk RNA-seq, 2-week time point. snRNA-seq: n = 3 per genotype at 1- and 2-week time points. (E) Differential Gene Ontology terms from bulk RNA-seq. FDR, false discovery rate; IFN, interferon; NES, normalized enrichment score. (F) Heatmap of differentially expressed genes associated with inflammation from bulk RNA-seq. (G) Left: Representative images of \textit{Ccl2} γH2AX staining for 1-, 2-, and 6-week CK-p25 cortex. Right: Quantification of number γH2AX\textsuperscript{+} and γH2AX\textsuperscript{−} cells with ≥2 Ccl2 puncta. Data points represent one image from one mouse. Three to four images were taken per mouse (1 week, n = 4; 2 weeks, n = 5; 6 weeks, n = 3). DAPI, 4′,6-diamidino-2-phenylindole. (H) Top: Representative image of p65 in 2-week CK-p25. Bottom: Quantification of p65 mean intensity for γH2AX\textsuperscript{+} and γH2AX\textsuperscript{−} nuclei. Data points represent 20 to 60 nuclei from one mouse. (I) UMAP (Uniform Manifold Approximation and Projection) of gated populations from CK and CK-p25 cortex at 1- and 2-week time points. (J) Marker gene expression for each cell type cluster. (K and L) Trajectory analysis of Ex0, Ex1, Ex2, Ex3, and stage 2 neurons. Smoothened gene signature expression across pseudotime. Immune = stage 2 immune genes. P_{adj} < 0.05, log fold change ≥ 1.0 (K). Individual genes across trajectory (L). Error bars represent SEM. ****P < 0.0001, ***P < 0.01, and **P < 0.05; ns, not significant. One-way analysis of variance (ANOVA) with Tukey’s test for multiple comparisons (B and H). Two-way ANOVA followed by Sidak’s test for multiple comparisons (G). Data are pooled from four independent experiments (B). Data are representative of two independent experiments (G and H).
nuclei are nearly exclusively excitatory neurons. RNAscope analysis also revealed that γH2AX\(^+\) nuclei were the only cells to express Cxcl10 and Ccl2 at the 1-week time point. In contrast, Cxcl10 and Ccl2 gene expression was highly enriched in γH2AX-immunonegative nuclei at the 2-week time point (Fig. 1G and fig. S2E). Both γH2AX\(^+\) and γH2AX\(^-\) nuclei had reduced Cxcl10 and Ccl2 expression by 6 weeks (Fig. 1G and fig. S2E). Analysis of the cell type markers associated with Cxcl10 expression revealed that most γH2AX\(^+\) nuclei expressing Cxcl10 were astrocytes and microglia (fig. S2F). Together, these data indicate that expression of Cxcl10 and Ccl2 in γH2AX\(^+\) cells precedes their expression in glial cells and that cytokine secretion from DSB-bearing neurons may be an early mechanism of glial cell recruitment and activation in the CK-p25 brain.

To identify the master regulators of DSB-associated neuronal immune signaling, we performed transcription factor enrichment analysis using Enrichr (29). A subset of immune pathway genes was extracted from the significantly up-regulated genes in stage 2 neurons for analysis. The overlap between this immune gene module and transcription factor target genes was then calculated. Multiple subunits of the NFKB complex were consistently enriched across Enrichr transcription factor libraries, including Rela, NFKb1, and Relb (table S1). Notably, the NFKB transcription factor plays a well-established role in SASP activation and the DSB response (30, 31). We chose to focus on Rela, also known as p65, because it is a core member of the canonical NFKB complex, and it was most frequently enriched in the Enrichr analysis. We stained for p65 in 1-, 2-, and 6-week CK and CK-p25 cortices. NFKB is normally sequestered in the cytosol but translocates to the nucleus to form an active complex upon cellular insult. Nuclear p65 intensity was significantly higher in γH2AX\(^+\) cells compared to other γH2AX\(^-\) cells at the 1- and 2-week time points (Fig. 1H and fig. S3A), supporting evidence of increased NFKB transcriptional activity. By 6 weeks, there was no significant difference between γH2AX\(^+\) and γH2AX\(^-\) nuclei in nuclear p65 intensity (fig. S3A).

**Single-cell RNA-seq analysis in DSB-bearing neurons**

In our bulk RNA-seq analysis, inflammatory gene expression was lower in stage 1 neurons compared to stage 2 neurons (Fig. 1F). We also performed a flow cytometry analysis of stage 1 and stage 2 populations over 1 to 6 weeks and found that 1 week after induction, stage 1 neurons made up 75% of all γH2AX\(^+\) neurons, while stage 2 neurons made up the remaining 25%. By 2 weeks, however, the total population of γH2AX\(^+\) neurons was approximately 50% stage 1 and 50% stage 2 (fig. S3, B and C). This suggested that the stage 2 population may develop after stage 1, which aligned with our initial prediction when defining these distinct cellular states based on NeuN expression. To better understand the relationship between stage 1 and stage 2 neurons, we performed snRNA-seq on each FANS-gated population at both 1- and 2-week time points (Fig. 1D). A total of 1357 single-nucleus libraries were prepared using SMART-seq2 chemistry. Following quality control measures (see Materials and Methods), 889 libraries remained for downstream analysis (Fig. 11 and fig. S3D). Cells were classified into major cell type clusters based on marker gene expression, resulting in the identification of 521 excitatory neurons, 71 inhibitory neurons, 131 oligodendrocytes, 33 oligodendrocyte precursor cells (OPCs), and 50 microglia. We did not detect any astrocytes.

Most nuclei sorted under the stage 2 gate formed their own cell type cluster (Fig. 11), accounting for the remaining 83 cells. While we did identify 13 stage 2–gated nuclei in microglia and oligodendrocyte clusters, all of the nuclei in question came from one CK-p25 mouse (fig. S3, E and F). None of the other five CK-p25 mice had stage 2–gated nuclei fall into glial clusters. Combined with our previous cell type immunostaining analysis (fig. S1, C and D), we concluded that the immune signature identified in the stage 2 population was not likely to be driven by contamination from microglia or oligodendrocytes. Compared to other neuronal clusters, the in silico stage 2 cluster was significantly enriched for the FANS-gated stage 1 and stage 2 gene signatures (fig. S3G). The stage 2 cluster expressed only moderate levels of the excitatory neuron cluster markers Camk2a, Gria1, and Syt2 and lacked marker genes of other canonical cell types such as inhibitory neurons (Gad1, Gad2), astrocytes (Gfap), microglia (C33, Csf1r), oligodendrocytes (Plp1, Mbp), and OPCs (Beanc). Instead, the stage 2 cluster expressed marker genes indicative of senescence, including Cdkn1a and Ubb (Fig. 1I). These distinctive cell type markers further suggested to us that the stage 2 cluster represented a population of DSB-bearing neurons engaged in a senescence-like inflammatory response.

To further examine stage 1 and stage 2 cell type heterogeneity, we subclustered all neuronal cells. This resulted in the identification of four excitatory neuron subclusters (Ex0 to Ex3) and two inhibitory neuron subclusters (In0 and In1) (Fig. 1I). Stage 1 cells were enriched in subclusters Ex2 and Ex3 compared to Ex0 and Ex1 (fig. S3F). In addition to excitatory neuron markers, subclusters Ex2 and Ex3 were also marked by genes associated with DNA repair and DNA synthesis, such as Holliday junction–recognizing protein (Hjurp), the helicase Zgrf1, and ligase I (Lig1). Both Hjurp and Zgrf1 are involved in homologous recombination, a form of DSB repair that requires entry into S phase of the cell cycle. Expression of these genes by postmitotic neurons may reflect erroneous cell cycle reentry in an effort to repair DSBs (32). Lig1 is involved in both DNA replication and numerous DNA repair pathways, including alternative end-joining of DSBs. These genes indicate that Ex2 and Ex3 cells were likely engaged in a DSB repair response and cell cycle reentry (Fig. 1J).

Hjurp, Zgrf1, and Lig1 were not expressed in the stage 2 cluster. Combined with the enrichment for the classical senescence marker Cdkn1a, and the fact that these cells are still γH2AX\(^+\), we hypothesized that stage 2 cells may be engaged in an even later stage of response to DSBs. This motivated us to perform trajectory analysis. The single-cell analysis package Monocle 3 (33) was used to order the trajectory neuron subclusters (In0 and In1) (Fig. 1I). Stage 1 cells were enriched in subclusters Ex2 and Ex3 compared to Ex0 and Ex1 (fig. S3F). In addition to excitatory neuron markers, subclusters Ex2 and Ex3 were also marked by genes associated with DNA repair and DNA synthesis, such as Holliday junction–recognizing protein (Hjurp), the helicase Zgrf1, and ligase I (Lig1). Both Hjurp and Zgrf1 are involved in homologous recombination, a form of DSB repair that requires entry into S phase of the cell cycle. Expression of these genes by postmitotic neurons may reflect erroneous cell cycle reentry in an effort to repair DSBs (32). Lig1 is involved in both DNA replication and numerous DNA repair pathways, including alternative end-joining of DSBs. These genes indicate that Ex2 and Ex3 cells were likely engaged in a DSB repair response and cell cycle reentry (Fig. 1J).

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we did not observe a significant difference in expression between Ex0 and stage 2 (fig. S3H). This indicates that reduction of NeuN in stage 2 neurons likely occurs at the posttranscriptional level and agrees with previous observations that reduced NeuN antigenicity or protein levels are observed in damaged neurons (21, 23). We also observed a significant increase in Rela expression in Ex1 and Ex2 neurons compared to Ex0, but we did not observe a significant difference in expression between Ex0 and stage 2 (fig. S3H).

**DSBs elicit innate immune signaling in neurons**

We next wanted to determine whether the gene signatures identified in CK-p25 mice could be recapitulated independent of p25 expression. We also wanted to test whether DSB induction is sufficient to trigger inflammatory gene expression in neurons. To accomplish this, we first treated murine primary neuron cultures with etoposide (ETP). ETP produces DSBs by inhibiting topoisomerase II function, thus preventing the religation of cleaved DNA-TopII complexes (Fig. 2A). The dose of ETP used was consistent with previous studies using ETP to investigate DSB-elicited immune activity (35, 36).

Neuron cell type purity was also confirmed in primary neuronal cultures (see Materials and Methods and fig. S4A).

RNA-seq was performed to profile the transcriptional changes occurring in primary neurons after ETP treatment (Fig. 2B). There was a significant up-regulation of 7532 transcripts and down-regulation...
of 7106 transcripts compared to vehicle-treated control neurons [dimethyl sulfoxide (DMSO)] (adjusted \( P < 0.05 \)) (fig. S4, B to D). Notably, GSEA revealed that biological pathways mediating senescence and antiviral activity such as “Interferon alpha response” and “Il6 jak stat3 signaling” were enriched in ETP-treated cultures (Fig. 2C). Furthermore, many of the cytokines up-regulated in stage 2 neurons were also up-regulated in ETP-treated neurons, including Ccl2, Cxcl10, Cxcl16, and Il6 (table 2). Stage 1 and stage 2 gene signatures were both significantly enriched in differentially expressed genes from ETP-treated neurons (\( P = 0.00064 \) and \( P = 0.0081 \), respectively) (Fig. 2D). Venn diagram analysis indicated that 12.29% of all protein-coding differentially expressed genes (DEGs) (total protein-coding DEGs from all datasets) were shared between ETP, stage 1, and stage 2 conditions (Fig. 2E). This overlap was enriched for DNA damage repair genes such as Parp10, Tdp2, Fanca, and Rad54l. A further 12.86% of all DEGs were shared between ETP and stage 2 conditions. This overlap was enriched for immune genes such as Rela, Nfkbia, and Irf1 (Fig. 2E). \( \gamma H2AX \) nuclear intensity was also significantly increased in ETP-treated neurons (Fig. 2A). We also used x-ray irradiation as an independent method to induce DSBs. Exposing primary neuronal cultures to 10-Gy irradiation was also able to increase nuclear \( \gamma H2AX \) intensity in neurons, as was treatment with a lower dose of ETP (25 \( \mu M \)) (fig. S4, E and F). Furthermore, both x-ray irradiation and 25 \( \mu M \) ETP significantly increased many of the genes shared by the higher-dose ETP treatment and stage 1 or stage 2 (fig. S4G). Together, these data indicated that stage 1 and stage 2 gene signatures can be recapitulated in primary neuron cultures independent of p25 expression.

**A DSB-associated immune signature is conserved in AD human neurons**

We next sought to determine whether signatures of DSB-bearing neurons could also be detected in the human brain. First, we used the stage 2 gene signature to identify neurons with DSB-associated immune activation in a previously published snRNA-seq dataset of individuals with and without AD pathology (Fig. 3A) (37). We assessed stage 2 gene expression in all major brain cell types: excitatory neurons, inhibitory neurons, astrocytes, oligodendrocytes, OPCs, and microglia. For each cell type, genes were ranked on the basis of their expression and correlation to “global pathology,” a variable quantifying three major AD pathologies: neuritic plaques, diffuse plaques, and neurofibrillary tangles (38). Genes that positively correlated with global pathology for each cell type were then sampled for the enrichment of stage 2 genes (Fig. 3A). The strongest enrichment of stage 2 genes was identified in excitatory neurons (\( P = 8.7 \times 10^{-19} \)) and inhibitory neurons (\( P = 7.7 \times 10^{-7} \)). Astrocytes and oligodendrocytes were also found to have a significant enrichment of stage 2 genes correlating with global pathology, albeit to a much lesser extent (Fig. 3B) (Bonferroni adjusted \( P < 0.01 \)). Therefore, while we cannot exclude the possibility that human astrocytes and oligodendrocytes may carry some disease-associated enrichment for stage 2 genes, the stage 2 signature is largely driven by neurons in this dataset. Furthermore, when assessing the stage 2 genes positively correlated with global pathology in excitatory neurons, we again observed an enrichment of immune-related biological pathways, including the genes Ccl2-like receptor (CCRL2), CD74, CXCL16, and APOE (Fig. 3, C and D).

To validate the enrichment of DSB-bearing neurons identified in our snRNA-seq analysis, we sorted \( \gamma H2AX^{hi} \) and \( \gamma H2AX^{lo} \) NeuN\(^{hi} \) and NeuN\(^{lo} \) nuclei from the postmortem temporal cortex of six individuals and then performed bulk RNA-seq (Fig. 3E and fig. S5, A to C). These individuals were part of the Massachusetts Alzheimer’s Disease Research Center (MADRC) cohort and were divided by Braak score as either AD (Braak 4 to 6) or non-AD (Braak 1 to 3). Age, sex, and other biological variables for each individual are available in table S3. To assess the cell type specificity of our sorted populations, we examined the expression of several cell type marker genes (fig. S5, D to F). Unlike the stage 2 nuclei from the CK-p25 mice, \( \gamma H2AX^{hi} \) NeuN\(^{hi} \) nuclei from the human brain were enriched for glial markers (fig. S5, E and F). This indicated to us that human glial cells also accumulate DSBs, as has been previously reported (6), and that \( \gamma H2AX^{hi} \) nuclei with low NeuN expression are likely to contain a heterogeneous population of many different cell types. Thus, to ensure that we were analyzing neuronal gene expression, we focused our analysis on \( \gamma H2AX^{hi} \) NeuN\(^{hi} \) nuclei.

We assessed stage 1 and stage 2 gene signature activity in NeuN\(^{hi} \) nuclei from the human brain (Fig. 3F). \( \gamma H2AX^{hi} \) neurons displayed modest enrichment for the CK-p25 stage 1 and stage 2 genes (\( P = 0.31 \) and \( P = 0.04 \), respectively) (Fig. 3G). Comparing samples by AD status and \( \gamma H2AX \) status further revealed that stage 1 and stage 2 gene activity was markedly enriched in \( \gamma H2AX^{hi} \) neurons from AD individuals (\( P = 0.0075 \) and \( P = 0.0021 \), respectively) (Fig. 3, H and I). Furthermore, when contrasting all \( \gamma H2AX^{hi} \) neurons against all \( \gamma H2AX^{lo} \) neurons, we found that the average log2 fold change values for both stage 1 and stage 2 gene sets were positive and significantly higher than the average log2 fold change values for all other genes (fig. S5G). We also assessed the transcriptional similarity between human \( \gamma H2AX^{hi} \) neurons and ETP-treated primary neurons. Compared to \( \gamma H2AX^{lo} \) neurons, we found that gene expression in \( \gamma H2AX^{hi} \) neurons tended to be more similar to gene expression in ETP-treated neurons (fig. S5H).

To further verify the enrichment of immune activation in DSB-bearing neurons from brains with AD pathology, we assessed nuclear \( \gamma H2AX \) expression in NeuN-expressing neurons with high versus low burdens of DSBs in the AD postmortem brain. We acquired four prefrontal cortex tissue samples from individuals belonging to the Religious Orders Study cohort. All individuals analyzed exhibited AD pathology, described in table S4. To bin neurons by DSB burden, we analyzed \( \gamma H2AX \) expression in NeuN-expressing nuclei. The median \( \gamma H2AX \) expression was calculated for each brain and used to split neurons into the high or low DSB condition (fig. S6A). Neurons with high DSB burden consistently expressed higher levels of nuclear \( \gamma H2AX \) compared to neurons with low DSB burden (Fig. 3, J and K, and fig. S6B). Furthermore, we observed a strong positive correlation between raw \( \gamma H2AX \) values and raw p65 values when grouping together all neurons from all individuals (\( P < 0.0001 \)) (fig. S6C).

In summary, through the analysis of an independent snRNA-seq dataset, FANS RNA-seq, and immunofluorescent microscopy, we found that stage 1 and stage 2 gene signatures were active in human DSB-bearing neurons. Furthermore, this neuronal immune signature was further amplified in the context of AD pathology, suggesting that it may serve a functional role in disease-associated neuroinflammation.

**DSB-bearing neurons stimulate glial activation**

We have previously published an in-depth characterization of reactive microglia in CK-p25 mice using snRNA-seq (16). We wondered if these microglia were responding to immune signaling from DSB-bearing neurons. To address this, we performed \( \gamma H2AX \)...
immunostaining followed by spatial transcriptomics on 2-week induced CK-p25 brains (Fig. 4A). The capture areas of 10X Visium spatial transcriptomics slides are large enough to encompass the transcriptional profile of three to five cells in mouse brain tissue. Therefore, we hypothesized that capture areas with γH2AX signal would also contain transcripts from nearby microglia. First, we identified 23 spatial clusters across three CK and four CK-p25 coronal sections (Fig. 4, B and C, and fig. S7, A and B). Next, we used a binary classification system to identify capture areas with γH2AX signal past a given threshold (i.e., γH2AX+ capture areas) (see Materials and Methods, Fig. 4, D and E, and fig. S7C). To determine whether the reactive microglia were enriched in γH2AX+ capture areas, we used a Wilcoxon test (G to I).
Fig. 4. Immune signaling in neurons recruits and activates microglia. (A) Schematic of spatial transcriptomics experiment. CK (n = 3) and CK-p25 (n = 4) were induced for 2 weeks. Coronal brain sections were stained and imaged for γH2AX and then sequenced. (B) UMAP of capture areas from all samples. Leiden clusters are indicated by color and number. Each dot represents one capture area. (C) Leiden clusters superimposed onto a CK-p25 brain slice used for spatial transcriptomics. (D) UMAP indicating the density of γH2AX capture areas. (E) γH2AX capture areas identified in one CK-p25 brain slice. (F) Spatial clusters, γH2AX capture areas, and reactive microglia signature gene expression in one CK-p25 sample. (G) Schematic of neuronal p65 knockdown experiment. CK-p25 mice received retro-orbital injections of scramble shRNA-red fluorescent protein (RFP) adeno-associated virus (AAV) or shp65-RFP AAV. CK mice received retro-orbital injections of phosphate-buffered saline (PBS). Mice recovered for 2 weeks before being taken off dox. Brains were collected at the 2-week time point. (H) Reverse transcription quantitative polymerase chain reaction of immune genes in sorted RFP+ γH2AX+ nuclei. (I) Representative images of Iba1 in CK, scramble, and p65kd cortex. (J) Quantification of (left to right) Iba1+ soma area, number Iba1+ per image, average branch length per Iba1+ cell, and number end points per Iba1+ cell. Each data point represents one image. Two images were taken per mouse. (K) Heatmap of differentially expressed genes from p65 versus scramble contrast. (L) Up-regulated and down-regulated Gene Ontology (biological pathway) terms identified through GSEA of p65kd versus scramble contrast. (L) Heatmap of significantly up-regulated and down-regulated genes in PU.1** nuclei from p65kd cortex compared to PU.1** nuclei from scramble cortex. Error bars represent SEM; ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05. Student’s t test (H). One-way ANOVA followed by Holm-Sidak’s test for multiple comparisons (I). (H) Scramble (n = 5), p65kd (n = 5). (I) CK (n = 7), scramble (n = 5), p65kd (n = 6).
expression levels and \( \gamma \)-H2AX by cortical layer also seemed to associate with and \( \gamma \) by microglia with larger soma area. The density of DSB-bearing neurons also have higher densities of mi-

ly in layers 2/3 (fig. S8, E and F), suggesting that regions with high 

density of \( \gamma \)-H2AX + neurons.

To determine whether suppressing neuron immune genes attenuates microglial activation, we performed immunofluorescent staining with the microglia marker Iba1. We found that microglia proliferation was suppressed in p65kd mice (Fig. 4I), which was confirmed through flow cytometry analysis of PU.1hi nuclei from the entire cortex (fig. S10H). We also found that microglia soma area was significantly reduced, but differences in branch length and end points were not statistically significant (Fig. 4I). These data show that suppressing neuron immune signaling significantly alters the number and morphology of microglia.

To determine whether changes in microglia number and morphology were accompanied with changes in gene expression, we performed RNA-seq on sorted PU.1hi nuclei from CK control, CK-p25-scramble, and CK-p25-p65kd cortex (Fig. 4G and fig. S10, H and I). Differential analysis comparing microglia from p65kd and scramble cortices revealed the up-regulation of 627 transcripts and down-regulation of 160 transcripts (Fig. 4I and fig. S10I). Genes that were down-regulated in microglia from p65kd mice were involved in cell killing and antigen processing, such as H2-Q2, Lag3, Ccr5, and Ccr2. Notably, the Ccr2 gene encodes the receptor for CCL2, further implying an alteration of the CCL2-CCR2 axis compared to scram-

ble mice (Fig. 4, K and L). We also observed a down-regulation of genes related to lipoprotein processing (Fig. 4, K and L). Pathways related to synaptic activity and membrane organization were significantly up-regulated in microglia from p65kd mice. This included calcium channels Cacna1g, Cacna1c, and Cacnb2, and sodium channel Scn1a, suggesting that p65kd microglia may return to a more ho-

mestatic state embodied by CK microglia (Fig. 4I). Together, these results suggest that neuron immune signaling disrupts microglia homeostatic activity and activate cell killing and antigen processing mechanisms in microglia.

To gain a better understanding of how microglia respond to neuronal p65kd over time, we generated a mir30-based shRNA driven by an hSyn promoter packaged in AAV PHP.eB to suppress p65 exclusively in neurons (fig. S11A). CK-p25 brains injected with mir30-

based shp65 were collected for analysis at 1, 2, and 6 weeks after p25 induction (fig. S11B). After confirming suppression of p65 expres-

sion (fig. S11, C and D), we assessed microglia number and morphol-

ogy at each time point (fig. S11E). We found that neuronal p65kd was able to suppress microglial proliferation for 1 to 6 weeks of p25 induction (fig. S11F). Neuronal p65kd was also able to ameliorate branch shortening and end-point reduction at 1 and 2 weeks, but not at 6 weeks (fig. S11G). While we did not observe any rescue in microglia morphology at the 6-week time point, we did observe a significant reduction in the percentage of microglia that were MHCIIm (fig. S11H). MHCIIm expression is a hallmark feature of late-

responding microglia in CK-p25 (16). While neuronal p65kd did not affect levels of endogenous amyloid-\( \beta \), phosphorylated tau, or neu-

ronal loss, we observed a marked rescue of synaptic density as mea-

sured by vGlut1 and synaptophysin (fig. S12, A to D). We also observed
Substantial evidence supports a causal role for microglia and neuroinflammation in AD pathogenesis (25, 44). For example, many AD risk genes and genomic loci are most active in microglia (45), and reactive microglia are thought to create a cytotoxic environment for neurons and exacerbate neurodegeneration (46, 47). Here, we provide evidence that neurons participate in this inflammatory signaling as well. Specifically, upon the accumulation of DSBs, neurons are able to engage microglia through the secretion of chemotactic and pro-inflammatory factors, thus facilitating neuroinflammation and disease progression. This is a previously undefined role for neurons in the context of neurodegenerative disease and provides a mechanistic link between genomic fragility and senescence in neurons and microglia activation.

The activation of inflammatory signaling in DSB-bearing neurons corresponds with a progressive erosion of cell identity. Using snRNA-seq, we found that DSB-bearing neurons engage DNA repair and DNA synthesis pathways first (stage 1) but transition to immune gene expression at later stages of genome toxicity (stage 2). These stage 2 cells are most enriched for senescent markers, such as Cdkn1a and Ubb, rather than neuronal markers. The age-dependent erosion of neuronal identity has been observed in both sporadic and familial AD patients, and coincides with the activation of a number of biological pathways identified in our study, including DNA damage response, cell cycle reentry, and NFκB signaling (19). Interpreted in the context of the present study, these observations suggest that the age-associated accumulation of DSBs may degrade chromatin integrity in neurons, ultimately resulting in the activation of immune signaling that engage microglia.

While we initially identified the stage 2 gene signature in CK-p25 mice by virtue of reduced NeuN immunoreactivity in a subset of DSB-bearing neurons, we also identified stage 2 characteristics in DSB-bearing neurons with NeuN expression, including neurons from the human postmortem brain and primary culture. These observations indicate that there are likely high levels of heterogeneity in the damaged neuron population and that a simple stage 1 versus stage 2 dichotomy may not fully encompass all transcriptional states. However, the stage 1 and stage 2 populations are able to serve as proof of principle that a heterogeneous response to DSBs in neurons does exist and that transitions between these transcriptional states may occur during the progression of neurodegenerative disease.

The SASP and canonical antiviral response are activated through the detection of nonendogenous nucleic acids within the cytosol. Thus, it is likely that loss of nuclear and genomic integrity may lead to the release of DNA fragments in DSB-bearing neurons. We observed increased expression of nucleic acid sensors in DSB-bearing neurons, including cGAS, which plays a significant role in innate immune association with senescence (12). Nevertheless, many other mechanisms may be at play, including derepression of transposable elements (48), epigenetic drift, and ATM-mediated NFκB transcription (30). There is also evidence that nucleic acids released from mitochondria play roles in neuron immune gene expression (49). It remains to be addressed whether DSB-induced immune gene expression is a product of age-associated decline in DNA repair or whether multiple cellular functions that are also known to decline with age engage immune signaling as a convergent mechanism of neuronal distress. Therefore, while the current study does not address the mechanism by which DSBs in the CK-p25 mouse activate immune gene expression, a rich foundation of evidence for DSBs activating immune gene expression through multiple pathways exists for future studies.
Our data do not exclude the possibility that other cell types in the brain are capable of initiating a SASP in response to DNA damage. cGAS-STING–mediated senescence has also been described in ATM knockout microglia (50). Additional senescent phenotypes described in microglia (51), astrocytes (52), and OPCs (53) have also been shown to play a functional role in mouse models of neurodegenerative disease. However, despite the fact that neurons are the brain cell type most vulnerable to DSB accumulation, few studies have examined the relationship between DNA damage and senescence in neurons. Observations of senescent neurons have been

Fig. 5. CCL2 and CXCL10 are secreted by DSB-bearing neurons to activate microglia. (A) Schematic for treating acute Cx3cr1-GFP slices with conditioned medium (CM) from ETP-treated primary neurons. Cultures were treated with 50 μM ETP or vehicle control (DMSO) ± 10 μM NFκB Activation Inhibitor VI (IKK2 inhibitor) for 6 hours. Cultures were washed with PBS, and the medium was replaced. After 24 hours, the medium was applied to acute Cx3cr1-GFP slices for 6 hours. (B) Representative images of GFP in Cx3cr1-GFP acute slices treated with CM. (C to F) Quantification of branch length (C), end points (D), soma area per microglia (E), and number of microglia per image (F). Each data point represents the average of two images in one acute slice. (G and H) Quantification of CXCL10 (G) and CCL2 (H) from CM from control and ETP-treated primary neurons. Each data point represents one biological replicate. (I) Schematic of ETP CM experiment. Primary neurons were treated with either ETP or DMSO for 6 hours and washed with PBS, and then the medium was replaced. Cultures recovered for 24 hours before CM was collected. Immunoglobulin G (IgG), CCL2, or CXCL10 antibodies were used to immunodeplete CM before they were applied to Cx3cr1-GFP acute slices for 6 hours. (J) Representative images of microglia from acute slices treated with different CM. (K to N) Quantification of (K) branch length (L) end points, soma area per microglia (M), and number of microglia per image (N). Each data point represents the average of two images in one acute slice. Error bars represent SEM. ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05. Two-way ANOVA followed by Sidak's test for multiple comparisons (C to F). One-way ANOVA followed by Tukey's test for multiple comparisons (K to N). Data are combined from two independent experiments (C to F and K to N). Data are combined from three independent experiments (G and H).
observed in other studies (54), indicating that an investigation of the effects of senescent neurons in neurodegenerative disease is merited. We build upon these foundational observations by providing a detailed transcriptional analysis of such a population of neurons, linking them to a late-stage DSB response, and mechanistically establishing their role in microglia recruitment and activation in neurodegeneration.

We identified NFκB as a major regulator of immune gene expression in DSB-bearing neurons from both CK-p25 mice, primary neurons, and postmortem human brain. In addition, we found that suppression of NFκB activity through p65 knockdown or small-molecule inhibition in neurons was sufficient to reduce activated microglia morphology and gene expression. NFκB has been previously identified as a therapeutic target for AD, albeit in the context of microglia and astrocytes (55). Our results now indicate that this transcription factor plays a pivotal role in DSB-bearing neurons as well. In rodent models of learning and memory, NFκB activity in neurons is neuroprotective. Suppression of NFκB in forebrain excitatory neurons impairs spatial learning and neuronal plasticity (56) and exacerbates cell death following exposure to neurotoxic stimuli (57). Therefore, the primary role of neuronal NFκB may be to regulate synaptic processes, but it can also orchestrate immune activation in response to cell stressors. It is possible that NFκB and inflammatory activity in DSB-bearing neurons may initially function as a neuroprotective process. While we observed a notable reduction in synaptic gene expression in stage 1 neurons, this down-regulation was virtually absent in stage 2 neurons (Fig. 1E). However, we also observed a notable rescue of synaptic loss in p65kd mice at the 6-week time point, indicating that there is a complex relationship at play between the neuroprotective effects of NFκB and the synaptic pruning activity it may promote in microglia.

Last, we identify CCL2 and CXCL10 as primary signaling molecules secreted from DSB-bearing neurons to recruit and activate microglia. In the CK-p25 model, DSB-bearing neurons are the first cell type to express CXCL10 and CCL2. Mainly astrocytes and microglia express these chemokines at a later time point, presumably in response to DSB-bearing neurons. Furthermore, spatial transcriptomics revealed that signatures of reactive microglia are closely associated with γH2AXX neurons, suggesting that DSB-bearing neurons are hubs for neuroimmune communication. Last, immunodepletion of CCL2 or CXCL10 in conditioned medium from ETP-treated neurons was able to prevent branch shortening and end-point reduction in microglia in acute slice culture. Notably, increased levels of both of these cytokines are implicated in the pathogenesis of AD and affect blood-brain barrier permeability to aid the infiltration of peripheral monocytes (28, 58). However, manipulation of either signaling axis seems to have varying effects on AD pathology. For example, CCR2 deficiency in murine models of AD aggravates amyloid pathology and cognitive decline, but CCL2 overexpression seems to also increase amyloid deposition (59–61). Another study demonstrates that deficiency of the CXCL10 receptor CXCR3 reduces amyloid deposition and behavioral deficits (62). These findings suggest that CCL2 and CXCL10 are crucial for effective microglia recruitment and activation, but imbalance in these signaling axes have detrimental effects on cognition and pathology clearance.

In summary, we leveraged bulk, single-nuclei, and spatial transcriptomic techniques in parallel with in vitro and in vivo manipulations of NFκB signaling to characterize DSB-bearing neurons and their relationship with microglia in the context of age-associated neurodegenerative disease. We demonstrate that DSB accumulation elicits senescent and antiviral-like signaling in neurons, which recruits and activates microglia in an NFκB-dependent manner. Our data posit that neurons play meaningful roles in neuroinflammation, which historically has been thought to be driven largely by glial cells. Crucially, this axis of neuron-microglia communication is mediated by DNA damage accumulation in neurons, revealing that two hallmarks of AD, genome fragility and neuroinflammation, are mechanically linked.

MATERIALS AND METHODS

Fluorescence-activated nuclei sorting

Frozen cortices were disrupted with a handheld homogenizer in ice-cold PBS with protease inhibitors (catalog no. 11836170001, Roche, Basel, Switzerland) and ribonuclease inhibitors (catalog no. E00382, Thermo Fisher Scientific, Waltham, MA). Samples were fixed with 1% paraformaldehyde for 10 min at room temperature and then quenched with 2.5 M glycine for 5 min. Nuclei were isolated through dounce homogenization followed by filtration with a 70-μm cell strainer (catalog no. 21008-952, VWR, Radnor, PA). The following antibodies were used to tag nuclei: anti-H2AX-phosphorylated (Ser139) antibody conjugated to allophycocyanin (APC) (catalog no. 613416, BioLegend, San Diego, CA), anti-ATM–phosphorylated (Ser1981) antibody conjugated to phycoerythrin (PE) (catalog no. 651203, BioLegend, San Diego, CA), anti-NeuN antibody conjugated to Alexa Fluor 488 (catalog no. MAB377X, EMD Millipore, Burlington, MA), anti-PU.1 antibody conjugated to Alexa Fluor 647 (catalog no. 22408, Cell Signaling Technology, Danvers, MA), anti-RFP antibody (catalog no. 600-401-379, Rockland Antibodies and Assays, Gilbertsville, PA), and anti–CαMKII-α (6G9) antibody (catalog no. 50049S, Cell Signaling Technology, Danvers, MA). Antibodies were incubated with nuclei in 1% bovine serum albumin (BSA)/PBS at 4°C for 1 hour or overnight. For nonconjugated antibodies, samples were washed once with 1% BSA/PBS and then resuspended with 1:1000 Alexa Fluor secondary antibody (Thermo Fisher Scientific, Waltham, MA) for 1 hour at 4°C. Samples were passed through a 40-μm filter (21008-949, VWR) and stained with 4’,6-diamidino-2-phenylindole (DAPI) (catalog no. D9542, Sigma-Aldrich, St. Louis, MO) before sorting. Sorting was performed on FACSAria at the Koch Institute Flow Cytometry Core (BD Biosciences, USA). At least 50,000 nuclei of each cell type were collected for RNA-seq. Nuclei were sorted into 1% BSA/PBS and then spun at 2000g for 15 min in a cooled centrifuge (catalog no. 97058-916, Eppendorf, North America) for downstream analysis. For snRNA-seq, nuclei were not fixed. Single nuclei were sorted into a 96-well plate and then transported immediately to the Massachusetts Institute of Technology (MIT) BioMicro Center for library preparation. Forty-eight nuclei were sorted for each gated CK-p25 population per mouse, and 32 nuclei were sorted for each gated CK population per mouse. Flow cytometry analysis was performed using FlowJo software (Ashland, OR).

Bulk RNA-seq

For bulk RNA-seq of sorted nuclei, samples were treated for 15 min with proteinase K at 50°C and then for 13 min at 80°C. RNA was extracted using a Direct-zol RNA MicroPrep kit according to the manufacturer’s instructions (catalog no. R2062, Zymo Research, Irvine, CA). Purified RNA samples underwent fragment analysis at

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the MIT BioMicro Center. Libraries were generated from samples passing quality control (DV200 < 50%). Library generation was performed using SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (catalog no. 634412, Takara Bio Inc.) and then submitted to the MIT BioMicro Center for quality control (fragment analysis and qPCR), followed by sequencing. Paired-end sequencing was performed using the Illumina NextSeq500 platform according to standard operating procedures.

For bulk RNA-seq of ETP-treated primary neurons, cultures were collected in TRIzol LS (catalog no. 10296028, Thermo Fisher Scientific, Waltham, MA). mRNA was purified and extracted using a Direct-zol RNA MicroPrep kit according to the manufacturer’s instructions. RNA was submitted to the MIT BioMicro Center for library preparation and sequencing. Libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina (catalog no. E7770, New England Biolabs, Ipswich, MA). Single-end sequencing was performed using the Illumina NextSeq500 platform according to standard operating procedures.

**Mouse bulk RNA-seq read cleaning and bulk RNA-seq pipeline**

For the paired-end fastq files, the first three nucleotides were trimmed off the second sequencing read using cutadapt version 1.16. TrimGalore version 0.4.5 was used in paired mode to trim adapters and low-quality portions of reads. Reads were aligned using Salmon version 0.12.0 to mouse genome version GRCh38.94 and human genome version GRCh38.94. For downstream analysis, TPM files from Salmon were imported into R version 3.6.1 using tximport version 3.0.1. We performed differential analysis using R version 3.6.1 and DESeq2 (countsFromAbundance = lengthScaledTPM).

**Mouse bulk RNA-seq differential analysis**

We performed differential analysis using R version 3.6.1 and DESeq2 version 1.26.0. For each corresponding cell type and condition, we performed differential expression using only the corresponding samples. Genes were categorized as significant if they met the cutoff threshold (log2 fold change ≥ |1.0|, adjusted P < 0.05).

**Mouse bulk RNA-seq GSEA**

We used the complete set of RNA-seq results for each differential analysis for downstream GSEA processing. Genes for which adjusted P value could not be calculated were excluded. We ran GSEA version 3.0 in ranked list mode with default settings. The Gene Ontology biological pathway gene sets from Molecular Signatures Database v7.3 (MSigDB) were used for analysis. Genes were ranked by the sign of the fold change times the negative base 10 log of the adjusted P value.

**Enrichr**

Significantly up-regulated genes from the stage 2 versus baseline contrast were filtered on the basis of their occupation in MSigDB Gene Ontology biological processes containing the keyword “immune.” This resulted in a stage 2 immune module consisting of 940 genes. The corresponding Entrace gene symbols were then entered into the Enrichr website: https://maayanlab.cloud/Enrichr/#. The resulting transcription factor enrichment data were downloaded as tables from the following transcription factor libraries: ChEA 2016, ENCODE and ChEA Consensus TFs from ChIP-X, TRRUST Transcription Factors 2019, Enrichr Submission TF–Gene Coocurrence, TRANSFAC and JASPAR PWMs, and ENCODE TF ChIP-seq 2015. These data are available in table S1. More information about the Enrichr libraries can be accessed on the Enrichr website.

**Smart-seq pipeline**

**SMART-seq2**

Single-nuclei library preparation was performed by the MIT BioMicro Center using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara Bio Inc.) according to the manufacturer’s instructions. Libraries were sequenced on a MiSeq Illumina sequencer according to standard operating procedures.

**Read processing**

We aligned 40–base pair (bp) paired-end reads to the mm10 genome for each of 1357 single nuclei from 12 mice (64 cells each for six CK controls and an average of 162 cells in each of six CK-p25 mice) using Burrows-Wheeler Alignment–maximal exact match (options: -k 15 -M) and filtered out improperly aligned reads and secondary alignments with samtools (options: -f -F 1280). We ran HTSeq-count10 on each cell’s filtered bam file to compute the cell’s transcriptomic coverage over each gene’s exons in vM25 GENCODE gene annotation.

**Transgene detection**

We first aligned 40-bp single-end reads from each sequenced single nucleus separately with the STAR aligner vers against the b37 genome with decoy contigs using a two-pass alignment (options: --outFilterMultimapNmax 20 --alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --alignIntronMin 20 --alignIntronMax 1000000). We then used Picard tools to revert and merge the alignment with unaligned reads and marked duplicates on the merged bam file. We identified and removed alignments on decoy contigs, sorted and fixed NM, MD, and UQ tags with Picard tools, filtered duplicates, unmapped, and nonprimary alignment reads, and split reads by Ns in their CIGAR string.

**Cell type annotation**

**Cell identities**

We used SCANPY11 to process and cluster the expression profiles and infer cell identities. We kept only 21,859 protein-coding genes detected in at least 3 cells and filtered out 349 cells with less than 100 expressed genes, leaving 1008 cells over the 12 mice. We used the filtered dataset to calculate the low-dimensional embedding of the cells [Uniform Manifold Approximation and Projection (UMAP)] from the log1p matrix principal components analysis (PCA) with k = 50 and nearest neighbor graph with N = 20 (UMAP default parameters, min_dist = 0.2) and clustered it with Leiden clustering (resolution = 2), giving 17 preliminary clusters. We then manually assigned clusters based on the following two to three major marker genes per class: excitatory neurons: Camk2a, Gria2, and Syt1; inhibitory neurons: Gad1 and Gad2; astrocytes: Gfap; microglia: Cd33 and Csf1r; oligodendrocytes: Plp1 and Mbp; OPCs: Bcan; stage 2: Cdkn1a and Ubb. We merged clusters sharing marker genes to obtain seven final clusters, defining two broad neuronal subtypes (484 excitatory and 108 inhibitory cells), three glial clusters (50 microglia, 131 oligodendrocytes, and 33 OPCs), 179 stage 2 cells, and 23 cells with both high read counts and broad, nonspecific marker gene expression, likely due to doublets or other sorting artifacts. The doublet cluster was removed from downstream analyses. We also identified a cluster of cells that expressed fewer than 500 genes and had high
expression of mitochondrial genes. Because of the questionable quality of these cells, this cluster was also removed from downstream analysis. We further sub-annotated neuronal subtypes from neuronal clusters with distinctive expression in the original Leiden clustering, resulting in four excitatory subclusters (314 Ex0, 91 Ex1, 32 Ex2, and 47 Ex3 neurons) and two inhibitory subclusters (71 In0 and 37 In1 neurons).

**Signature analysis on single cells**

For each signature, a joint expression value was calculated as the number of reads per 10,000 reads in each cell coming from all of the signature’s genes (genes with adjusted $P < 0.05$). Signature expression was transformed by log1p and averaged across all cells in a given neuronal subtype and mouse to obtain average signature values for plotting. We used two-sided t tests to compare the signature expression levels of pairs of excitatory neuronal subtypes (fig. S3G).

**Trajectory analysis**

**Pseudotime**

We performed pseudotime analysis using Monocle3 (v0.2.3.0) on the in silico Ex0, Ex1, Ex2, Ex3, and stage 2 neuronal populations. We normalized total counts per cell in the read count matrix to the median number of counts, log1p transformed the matrix, and regressed out the counts per gene using Monocle. We clustered the subsets data in a new UMAP, clustered cells, learned a trajectory graph, and ordered cells by choosing the initial node as the Ex0 end of the graph to get a pseudotime across the graph. We plotted the log1p read counts across the gene set as well as the generalized additive model smoothed fits of each signature’s per-cell expression values across pseudotime (Fig. 1K).

**Human snRNA-seq analysis**

Single-nucleus transcriptomic sequencing data from postmortem cortical samples (prefrontal cortex, Brodmann area 10) of 48 subjects with varying levels of AD pathology were obtained from a previous publication (37). Individual-level cell type expression profiles were computed by averaging for each individual the normalized gene expression profiles across cells of the same cell type. Average profiles were subsequently mean-centered and scaled to compute gene-wise correlation coefficients of gene expression versus individual-level measures of global AD pathology burden reported as part of the Religious Orders Study and Memory and Aging Project (ROSMAP) cohort. Briefly, global burden of AD pathology is a quantitative summary of AD pathology derived from counts of three AD pathologies: neuritic plaques (n), diffuse plaques (d), and neurofibrillary tangles (nt), as determined by microscopic examination of silver-stained slides (37).

Global consistency between gene signatures observed in CK-p25 mice and a neuronal-specific association between gene expression and AD pathology in human tissue was assessed statistically using a nonparametric resampling test. To test whether cell type–specific expression of CK-p25 stage 2 signature genes tends to correlate with pathology in the human brain, a z-score statistic was computed to quantify the deviation of their correlation coefficient rank scores, relative to random expectation. Expected scores were estimated by randomly sampling same-sized gene sets ($n = 1000$ replicates). This analysis was performed for excitatory neurons, inhibitory neurons, and microglia cells independently.

**Stage 1 and 2 signature generation**

Stage 1 and 2 gene signatures were curated by performing differential expression analysis on the CK-p25 bulk RNA-seq dataset. Stage 1 versus baseline provided the genes for the stage 1 signature, and stage 2 versus baseline provided the genes for the stage 2 signature. Only genes that met the cutoff threshold (log2 fold change $\geq 1.0$, adjusted $P < 0.05$) were retained.

**Visium spatial transcriptomics library generation**

Mice were transcardially perfused with ice-cold saline, and then brains were dissected and flash-frozen in Optimal Cutting Temperature (OCT) Compound. A cryostat was used to generate 10-μm coronal sections of the hippocampus. These sections were applied to 10X Visium Spatial Gene Expression slides. Sections were immunostained with γH2AX and DAPI following the manufacturer’s instructions. Sectioning and staining was performed at the MIT Hope Babette Tang (1983) Histology Core Facility. Sections were imaged immediately after staining using the Olympus FV1200 Laser Scanning Confocal Microscope at the MIT Microscopy Core Facility. Sections were then used to generate 10X Visium Spatial Gene Expression Libraries according to the manufacturer’s instructions at the MIT BioMicro Center. Libraries were sequenced on a NovaSeq6000 Illumina sequencer according to standard operating procedures.

**Visium spatial transcriptomics data processing**

Samples were processed using Scanpy 1.7.2. The seven sample data matrices were merged into one matrix, which was then processed. The sample id and location of each capture area of the resulting matrix were saved and used for visualization. Counts were normalized (total count of 10,000 per capture area) and logarithmized (using Scanpy’s log1p function). The resulting counts matrix, called raw normalized, was used for expression visualization and differential expression analysis. For dimension resolution purposes, the raw normalized matrix was further processed: Genes that were not characterized as highly variable enough were filtered out (minimum mean of 0.0125, max mean of 3, minimum dispersion of 0.5), and linear regression was performed to eliminate the effect of covariates (total_counts and mitochondrial gene percentage). The data were then scaled (standard scaling, max value of 10). Afterward, PCA was performed, as well as sample-level batch correction, using Harmony. Then, a knn network was constructed for the creation of a UMAP embedding. Clusters were found using the Leiden algorithm.

**Visium immunohistochemistry image processing**

Immunostaining images were first processed as shades of gray pictures. The largest autofluorescence artifacts were removed manually. The signal was then amplified and cleaned using an 85% contrast increase on each image.

As the immunohistochemistry images of the tissue align perfectly to the pictures taken for 10X Visium purposes, calibration was performed to reconstruct the capture areas grid on the immunostaining images. In that image, for each capture area, the mean signal within a circle maximizing image coverage is calculated and recorded as the DNA damage signal. After standard scaling of this variable, a threshold of 0.4 SD was set to assign capture areas as positive or negative for DNA damage.

**Visium differential expression and reactive microglia signature analysis**

Differential expression analysis was performed using Wilcoxon rank sum test, and the resulting $P$ values were corrected using Benjamini-Hochberg false discovery rate correction. For GSEA, the package gsea was used, with 100 permutation and the signal-to-noise method. As input, the raw normalized matrix was used, though only containing the genes considered as highly variable in the dataset.
Reverse transcription qPCR
RNA was extracted from primary tissue cultures using the RNeasy Plus Mini Kit (catalog no. 74136, Qiagen, Hilden, Germany). Reverse transcription was performed using Invitrogen SuperScript IV First Strand Synthesis System with Oligo dT primers according to the manufacturer’s protocol (catalog no. 18091050, Thermo Fisher Scientific, Waltham, MA). Complementary DNA (cDNA) was quantified with a NanoDrop spectrophotometer. qPCR was performed using a Bio-Rad CFX-96 quantitative thermal cycler (catalog no. 1855195, Bio-Rad, Hercules, CA) and SsoFast EvaGreen Supermix (catalog no. 1725202, Bio-Rad, Hercules, CA). Relative changes in gene expression were determined using the 2⁻ΔΔCT method. Cycle numbers for the gene Gapdh or Rp11 were used for housekeeping C values.

Immunofluorescent microscopy
Mice were transcardially perfused with ice-cold PBS and then fixed with ice-cold 4% paraformaldehyde in PBS. Dissected brains were drop-fixed overnight in 4% paraformaldehyde in PBS at 4°C. Forebrains were sectioned with a vibrating microtome (Leica Biosystems, Wetzlar Germany) to generate 40-μm coronal slices. Slices were blocked for 2 hours at room temperature in blocking buffer (10% normal goat serum, 0.3% Triton X-100, and PBS) and then incubated with primary antibody overnight at 4°C. Slices were washed 3 × 10 min with PBS, and Alexa Fluor secondary antibodies (Thermo Fisher Scientific) were added at a 1:1000 dilution for 1 hour at room temperature. Slices were washed again 3 × 10 min with PBS, then stained with DAPI (catalog no. D9542, Sigma-Aldrich, St. Louis, MO), and mounted onto Fisherbrand Superfrost Plus Microscope Slides (catalog no. 12-550-15, Thermo Fisher Scientific, Waltham, MA) with Fluoromount-G slide mounting medium (catalog no. 100502-406, VWR, Radnor, PA).

Primary neuron culture
Cultures were dissected from E15 Swiss-Webster embryos in ice-cold Hanks’ balanced salt solution (HBSS) (catalog no. 14175103, Thermo Fisher Scientific, Waltham, MA) and dissociated with papain (catalog no. L5003126, Worthington Biochemical Corp., Lakewood, NJ) and deoxyribonuclease I (catalog no. 1014159001, Roche, Basel, Switzerland). Cells were resuspended in plating medium [neurobasal medium (catalog no. 21030049, Thermo Fisher Scientific, Waltham, MA), 1% penicillin/streptomycin solution (catalog no. 400-109, Gemini Bio-Products, Sacramento, CA), and 10% fetal bovine serum] and filtered through a 100-μm cell strainer (catalog no. 21008-950, VWR, Radnor, PA). Cell density was quantified using the Countess II Automated Cell Counter (catalog no. AMQAX1000, Thermo Fisher Scientific, Waltham, MA) and then plated on poly-d-lysine–coated 12-well culture dishes, 0.5 × 10⁶. Cultures were maintained in 5% CO₂ at 37°C in a cell culture incubator. After allowing 4 hours for the cells to adhere to the plate, the medium was replaced and maintained with neurobasal medium supplemented with B-27 (catalog no. 17504-044, Invitrogen, Carlsbad, CA), 1% penicillin/streptomycin, and 1% GlutaMAX supplement (catalog no. 35050-079, Thermo Fisher Scientific, Waltham, MA).

We also performed cell type immunostaining analysis to assess the cell type composition of our primary cultures. In total, 82.87% of cells were identified as mature neurons (NeuN expression), and another 9.31% were neural precursor cells (nestin expression) (fig. S4A). A trace number of cells were also identified as astrocytes (GFAP expression, 2.46%) and oligodendroglia (Olig2 expression without nestin expression, 1.35%) (fig. S4A).

Primary neuron treatments
Primary cortical neuron cultures (DIV11 to DIV13) were treated with 50 μM ETP prepared from 20 mM stock (catalog no. E1383-250MG, Sigma-Aldrich, St. Louis MO). Cultures were treated for 6 hours before collection for downstream experiments. RT-qPCR was used to measure gene expression. We were also able to observe increased expression of nuclear p65 and immune genes in primary neuron cultures treated with 25 μM ETP.

X-ray irradiation. Primary cortical neurons (DIV11 to DIV13) were exposed to 10- or 0-Gy x-ray irradiation using X-Rad320 from Precision X-Ray. Cultures were given 24 hours to recover before proceeding with experiments.
NFκB activation inhibitor

Primary neuron cultures were treated with 10 μM NFκB Activation Inhibitor VI, a benzo xathiole compound from Abcam (catalog no. ab145954). Cultures were pretreated for at least 30 min before ETP exposure.

RNAscope in situ hybridization

Fluorescent in situ hybridization was performed using RNAscope Multiplex Fluorescent Reagent Kit v2 according to the manufacturer’s instructions (catalog no. 323100, Advanced Cell Diagnostics, Newark, CA). Probes targeting murine Ccl2 (catalog no. 311791), Cxcl10 (catalog no. 408921), Aif1-C2 (catalog no. 31941-C2), Olig2-C3 (catalog no. 447091-C3), GFAP-C3 (catalog no. 313211-C3), and Rbfox3-C2 (catalog no. 313311-C2) were purchased from Advanced Cell Diagnostics. Following the RNAscope protocol, slices were stained for γH2AX (catalog no. 05-636, EMD Millipore, Burlington, MA). Samples were imaged with a Zeiss LSM 710 confocal microscope at 40× objective. Images were analyzed with ImageJ.

RNA scope analysis

ImageJ version 2.1.0 thresholding was used to identify γH2AX+ nuclei and generate regions of interest (ROIs). Process -> “Find Maxima” was used to count mRNA puncta within ROIs. Prominence > 100.00 with “Strict” setting. The number of γH2AX+ nuclei with ≥2 maxima, as well as the number of maxima per γH2AX+ nucleus, was quantified. γH2AX+ nuclei were identified by thresholding for individual nuclei using the DAPI channel and then excluding ROIs that overlapped with γH2AX+ ROIs. Individual nuclei were identified following the Nuclei Watershed Separation process described on the ImageJ website (https://imagej.net/imaging/watershed).

Brain tissue samples

MADRC brain tissue samples

Fresh-frozen postmortem brain samples were provided by the MADRC. These samples were used for bulk RNA-seq. Individuals were selected on the basis of clinical diagnosis and Braak score. The three samples labeled as AD all had a clinical diagnosis of AD and a Braak score of VI. The three samples labeled as non-AD did not have a clinical diagnosis of AD and had Braak scores of I, II, and II. Sample metadata is available in table S3.

ROSMAP brain tissue samples

Fixed-frozen postmortem brain samples were chosen from the ROSMAP cohort. ROSMAP is a longitudinal cohort study of aging dementia in elderly nuns, brothers, and priests. Sample metadata is available in table S4. In-depth description of metadata variables is available at the Rush Alzheimer’s Disease Center website (www.radc.rush.edu/docs/var/variables.htm).

p65 knockdown

Custom p65 and scramble shRNA oligos cloned into an AAV backbone (pAV-U6-RFP) were purchased from ViGene Biosciences (Rockville, MD). p65 shRNA sequences were as follows: GATCCGCGAGCTAT-CAGTCCGAGCTATGTGCTATATTGCTGAGATGATGCT- GCTTTTTA, GATCCGCGAGCTATGTGCTATATTGCTGAGATGATGCT- GCTTTTTA, GATCCGCGAGCTATGTGCTATATTGCTGAGATGATGCT- GCTTTTTA, GATCCGCGAGCTATGTGCTATATTGCTGAGATGATGCT- GCTTTTTA, GATCCGCGAGCTATGTGCTATATTGCTGAGATGATGCT- GCTTTTTA, GATCCGCGAGCTATGTGCTATATTGCTGAGATGATGCT- GCTTTTTA, GATCCGCGAGCTATGTGCTATATTGCTGAGATGATGCT- GCTTTTTA, GATCCGCGAGCTATGTGCTATATTGCTGAGATGATGCT- GCTTTTTA, GATCCGCGAGCTATGTGCTATATTGCTGAGATGATGCT- GCTTTTTA, GATCCGCGAGCTATGTGCTATATTGCTGAGATGATGCT- GCTTTTTA. Or PBS was delivered retro-orbitally to anesthetized CK-p25 mice, 2 × 10^11 IU/ml. For neuron-specific knockdown of p65, the plasmid SIN40C.SFFV.dTomato.mir30n (a gift from D. Heckl; Addgene, plasmid #169277) was modified with an hSyn, a neuron-specific promoter, and then cloned into a AAV backbone for generation of PHP.eB AAV.

Mir30-based p65 shRNA: AGCGAGCCTCATCCACAT-GAATTTTGATTAAGCCACAGATGTAACAGAGTTCATGT-GGATGAGGCC.

Mir30-based scramble: AGCGGCTTAAGGTTAAGTCGCCCTC-GTATGTAAGCCACAGATGTAACAGAGTTCATGT-GGATGAGGCC.

Mice received bilateral intracranial injections [-2.00 Anterior-Posterior (AP), 1.5 Medial-Lateral (ML), –0.5 Dorsal-Ventral (DV)] of 500 nl of AAV-PHP.eb-hSyn-RFP-mir30-shp65 or AAV- PHP.eb-hSyn-RFP-mir30-scramble at a rate of 100 nl/min (both 2 × 10^11 IU/ml). Two weeks after injection, mice were induced by removing dox diet. Following induction, mice were transcardially perfused with ice-cold PBS. One hemisphere was drop-fixed overnight in 4% paraformaldehyde/PBS at 4°C for immunostaining. The other hemisphere was flash-frozen in liquid nitrogen and stored at –80°C for RNA-seq.

CK-p25 mice

All mouse experiments were approved by the Committee for Animal Care of the Division of Comparative Medicine at the MIT, as well as the MIT Institutional Animal Care and Use Committee. All animal experiments were carried out at MIT. CK-p25 double transgenic mice were raised and maintained on a dox diet. All mice were induced by removing dox from their diet to drive the expression of p25-GFP in forebrain excitatory neurons. All mice were induced at 3 to 4 months old.

Conditioned medium and immunodepletion

Following ETP treatment, cultures were washed once with PBS. Cultures then recovered in fresh medium for 24 hours. The medium was stored at –80°C for future experiments. Conditioned medium from ETP-treated neurons was incubated with IgG, Ccl2, or Cxcl10 antibodies (all 40 μg/ml) for 4 hours at 4°C. Dynabeads Protein G were added to pull down the antibody complex.

Organotypic brain slice culture

Eight- to 12-week-old male Cx3cr1-GFP mice were anesthetized with isoflurane and transcardially perfused, dissected, and sliced in ice-cold N-methyl-d-glucamine (NMDG) cutting solution containing 2.5 mM KCl, 0.5 mM CaCl2, 10 mM MgSO4, 1.25 mM Na2HPO4, 20 mM Hepes, 2 mM thiourea, 5 mM sodium ascorbate, 3 mM sodium pyruvate, 92 mM NMDG, 30 mM NaHCO3, and 25 mM d-glucose (pH 7.3 to 7.4) with HCl. The slicing chamber was bubbled with 95% O2/5% CO2 and coronal slices were cut at 250-μm thickness using a vibratome (Leica, VT1000s). After the last slice was collected, slices were transferred to a well plate containing fresh artificial cerebrospinal fluid (ACSF) and placed in an incubator set at 37°C with 95% O2/5% CO2 for 30 min. The ACSF solution contained 125 mM NaCl, 2.5 mM KCl, 1.2 mM Na2HPO4, 1.2 mM MgCl2, 2.4 mM CaCl2, 26 mM NaHCO3, and 11 mM d-glucose. Afterward, 100% of ACSF was removed and a 1:1 mixture of fresh ACSF and conditioned medium was added to the slices and placed back into the incubator for 6 hours. At the completion of the experiment, slices were fixed overnight at 4°C with 4% paraformaldehyde. Slices were then incubated in 30% glucose overnight at 4°C. Slices were subsectioned into 25-μm slices using a cryostat and then coverslipped for imaging.
Enzyme-linked immunosorbent assay
The Mouse MCP1 ELISA Kit and Mouse IP-10 ELISA Kit from Abcam (catalog nos. ab208979 and ab214563, respectively) were used to quantify CCL2 and CXCL10 in conditioned medium from primary neurons. Assays were quantified on a plate reader, and protein concentration was calculated according to manual instructions.

Microglia morphological analysis
Microglia morphology from CK-p25 and acute slice cultures was analyzed according to the protocol described by Young and Morrison (2016) with minor alterations. Gray Scale Attribute Filtering (default settings, connectivity: 8) from MorphoJ plug-in version 1.4.1 was used to reduce background noise when thresholding images for skeleton analysis. Following skeleton analysis, Morphological Filters (Operation: Opening, Element: Octagon, Radius (in pixels): 2) from MorphoJ were used to quantify soma area. This analysis was performed for both Iba1 and Cx3cr1-GFP imaging experiments.

Statistical methods and reproducibility
All imaging, RT-qPCR, and enzyme-linked immunosorbent assay (ELISA) data were analyzed using either Student’s t-test, one-way analysis of variance (ANOVA), two-way ANOVA, or simple linear regression. Data were plotted and statistical tests were performed on GraphPad Prism 9. Detailed statistical procedures for sequencing analysis are described in the sequencing sections of Methods and Materials.

SUPPLEMENTARY MATERIALS
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View/request a protocol for this paper from Bio-protocol.
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The authors declare that they have no competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. All mouse sequencing data are available through GEO accession number GSE174265 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE174265). Human bulk RNA-seq data generated for this manuscript are available at Synapse (www.synapse.org). All source code and software used for this study are available at Github (https://github.com/mikobamba/CellDeathHeaders). All code and software are made available under an open-source license. All data used in this study are available in the Synapse repository. All data used in this study are publicly available.

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Neurons burdened by DNA double-strand breaks incite microglia activation through antiviral-like signaling in neurodegeneration

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