

Single-cell transcriptomic and genomic changes in the ageing human brain


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Over time, cells in the brain and in the body accumulate damage, which contributes to the ageing process¹. In the human brain, the prefrontal cortex undergoes age-related changes that can affect cognitive functioning later in life². Here, using single-nucleus RNA sequencing (snRNA-seq), single-cell whole-genome sequencing (scWGS) and spatial transcriptomics, we identify gene-expression and genomic changes in the human prefrontal cortex across lifespan, from infancy to centenarian. snRNA-seq identified infant-specific cell clusters enriched for the expression of neurodevelopmental genes, as well as an age-associated common downregulation of cell-essential homeostatic genes that function in ribosomes, transport and metabolism across cell types. Conversely, the expression of neuron-specific genes generally remains stable throughout life. These findings were validated with spatial transcriptomics. scWGS identified two age-associated mutational signatures that correlate with gene transcription and gene repression, respectively, and revealed gene length- and expression-level-dependent rates of somatic mutation in neurons that correlate with the transcriptomic landscape of the aged human brain. Our results provide insight into crucial aspects of human brain development and ageing, and shed light on transcriptomic and genomic dynamics.

Bulk RNA-sequencing studies of ageing have revealed disruptions to essential cellular processes such as transcription, translation and growth-factor signalling³, with processes involved in mitochondrial function, neuronal activity and DNA damage being dysregulated in the ageing brain^{2,4}. Cell-type-specific changes during ageing are obscured in bulk analyses and are poorly understood. This represents a major knowledge gap in the human brain, in which molecularly distinct cell types perform specific functions throughout life. The advent of single-cell genomics has allowed high-resolution analysis of both DNA and RNA. scWGS and other techniques have shown that somatic mutations accumulate in human neurons during ageing and in age-related diseases, raising the possibility that such variants contribute to transcriptional dysregulation and the concomitant increased susceptibility to dysfunction and disease that accompanies advanced age^{5–10}. Single-cell RNA sequencing and snRNA-seq have refined the understanding of brain cell states^{11–14}, and have been used to identify age-related and disease-related changes in several organs¹⁵, including the human brain^{16,17}. Despite this progress, our understanding of the transcriptional and genomic changes associated with healthy ageing—which might lay the groundwork for certain brain diseases—remains incomplete.

Here, to begin to capture the dynamics of human brain ageing in a cell-type-specific manner, we generated droplet-based snRNA-seq and scWGS libraries of fresh-frozen human prefrontal cortex (PFC) (Fig. 1a) from 19 neurotypical donors ranging in age from infant to centenarian

(Table 1 and Supplementary Table 1). As orthogonal validation of our snRNA-seq results, we performed multiplexed error-robust fluorescent in situ hybridization (MERFISH), a quantitative spatial-transcriptomic technique with single-molecule resolution, on a subset of donors. In the snRNA-seq experiments, 367,317 nuclei remained after quality control and artefact filtering¹⁸, with a mean of 19,332 per donor (Supplementary Fig. 1), and dimensionality reduction and hierarchical clustering yielded 31 clusters (Fig. 1b). We annotated these clusters using a previously published human PFC dataset¹⁹ as a reference (Fig. 1c and Supplementary Table 2), and identified clusters of excitatory neurons from various cortical layers, four subtypes of inhibitory neurons (IN-PV, IN-SST, IN-SV2C and IN-VIP), microglia, oligodendrocytes, oligodendrocyte precursor cells (OPCs), astrocytes and endothelial cells. The expression of canonical marker genes for each cell type was cluster-specific (Supplementary Fig. 2). Within these broad classes, we identified subclasses of cells that, despite their similarity, populated distinct clusters (Fig. 1b). On average, excitatory neurons expressed more than twice as many genes as did glial and endothelial cells (Fig. 1d).

Brain cell-type proportions during life

We detected no difference in the overall ratios of neurons to glia or excitatory neurons to inhibitory neurons. In addition, we did not observe the loss of any neuron subtype during non-pathological ageing, nor did we see evidence of the expansion of reactive microglia in the

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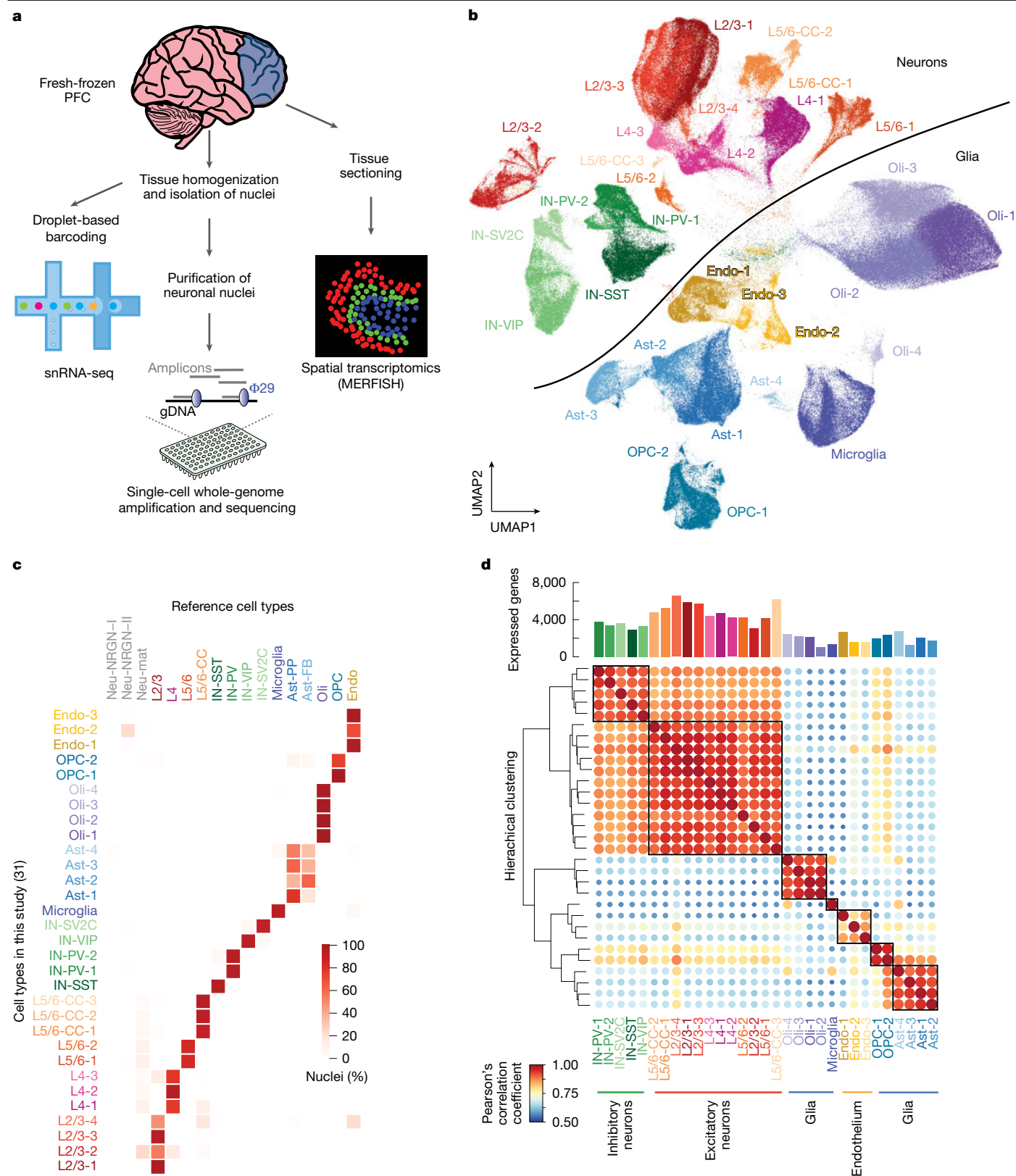


Fig. 1 | Study design and characterization of droplet-based snRNA-seq in human PFC. **a**, Overall study design. Human PFC was analysed by three single-cell genomic techniques in parallel. **b**, Dimensional reduction and clustering of all snRNA-seq nuclei after filtration yielded several clusters for each cell type (Ast, astrocyte; CC, cortico-cortico; Endo, endothelial; L, layer; Oli, oligodendrocyte; UMAP, uniform manifold approximation and projection).

c, Percentage of nuclei in each cluster of our data that correspond to the annotated reference cluster. **d**, Gene-expression profiles for each subcluster within a cell type correspond most closely to the cells of the same lineage based on Pearson's correlation coefficient. The bar plot above the heat map shows the number of genes expressed in each cluster.

Table 1 | Sample information for snRNA-seq and scWGS

Group	Age (years)	Sex	Nuclei		Segmented cells
			snRNA-seq	scWGS	MERFISH
Infant	0.4	M	23,091	7	26,036 ^a
	0.6	M	18,399	9	–
Adult	15	F	29,317	7	58,328 ^a
	17	M	11,305	4	–
	27	M	28,171	5	34,850 ^b
	28	M	20,425	5	63,394 ^a
	38	M	21,135	4	–
	42	F	36,921	7	21,162 ^b
	44	M	11,097	3	–
	49	F	22,958	5	28,292 ^b
	53	M	9,268	3	–
	57	M	20,751	7	73,406 ^a
Elderly	66	F	14,746	5	–
	70	F	8,710	3	–
	82	M	28,888	7	12,760 ^b
	82	F	17,382	3	15,451 ^b
	87	M	14,351	4	11,802 ^b
	93	M	16,781	5	–
	104	F	13,621	7	–
Total	19 donors	12 M, 7 F	367,317	100	345,481

Group, age and sex information for each donor brain. We report the numbers of unsorted nuclei input for snRNA-seq and sorted NeuN⁺ neuronal nuclei used for scWGS from each brain, as well as the number of segmented cells derived from MERFISH when applicable. Dorsolateral PFC was used for all experiments. ^aMERFISH samples prepared on the MERSCOPE Ultra instrument; ^bMERFISH samples prepared on the MERSCOPE instrument.

elderly (aged >65 years) brain (Extended Data Fig. 1 and Supplementary Table 3). However, we did identify subclusters of neurons and astrocytes that were composed exclusively or nearly exclusively of nuclei from infant donors (Fig. 2a). As a whole, the infant-specific neuron cluster resembled L2/3 neurons, but closer examination identified groups of cells in this cluster expressing markers of L4 or L5/6 neurons (Extended Data Fig. 2), and revealed that genes involved in development and neuron migration (Fig. 2b), such as *SLIT3* and *ROBO1*, were also expressed in this group (Supplementary Tables 4 and 5). An analysis of MERFISH data generated using the Ultra platform from a subset of four donors (a 0.4-year-old male individual, a 15-year-old female individual, a 28-year-old male individual and a 57-year-old male individual) showed that infant neurons mostly exhibited correct laminar positioning, with *CUX2*⁺ L2/3 neurons, *RORB*⁺ L4 neurons and *HS3ST4*⁺ L5/6 neurons²⁰ showing similar distributions across donors (Fig. 2c,d and Extended Data Fig. 3). *HS3ST4* also seems to mark white-matter neurons in all donors, similar to *TLE4*, a canonical L5/6 marker²¹. These data suggest that cluster L2/3-2 represents immature excitatory neurons that populate the various layers of the infant neocortex. Infant-specific astrocytes expressed neurodevelopmental genes that mark immature astrocytes; for example, *HESS*, *ID4*, *MFGE8* and *DCC* (refs. 22–24) (Fig. 2b, Supplementary Tables 4 and 5). Our reanalysis of a published snRNA-seq dataset of human PFC examining fetal development through adulthood²⁵ confirmed the patterns of down- and upregulated genes that we observed in infant neurons and astrocytes (Extended Data Fig. 4 and Supplementary Fig. 3).

The abundance of OPCs decreased during ageing ($P = 1.31 \times 10^{-2}$, Wilcoxon rank-sum test), being highest in infant donors and decreasing over lifespan (Fig. 2e), whereas mature oligodendrocytes increased during ageing in the brain ($P = 1.31 \times 10^{-2}$, Wilcoxon rank-sum test comparing infant with adult and elderly). These data suggest that the pool

of OPCs differentiates into mature oligodendrocytes during life with incomplete replacement; thus, the capacity to generate new oligodendrocytes might diminish in elderly people.

Increased cell-to-cell transcriptional variability during ageing has been identified in non-brain tissues^{26–28}, and is thought to be a consequence of ageing-related disruptions to the genome, epigenome and transcriptome. In our data, we detected only one cell type—IN-SST neurons—with a significant increase in the coefficient of variation in the transcriptome in elderly brains (Fig. 2f; $P = 4.30 \times 10^{-2}$, Wilcoxon rank-sum test). We observed similar trends when analysing our cohort in three age groups (15–39, 40–69 and 70 and over; Supplementary Fig. 4). Furthermore, the expression of *SST* and *VIP*, which are markers of two distinct classes of inhibitory neurons, decreased significantly with age (fold changes of –2.63 and –1.46; corrected P values $< 2.2 \times 10^{-16}$) in elderly IN-SST and IN-VIP cells, respectively (Fig. 2g). The loss of these functionally important marker genes, combined with increased transcriptional variability, suggests that inhibitory neurons are changing in fundamental ways during ageing. A previous report described a decrease in IN-SST and IN-VIP inhibitory neurons during ageing in the human brain¹⁶. Although we did not detect this phenomenon (Extended Data Fig. 1c,d), our data are consistent with the notion that inhibitory signalling is compromised in the elderly brain.

Housekeeping genes decrease in ageing

Differential expression analysis by cell type, comparing the 7 elderly cases with the 10 adult cases, yielded 2,803 genes that changed significantly with age ($\log_2(\text{elderly/adult}) > 0.5$, corrected $P < 0.05$) (Fig. 3a and Supplementary Table 6). We obtained similar results when our cohort was binned into three groups, or when using an alternate linear model method (Extended Data Fig. 5 and Supplementary Table 7). Reanalysis of published data from control donors spanning 38–93 years of age²⁹, and from a cohort of elderly donors³⁰, confirmed our results (Extended Data Fig. 5). In every cell type, more genes were downregulated during ageing than upregulated (Wilcoxon signed-rank test, $P = 2.44 \times 10^{-4}$), and most downregulated genes were identified in neurons. L2/3 excitatory neurons had the most up- and downregulated genes (201 and 1,273 respectively) of all cell types. A total of 124 genes that were downregulated in ageing were commonly downregulated across multiple cell types (Fig. 3b and Supplementary Table 8), reflecting an increase relative to random chance ($P < 0.001$, random permutation test). For example, the heat-shock protein *HSPA8*, the cytoskeletal protein *TUBA1A* and eight other genes were significantly downregulated in all 13 brain-cell types during ageing. Other commonly downregulated genes across cell types included other cytoskeletal genes such as *TUBB3* (down in 12/13 cell types), *TUBA4A* (10/13) and *TUBB* (9/13); the calmodulin genes *CALM2* and *CALM3* (9/13 and 12/13, respectively); and the vesicle protein *VAMP2* (13/13). By contrast, only two transcripts—the antisense transcript of *UBA6*, a ubiquitin-modifying enzyme, and *TMTCL1*, an endoplasmic-reticulum protein involved in calcium homeostasis—were commonly upregulated in multiple types of neuron and glia.

A common feature seen across cell types in the ageing brain was the widespread downregulation of ‘housekeeping’ genes. Indeed, gene ontology (GO) analysis of downregulated genes yielded common terms across all cell types except endothelial cells (Fig. 3c and Supplementary Table 9). This result was robust to evenly down-sampling lists of differentially expressed genes across cell types (Supplementary Table 10). In non-endothelial cells, terms related to housekeeping functions such as translation, metabolism, homeostasis, ribosomes, intracellular localization and intracellular transport were significantly enriched in the downregulated genes. To assess the expression changes of genes with common cellular functions further and in an unbiased manner, we defined a set of housekeeping genes in our dataset as those genes that were stably expressed in all brain cell types (average log(counts

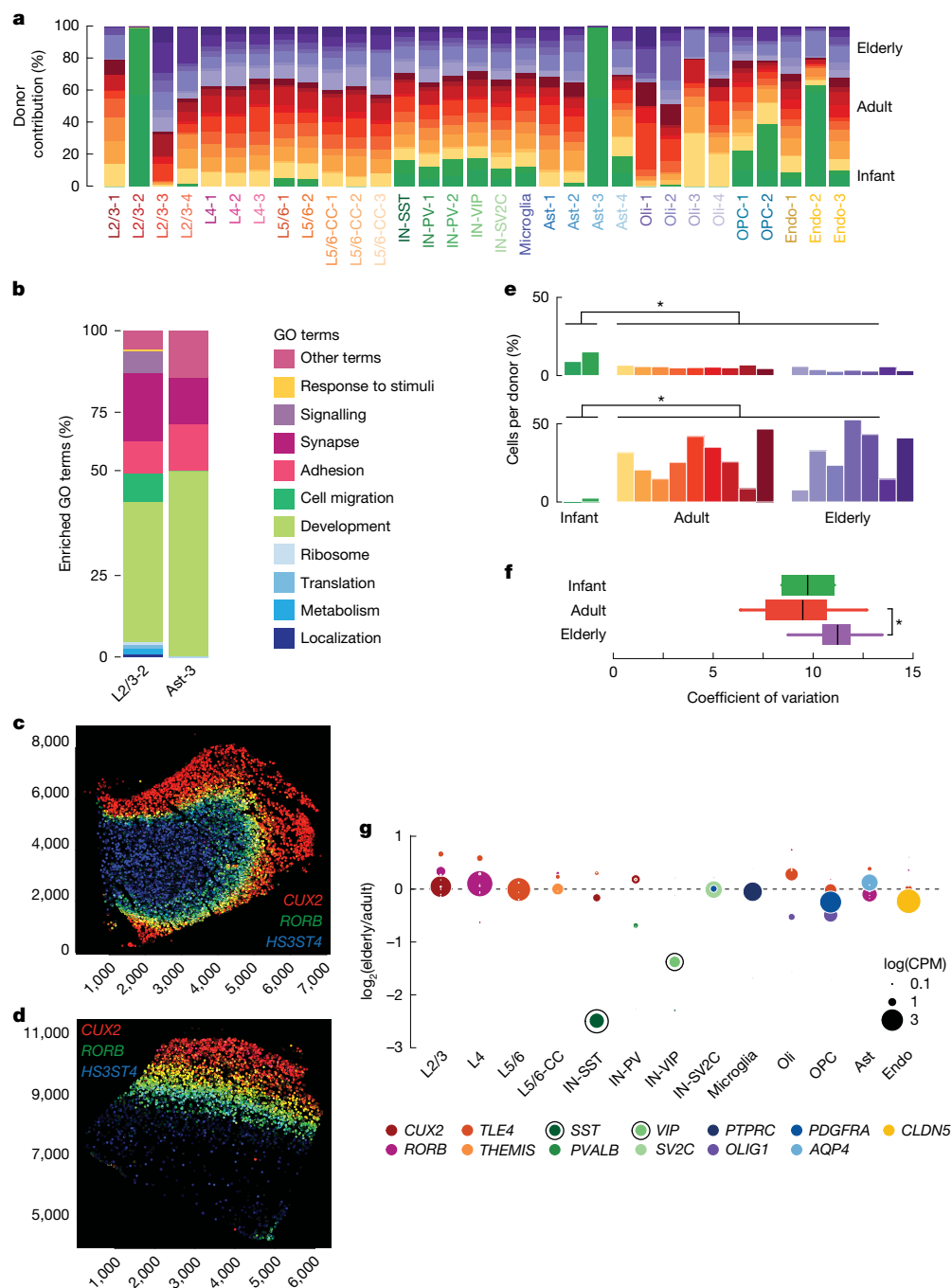


Fig. 2 | Changes in the transcriptional state of brain cells across the human lifespan. **a**, Clusters plotted by donor contribution as a percentage of total cells in the cluster. L2/3-2 and Ast-3 are composed nearly completely of nuclei from infant donors. **b**, GO terms derived from differentially expressed genes upregulated in infant-specific clusters plotted as general categories (see Supplementary Table 5 for a full list of terms and category designations). Development-related terms (shades of green) are most common. **c, d**, MERFISH section from a 0.4-year-old male donor (**c**) and a 15-year-old female donor (**d**), showing correct laminar positioning. Circles correspond to excitatory neurons and are coloured according to marker-gene expression (red, *CUX2* (L2/3); green, *RORB* (L4); blue, *HS3ST4* (L5/6); yellow, *CUX2* and *RORB* co-expression);

teal, *RORB* and *HS3ST4* co-expression). *x*- and *y*-axis values reflect pixel positions **e**, Contribution of OPCs (top) and oligodendrocytes (bottom) to the total nuclei identified in each donor (* $P < 0.05$). **f**, Transcriptional variability in IN-SST neurons. Variability significantly increases in neurons from elderly donors. Box plots depict median and first and third quartiles. Whiskers show 1.5 times the interquartile range (IQR) beyond the first and third quartiles ($P = 4.30 \times 10^{-2}$, two-sided Wilcoxon rank-sum test; elderly $n = 7$, adult $n = 9$). **g**, $\log_2(\text{elderly/adult})$ fold change plotted for each marker gene. Dot size corresponds to expression in each cell type. Dots circled in black have statistically significant fold changes, meeting our criteria for differential expression.

per million (CPM)) > 0.1 in each cell type and with differences of less than 0.1 between cell types), including endothelial cells and microglia that derive from a distinct embryological origin from that of neurons and other glia (Supplementary Table 11), and measured their changes in expression during ageing (Supplementary Fig. 5a). By the same logic, we

defined neuron-specific genes as those detected in all neuron subtypes but absent in non-neuronal cells (Supplementary Table 11). Expression of these housekeeping genes decreased in elderly relative to adult neurons across subtypes (Fig. 3d). By contrast, neuron-specific genes did not decrease in neurons during ageing (Supplementary Fig. 5b). Thus,

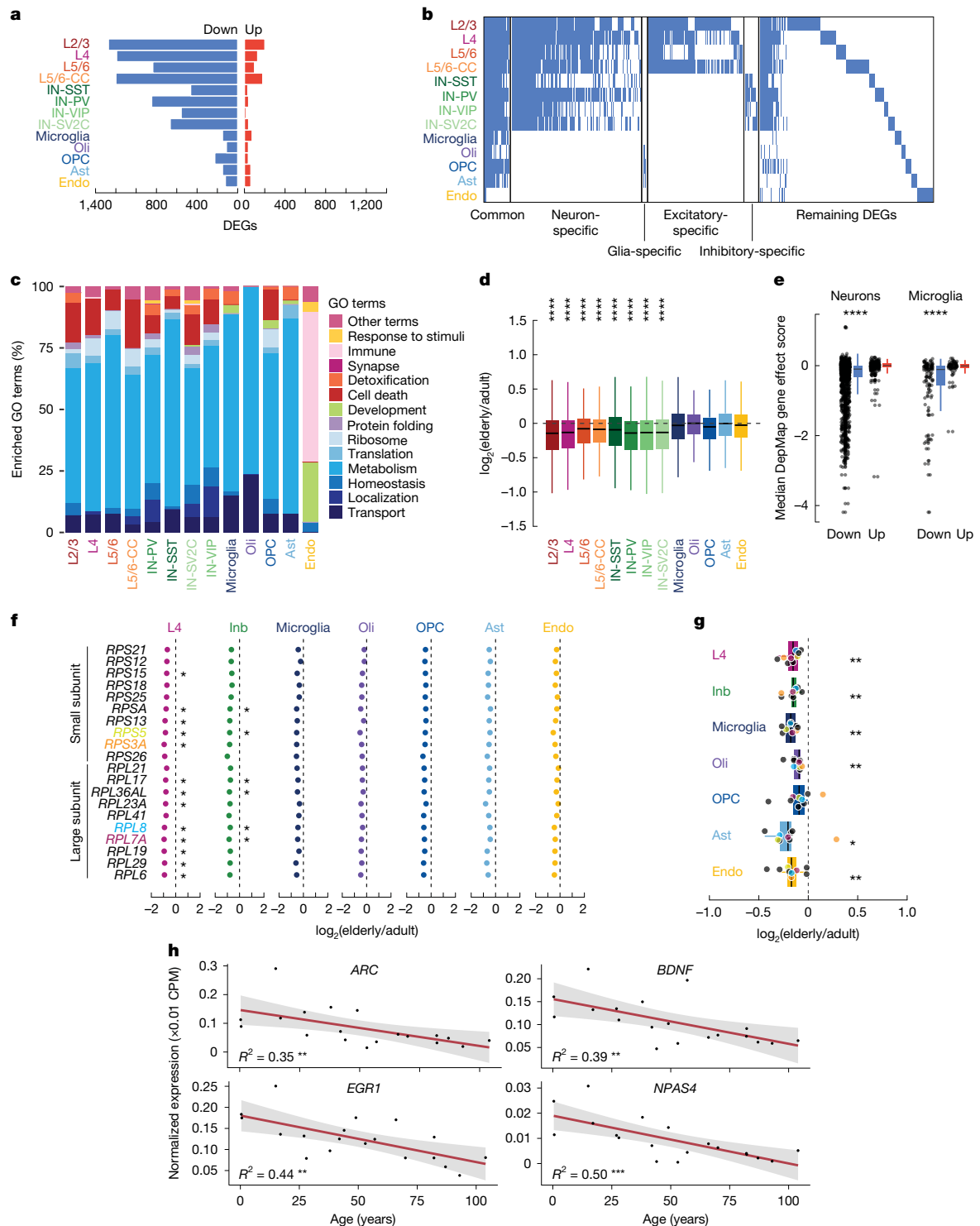


Fig. 3 | Common downregulation of genes across cell types. **a**, Number of downregulated (blue) and upregulated (red) genes for each cell type in elderly donors. DEGs, differentially expressed genes. **b**, Heat map of significantly downregulated differentially expressed genes in elderly donors. Genes not differentially expressed are in white. The leftmost genes are defined as common across cell types (down in one or more excitatory, one or more inhibitory and two or more non-neuronal cell types). **c**, GO terms of genes downregulated in ageing plotted as general categories (see Supplementary Table 8 for full GO results). Housekeeping functions (shades of blue) are commonly downregulated. **d**, Housekeeping genes are significantly downregulated in elderly relative to adult brains in all neuron types. Boxes show median, first and third quartiles. Whiskers show $1.5 \times \text{IQR}$ beyond the first and third quartiles ($****P < 0.0001$ and fold change < -0.05 , two-sided Wilcoxon rank-sum test; elderly $n = 7$, adult $n = 9$).

e, Mean gene effect score for all of the downregulated (blue) and upregulated (red) genes (in elderly versus adult donors) in the DepMap database. The downregulated genes for both neurons (left) and microglia (right) are more essential than the upregulated genes (two-sided t -test; neurons down $n = 1,954$, neurons up $n = 455$, microglia down $n = 149$, microglia up $n = 75$; neurons $****P = 7.33 \times 10^{-7}$, microglia $****P = 9.09 \times 10^{-7}$). Boxes and whiskers as in **d**. Points beyond whiskers are outliers. **f, g**, Fold change in elderly versus adult ribosomal-protein genes from both the small and the large subunit by snRNA-seq (two-sided t -test; elderly $n = 7$, adult $n = 9$) (**f**) and MERFISH (two-sided Wilcoxon rank-sum test; elderly $n = 3$, adult $n = 3$) (**g**). Inb, inhibitory. Genes shown in both **f** and **g** are colour-coded. Boxes and whiskers as in **e**. **h**, Expression of immediate early genes in excitatory neurons decreases with age. Grey shading, 95% confidence intervals. All data points shown ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

neurons lose the expression of genes related to general cell function, but maintain cell identity in the ageing brain.

The DepMap database scores gene essentiality on the basis of survival rates after knockout in hundreds of cancer cell lines. Using DepMap, we found that genes that were downregulated with age in neurons and microglia were more often essential for cell survival than were genes that were upregulated (neurons: $P = 7.33 \times 10^{-7}$; microglia: $P = 9.09 \times 10^{-7}$, two-sided *t*-test) (Fig. 3e and Supplementary Table 11), suggesting that genes that are downregulated in ageing reduce brain-cell viability.

RPS3A, *RPL26* and *RPL15* (all encoding ribosomal proteins) were significantly downregulated during ageing in 11 out of 13 cell types (Supplementary Table 8), and 14 other ribosomal-protein genes were commonly downregulated. This prompted us to examine the expression level of all ribosomal genes. We observed a near-universal trend of a decrease in the expression of genes encoding the small and large ribosomal subunits during ageing—much more than would be expected by chance (P values $< 3.76 \times 10^{-6}$; Fisher's exact test) (Fig. 3f and Supplementary Fig. 6). To validate this finding, we performed MERFISH experiments on three elderly brains (82 years (male), 82 years (female) and 87 years (male)) and three adult brains (28 years (male), 42 years (female) and 49 years (female)). Our results showed that across cell types, the expression of nine ribosomal proteins decreased in elderly brains, with significant decreases in all but OPCs (Fig. 3g, Extended Data Fig. 6 and Supplementary Table 11). Nuclear-encoded proteins of the mitochondrial electron transport chain, except for complex II genes, also showed coordinated downregulation by both snRNA-seq and MERFISH (Extended Data Fig. 6d,e and Supplementary Fig. 7). Analysis of our snRNA-seq cohort in three age groups instead of two indicates that both ribosomal and mitochondrial genes decrease significantly after the age of 40 years, with donors aged 40–69 years showing similar expression of these genes to that of donors aged 70–104 years (Extended Data Fig. 7). These data suggest that neurons become less metabolically active during life. Along these lines, the expression of immediate early genes, which are activated rapidly during neuronal stimulation³¹, decreases during brain ageing (Fig. 3h).

Mutation patterns reflect transcription

Somatic mutations accumulate in cells during life for many cell types throughout the human body^{9,32–35}, including in post-mitotic neurons of the human brain^{6,8,9}. Neuronal rates of somatic mutation correlate with transcription as measured by bulk RNA-seq in the brain^{5–7,9}, suggesting that somatic mutations can affect important brain gene-regulatory programs. Mutational signature analysis has implicated the activity of several DNA-repair genes in generating somatic mutations in neurons^{5,7,10}. Thus, both the upstream causes and downstream effects of single-cell somatic mutations can be studied using single-cell gene expression.

To link changes in the neuronal transcriptome to changes in the somatic mutation burden of individual neurons, we performed scWGS using primary template-directed amplification (PTA)^{7,36} on neurons from the same brain region and donors analysed by snRNA-seq (Supplementary Table 12). We used the SCAN2 algorithm⁶ to identify somatic single-nucleotide variants (sSNVs) in scWGS data from each sample (Supplementary Table 13). In agreement with previous reports^{6,7,9}, our analysis suggested that sSNVs accumulate at a rate of 15.1 per neuron per year ($R^2 = 0.87$, $P = 2.20 \times 10^{-16}$) (Extended Data Fig. 8a). The overall pattern of mutations resembles a known signature called SBS5 (cosine similarity 0.96), first identified by the Catalogue Of Somatic Mutations In Cancer (COSMIC) consortium, which accumulates during life across many tissues³⁷ (Extended Data Fig. 8b,c).

We compared the changes in neuronal gene expression with the age-related patterns of somatic mutation in neurons to investigate the relationships between the genome and the transcriptome in ageing. We found that the overall, SBS5-like spectrum of neuron sSNVs was composed of two distinct signatures, which we name A1 and A2

(Fig. 4a, Supplementary Fig. 8 and Extended Data Fig. 8c). Signature A1 resembled SBS5 (cosine similarity 0.88), and correlated strongly with the age of the donor ($R^2 = 0.88$, $P = 3.30 \times 10^{-50}$) (Fig. 4b), accounting for 12.1 of the 15.1 mutations per year. The burden of signature A1 also correlated strongly with neuronal gene-expression levels (Fig. 4c and Supplementary Fig. 8; chi-squared test), demonstrating that transcription in neurons sensitizes some loci to specific types of somatic mutation. In line with this, significant transcriptional strand bias in sSNVs, which is thought to result from asymmetrical damage and repair rates on template and non-template strands at transcribed loci³⁸, was observed in medium to highly expressed genes but not in genes expressed at low levels (Fig. 4d; asterisks denote significant deviations from 50:50). Furthermore, signature A1 was enriched in active chromatin states in the human brain at active transcription start sites (TSSs), enhancers, bivalent TSSs and weakly repressed polycomb sites, but depleted at quiescent and weakly transcribed loci (Fig. 4g and Supplementary Table 14; chi-squared test).

Signature A2 accounted for fewer age-related mutations per year (3; $R^2 = 0.42$, $P = 6.60 \times 10^{-14}$), and most sSNVs in infant neurons were derived from signature A2 (Fig. 4b,e and Supplementary Table 13). Signature A2 showed high similarity to developmental mosaic mutations identified in three separate studies that used orthogonal methods to scWGS^{39–41} (cosine similarity 0.77, 0.81 and 0.83; Extended Data Fig. 9a). The sSNVs identified in our infant donors were also similar to those confirmed developmental mosaics (cosine similarity 0.82, 0.85 and 0.88, Extended Data Fig. 9a). Signature A2 clustered with COSMIC signature SBS30 (cosine similarity 0.82) (Extended Data Fig. 8c). Signature A2 mutation rates anticorrelate with neuron gene-expression levels and are enriched in intergenic regions (Fig. 4f), in agreement with trends observed for SBS30 (COSMIC database). In accordance with its enrichment in genes expressed at low levels, signature A2 is enriched in the human brain in chromatin states found at sites of weak transcription, and is depleted at repressed and weakly repressed polycomb sites (Fig. 4h and Supplementary Table 14; chi-squared test).

Nevertheless, signature A2 differs from SBS30 in some key ways. SBS30 comprises C>T variants almost exclusively, and these variants are depleted at CpG dinucleotides (Extended Data Fig. 9c). By contrast, signature A2 contains substitutions in addition to C>T, such as C>A, which we previously linked to increased oxidative DNA damage during ageing, and T>C, which increases with age^{7,8}. Similarly to confirmed developmental clonal mosaic mutations identified in other studies using non-scWGS methods^{39–41}, signature A2 shows contributions of SBS1 and SBS5 in addition to SBS30 (Extended Data Fig. 9b). Signature A2 shows higher CpG>TpG variants than does SBS30, suggesting that deamination of methylated cytosines has a role in the genesis of signature A2, as it does in confirmed mosaics (Extended Data Fig. 9c). A high burden of C>T at CpG dinucleotides distinguishes biological from technical mutational signatures in single-cell genomics^{7,42}.

The differences observed between signatures A1 and A2 with respect to their rate of accumulation per year, their differential correlation with neuron gene expression, their distinct relative burden in genic versus non-genic regions and their differential correlation with brain chromatin states support the notion that these signatures represent biologically distinct components of the overall, SBS5-like mutation spectrum observed in single human neurons. Signature A1 is the predominant source of age-related sSNVs in neurons and correlates with neuron gene expression, confirming that transcription directly determines the neuronal sSNV rate. Signature A2 seems to be more active in development and early life, but signature A2 mutations continue to accumulate during ageing, at transcriptionally inactive loci.

Gene length, transcription and mutation in ageing

Somatic mutations arise from DNA damage that occurs through a variety of mechanisms. Long genes are downregulated in ageing across

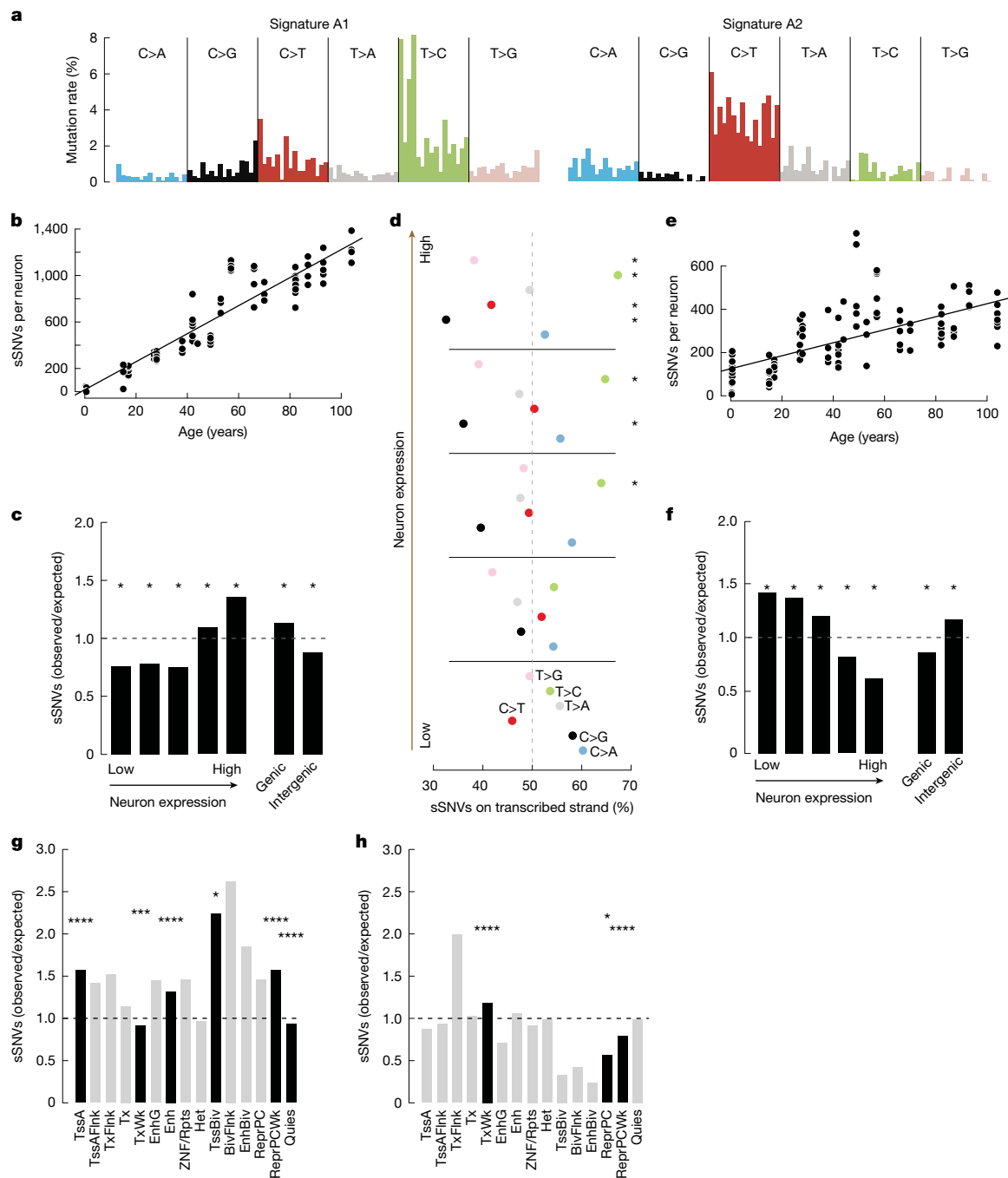


Fig. 4 | scWGS reveals sSNV mutational signatures linked to expression.

a, De novo mutational signature analysis of sSNVs in human neurons revealed two signatures: A1 dominated by T>C mutations and A2 dominated by C>T mutations. Trinucleotide contexts are the same as shown in Extended Data Fig. 6c. **b**, Number of signature A1 sSNVs in each neuron plotted by age. Signature A1 strongly correlates with age ($R^2 = 0.88$, $P = 3.30 \times 10^{-50}$) with an extrapolated mutation rate of 12.1 SNVs per year. **c**, sSNV enrichment of signature A1 in coding regions plotted by neuron expression quantile (left) and genic versus intergenic regions (right). Signature A1 is enriched in the highest-expressed genes and genic regions (chi-squared test). **d**, Percentage of total sSNVs derived from the transcribed strand broken down by expression quantile. T>C and C>T strand bias increases with expression (chi-squared test; *multiple-testing-corrected false discovery rate (FDR) < 0.05; **multiple-testing-corrected FDR < 0.01). **e**, Number of signature A2 sSNVs in each neuron plotted by age. Signature A2

correlates with age ($R^2 = 0.42$, $P = 6.60 \times 10^{-14}$) with an extrapolated mutation rate of 3 SNVs per year. **f**, sSNV enrichment of signature A2 in coding regions plotted by neuron expression quantile (left) and genic versus intergenic regions (right). Signature A2 is depleted in the highest-expressed genes and enriched in the lowest-expressed genes as well as intergenic regions (* $P < 0.05$, chi-squared test). **g, h**, Mutation enrichment in human brain chromatin states for signature A1 (**g**) and signature A2 (**h**) (chi-squared test; * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$). TssA, active TSS; TssAFlnk, flanking active TSS; TxFlnk, transcription at gene 5' and 3'; Tx, strong transcription; TxWk, weak transcription; EnhG, genic enhancers; Enh, enhancers; ZNF/Rpts, ZNF genes and repeats; Het, heterochromatin; TssBiv, bivalent or poised TSS; BivFlnk, flanking bivalent TSS or enhancer; EnhBiv, bivalent enhancer; ReprPC, repressed polycomb; ReprPCWk, weak repressed polycomb; Quies, quiescent or low expression.

many organs—an effect that is attributed to their naturally increased likelihood of acquiring transcription-blocking DNA damage owing to random chance^{43–45}. We find that sSNV rates correlate with neuronal

gene expression^{6,7} (Fig. 4c), suggesting that transcription and DNA damage are linked. How gene length and expression level relate to transcriptional changes in ageing, and whether differences in somatic mutation

patterns based on gene size or transcription correlate age-associated changes, are unknown.

To investigate the effects of gene length and expression level on transcriptional changes in ageing, we performed a multiple linear regression analysis. We found that high basal expression predicts decreased expression in aged donors (Fig. 5a, Supplementary Fig. 9a and Supplementary Table 15). We confirmed this relationship using bulk brain expression data from the GTEx consortium. However, a more robust effect was observed for gene size. There was a positive correlation between gene length and expression in elderly neurons compared with adult neurons. In other words, longer genes are more likely to maintain or increase their expression during ageing, and, unlike in other organs, downregulated genes in neurons are more likely to be short. A significant but lower magnitude effect was also observed for exon length and expression, suggesting that this effect was driven mostly by gene length, not transcript length. This length effect was stronger in excitatory and inhibitory neurons ($R = 0.59$ and 0.57 , respectively) than in glia (average $R = 0.35$), and downregulated genes in neurons were shorter than those in glia (Fig. 5b), highlighting a cell-type-specific effect. Although in opposition to the relationship observed in many tissues, our data agree with data from the mouse frontal cortex⁴⁵ and bulk-sorted retinal ganglion cells, in which long gene expression is preserved during ageing⁴⁵.

A larger percentage of neurons than non-neurons expresses the topoisomerases *TOP1* and *TOP2B*, and the topoisomerase interactors *PARP1*, *TDPI* and *BTBD1* ($P = 8.17 \times 10^{-5}$; Wilcoxon rank-sum test) (Fig. 5c). Neurons rely on topoisomerase activity to mitigate the torsional stress generated when unwinding neuronal genes during transcription⁴⁶, which tend to be longer than broadly expressed housekeeping genes⁴⁷ (Fig. 5d), suggesting that high topoisomerase expression protects long genes in neurons.

Our multiple linear regression model results are in general agreement with the results from GO analysis that suggested that housekeeping genes are downregulated in ageing (Fig. 3c), because housekeeping genes are generally short (Fig. 5d) and highly expressed^{48–50} (Fig. 5e and Supplementary Fig. 9b,c).

Our combined single-cell genomic and transcriptomic dataset allowed us to probe the relationship between gene size, genome damage and age-related expression changes in depth at the single-neuron level. Because gene length and gene function are related in the brain (neuronal genes tend to be long), we separately analysed the relationship between gene length and expression change during excitatory neuron ageing in neuron-specific genes and housekeeping genes. Housekeeping genes showed a positive correlation between gene length and expression change in ageing ($R^2 = 0.50$, $P = 1.35 \times 10^{-281}$) (Fig. 5f and Supplementary Fig. 10), such that the shortest genes were the most downregulated, whereas the longest showed no change or slightly increased in aged cells. This pattern resembled the downregulation of short genes observed in the overall transcriptome (Fig. 5b). However, across neuron-specific genes, there was a significantly weaker relationship (Fisher's r -to- z transformation, $P = 2.08 \times 10^{-11}$) between gene length and expression change in aged brains ($R^2 = 0.20$, $P = 1.24 \times 10^{-3}$) (Fig. 5g and Supplementary Fig. 11). These conclusions were validated by analyses of previously published datasets and by analysis of our data using different groupings or using a linear model method (Extended Data Fig. 10). MERFISH analysis of 33 short housekeeping genes, 33 long housekeeping genes, 24 short neuron-specific genes and 21 long neuron-specific genes confirmed the downregulation of short housekeeping genes in samples from elderly donors relative to samples from adult donors ($P = 3.4 \times 10^{-5}$, Wilcoxon rank-sum test), and did not identify any significant changes in long housekeeping genes or neuron-specific genes of either size (Fig. 5h).

Within gene classes, the sSNV rate mirrored changes in expression during ageing; in housekeeping genes, the sSNV rate decreased as gene length increased ($R^2 = 0.44$, $P = 3.52 \times 10^{-2}$) (Fig. 5i and Supplementary

Table 16), whereas in neuron-specific genes there was no significant relationship between gene length and sSNV rate ($R^2 = 0.02$, $P = 0.706$) (Fig. 5j and Supplementary Table 16). These data suggest that there are distinct patterns of DNA damage and repair in housekeeping and neuron-specific genes (Fig. 5k). Thus, gene length, gene function and genome damage combinatorially affect the transcriptome of the ageing brain.

Discussion

Here we used snRNA-seq, scWGS and spatial transcriptomics to study genomic and transcriptomic changes in the brain during life. We conclude that short, highly expressed housekeeping genes show high rates of sSNV accumulation during life that correlate with reduced expression. Several lines of evidence lead us to this conclusion. First, housekeeping functions were the most commonly enriched GO terms for downregulated genes, dominating the neurons in particular, whereas neuron-specific genes remained flat during ageing in general, with no significant changes in expression. Second, housekeeping genes were short and highly expressed, in agreement with previous literature. Third, sSNV rates in neurons correlated with neuron gene-expression levels. Indeed, the shortest housekeeping genes, which showed high levels of expression, showed the highest sSNV rates. Finally, a multiple linear regression model showed that high expression correlated with the likelihood of transcriptional downregulation in ageing, and that long gene length correlated with the maintenance or an increase of transcript levels in ageing. The relationship between gene length and the ageing transcriptome has been a point of curiosity in the field, but thus far, this association has varied across tissues^{43–45}. Our analysis suggests that in neurons, long genes related to cell identity are preserved in ageing, whereas short housekeeping genes accumulate somatic mutations and decrease in abundance during life.

Several mechanisms could explain this relationship. First, mutations might directly generate premature stop codons or change patterns of RNA splicing, inducing nonsense-mediated decay of mutant transcripts. Second, aberrant DNA-repair processes involved in generating somatic mutations cause local epigenetic dysregulation⁵¹, affecting transcript levels. Third, differential repair of housekeeping and neuron-specific genes could have a role in differential sSNV burdens. Recently, single-stranded DNA lesions were shown to endure for long periods of time—up to years—in human cells, in the absence of active DNA repair⁵². sSNV rates might be high in short, highly expressed genes because they show preferential transcription-coupled DNA repair^{53–55}, meaning that DNA damage that occurs during transcription⁵⁶ might be efficiently made into permanent, double-stranded mutations owing to repair errors. Neurons might differ from cells in other organs because of their post-mitotic nature, or owing to the high expression of topoisomerase genes, which protect long genes.

Our work also defined other changes in the human brain during healthy life. In the infant brain, we identified populations of immature neurons and astrocytes, and an increased ratio of oligodendrocyte precursors to mature oligodendrocytes, in support of the notion that brain-cell development continues after birth. In agreement with previous work, scWGS showed that sSNVs with an overall spectrum resembling COSMIC SBS5 increased in ageing neurons. De novo signature analysis revealed two signatures, A1 and A2, dominated by T>C and C>T transitions, respectively, that clustered with known somatic mutational signatures in cancer, SBS5 and SBS30, respectively. The aetiology of SBS5 is unknown, but it has been reported to behave in a clock-like manner in brain and other tissues^{8,9,32,37}. Signature A2 somewhat resembles SBS30 and contains C>A and T>C variants—mutation types that are linked with oxidative DNA damage and ageing, respectively. SBS30 has been linked with⁵⁷ decreased activity of the base excision repair protein NTHL1, and our previous work linked neuron C>A variants to the base excision repair protein OGG1. Our snRNA-seq data revealed

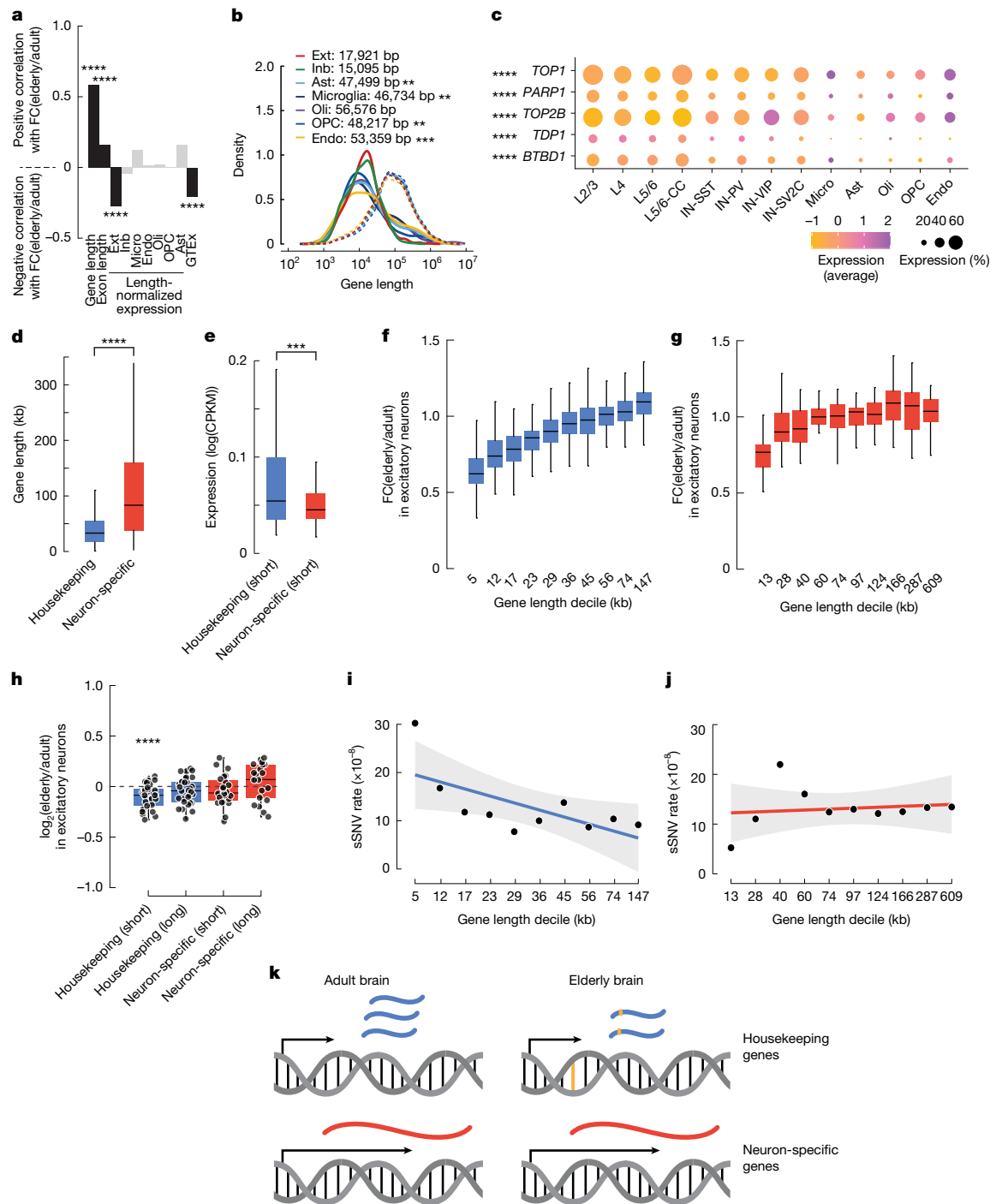


Fig. 5 | Gene downregulation during ageing relates to gene size, expression level, gene type and sSNV burden. **a**, Mixed-effects linear model identifying determinants of downregulation in excitatory neurons (model performance $R^2 = 0.54$). Gene and exon length positively correlated with ageing-related fold change (FC) in expression. Length-normalized expression in excitatory neurons and frontal cortex expression (GTEx database) negatively correlated with ageing-related fold change. Significance of linear model correlations was determined by two-sided t -test. **b**, Density plots of the length of downregulated genes (solid lines) and all expressed genes (dashed lines) for each cell type. Mean lengths for downregulated genes are shown; asterisks denote significant differences from the mean neuronal downregulated length (two-sided t -test). **c**, Expression of topoisomerase complex genes across cell types. Asterisks denote significant differences in the percentage of cells expressing between neurons and non-neurons (two-sided Wilcoxon rank-sum test). **d**, Housekeeping genes ($n = 1,802$) are significantly shorter than neuron-specific genes ($n = 288$) ($P = 2.2 \times 10^{-16}$, two-sided t -test). **e**, Short (decile 1) housekeeping ($n = 180$) and

neuron-specific ($n = 28$) genes showed differential expression in adult excitatory neurons ($P = 6.5 \times 10^{-4}$, two-sided t -test). CPKM, counts per kilobase per million. **f, g**, Fold change (elderly/adult) of housekeeping genes (**f**) and neuron-specific genes (**g**) by length decile in excitatory neurons (housekeeping $R^2 = 0.50$, $P = 1.35 \times 10^{-281}$; neuron-specific $R^2 = 0.20$, $P = 1.24 \times 10^{-3}$; elderly $n = 7$, adult $n = 9$). **h**, Fold change in the expression of the indicated gene sets in excitatory neurons, from MERFISH data ($P = 3.4 \times 10^{-5}$, two-sided Wilcoxon rank-sum test; elderly $n = 3$, adult $n = 3$). **i, j**, The sSNV rate per base pair negatively correlates with gene length in housekeeping genes ($R^2 = 0.44$, $P = 3.52 \times 10^{-2}$, Pearson's correlation) (**i**), but not in neuron-specific genes ($R^2 = 0.02$) (**j**). Simple linear model trend line with grey 95% confidence intervals shown. **k**, The relationship between gene length (black arrow), mRNA expression (blue and red), and mutations (yellow) identified in this work. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. All box plots depict median and first and third quartiles. Whiskers show $1.5 \times \text{IQR}$ beyond the first and third quartiles.

that both *NTHL1* and *OGG1* were expressed in neurons, and that this expression was dynamic during ageing, but further studies are needed to link these changes to signature A2. We note that, despite the high similarity between signature A2 and SBS30, A2 in neurons is distinguished from this tumour signature by higher levels of C>T at CpG dinucleotides. Signature A1 was enriched in coding regions, highly expressed genes and known open chromatin sites, whereas A2 showed the opposite pattern, being enriched in non-coding regions, highest in repressed genes and enriched in loci bearing repressive chromatin marks.

As the application of scWGS technologies expands to include other cell types in the brain, it will become possible to further elucidate the relationship between somatic mutations and gene expression during ageing. This will increase researchers' understanding of the genomic and transcriptomic landscape in the ageing brain.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-025-09435-8>.

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Methods

Tissue procurement

All tissue was provided by the National Institutes of Health (NIH) NeuroBioBank and Banner Sun Health Research Institute Brain and Body Donation Program, which obtained written authorization and informed consent for all donors. Tissue collection and distribution for research purposes were done in accordance with protocols approved by the NIH NeuroBioBank (IRB protocol number: HM-HP-00042077) or the Human Brain and Spinal Fluid Resource Center (managed by the Sepulveda Research Corporation; IRB protocol number: PCC: 2015-060672, VA project number: 0002) and by the Banner Sun Health Research Institute Brain and Body Donation Program (WCG IRB protocol number 20120821). Tissue was collected from post-mortem, de-identified donors and thus this work is not considered by our Institutional Review Board to be research using human subjects. Cases were selected on the basis of RNA quality, age at time of death and absence of a history of neurological disease or evidence of neuropathology in the tissue. Brodmann area 9 or adjacent Brodmann area 46 of PFC was provided for each donor and used for both snRNA-seq and scWGS. To obtain the donor reference genomes, bulk DNA samples were collected from donor-matched tissues, which included heart, liver, muscle, cerebellum or cortex. Bulk DNA whole-genome sequencing (WGS) data for donors 1278, 4638, 1465, 4643, 5657 and 5817 (0.4-year-old male, 15-year-old female, 17-year-old male, 42-year-old female, 82-year-old male and 0.6-year-old male individuals, respectively) were obtained from previous studies^{8,58}, along with bulk DNA WGS data for donor 5572 (70-year-old female individual)⁷.

Isolation of nuclei from fresh-frozen tissue samples

The nuclei isolation protocol was adapted from two previous publications^{59,60}. All procedures were performed on ice or at 4 °C. Fresh-frozen samples were processed using a 7-ml glass Dounce homogenizer with approximately 20 mg tissue in 5 ml of filter-sterilized tissue lysis buffer (0.32 M sucrose, 5 mM CaCl₂, 3 mM MgAc₂, 0.1 mM EDTA, 10 mM Tris-HCl (pH 8), 0.1% Triton X-100 and 1 mM fresh DTT). The homogenized solution was loaded on top of a filter-sterilized sucrose cushion (1.8 M sucrose, 3 mM MgAc₂, 10 mM Tris-HCl (pH 8) and 1 mM DTT) and spun in an ultracentrifuge in an SW28 rotor (13,300 rpm, 2 h, 4 °C) to separate nuclei.

For nuclei isolated for snRNA-seq, after spinning, the supernatant was removed and nuclei were resuspended (1% BSA in PBS plus 25 µl 40 U µl⁻¹ RNase inhibitor), then filtered through a 40-µm cell strainer. After filtration, nuclei were counted using trypan blue and an automated haemocytometer (Countess II; Invitrogen) and diluted to a concentration of 1,000 cells per µl.

For nuclei isolated for scWGS, the supernatant was removed and nuclear pellets were resuspended in ice-cold resuspension buffer (8.5 ml 1× PBS with 3 mM MgCl₂ + 1 ml 1× PBS with 3 mM MgCl₂ and 1% BSA + 500 µl sucrose cushion), filtered with a 40-µm cell strainer and then stained with an anti-NeuN antibody (directly conjugated to Alexa Fluor 488; Millipore MAB377X, clone A60; 1:1,250) and an anti-rabbit IgG Alexa Fluor 647 antibody as a negative control for 30 min. Using a BD Biosciences FACSARIA Fusion machine and BD FACSDiva Software, forward scatter A (FSC-A) was first used to isolate large non-replicating cells. NeuN staining produced a bimodal signal distribution, distinguishing NeuN⁺ and NeuN⁻ nuclei (Supplementary Fig. 13). Large neuronal nuclei, representing excitatory pyramidal neurons, were further identified by collecting the nuclei with the highest NeuN signal among the NeuN⁺ neuronal fraction, and gating for the population with the highest FSC-A signal and excluding Alexa-Fluor-647-high events⁷. This non-replicating high-FSC-A and high-NeuN population was confirmed to be an excitatory neuron population, comprising 2–5% of the total population of nuclei in each sample⁷.

Droplet-based snRNA-seq

Droplet-based libraries were generated using the Next GEM Single Cell 3' v.3 or v.3.1 reagent kits (10x Genomics) and the Chromium Controller according to the manufacturer's instructions. The resulting libraries were indexed with the KAPA Unique Dual-Indexed Adapter Kit (Roche KK8726) and sequenced on an Illumina NovaSeq 6000 with 150 paired-end reads by Genuity Science. Samples were prepared in batches of up to six donors at a time that always included male and female donors as well as mixed ages (Supplementary Table 3). To prevent age or gender bias in our batches, some samples have multiple biological replicates, prepared on different dates. A single replicate each from three distinct donors clustered abnormally during downstream analysis and was therefore excluded from analysis. After filtering, the only clusters exhibiting batch bias are those that are infant-specific and biologically driven (Supplementary Fig. 1). Because those cells were present only in infant donors, the only batches contributing to those clusters are those that included an infant.

In addition to data generated for this manuscript, we also included data that were previously published⁷: case 1465, a 17-year-old male individual. Single nuclei from the PFC were isolated by fluorescence-activated nuclear sorting using three gates (large NeuN⁺ nuclei, NeuN⁺ nuclei and DAPI⁺ nuclei) to generate three populations (large neurons, neurons and all nuclei). For each population, 16,000 nuclei were sorted into one well of a 96-well plate, which were then used to perform snRNA-seq using the Next GEM Single Cell 3' GEM kit v.3.1 and the Chromium Controller (10x Genomics). The three resulting libraries were indexed using the 10x Genomics Dual Index Plate and sequenced on an Illumina NovaSeq S4. For our downstream differential expression analysis, all three populations were grouped together. Donor 1465 was excluded from analyses of cell-type proportion because the tissue had been subjected to fluorescence-activated cell sorting, which skewed the cell-type ratios.

scWGS of neurons using PTA

Single neuronal nuclei, prepared as described above, were whole-genome-amplified by PTA^{6,36} using the ResolveDNA Whole Genome Amplification kit (BioSkrby Genomics). First, nuclei were sorted into cold 96-well plates pre-loaded with 3 µl cold cell buffer (BioSkrby) one per well. Nuclei were lysed as per the kit protocol by the addition of 3 µl MS mix, followed by a brief spin-down, then 1 min of agitation at room temperature at 1,400 rpm on a plate mixer, then 10 min on ice. Next, 3 µl SN1 buffer was added to each well and the plate was again spun down and agitated at 1,400 rpm for 1 min. Next, 3 µl SDX buffer was added, and the plate was again spun and agitated at 1,400 rpm for 1 min. Then, the plate was incubated at room temperature for 10 min. Next, reaction mix and enzyme were added to each well, for a total reaction volume of 20 µl per well. PTA was performed for 10 h at 30 °C, followed by enzyme inactivation at 65 °C for 3 min. Amplified DNA was cleaned up using an in-house carboxyl magnetic bead clean-up solution (0.024 M PEG-8000, 1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 8, 0.055% Tween 20 and 1.5 ml Cytiva Sera-Mag SpeedBeads Carboxyl Magnetic Beads, hydrophobic per 50 ml). DNA yield was determined using the QuantiFluor dsDNA System (Promega). Samples were subjected to quality control by multiplex PCR for four genomic loci on different chromosomes as previously described⁸. Amplified genomes showing positive amplification for all four multiplex PCR loci were prepared for Illumina sequencing.

Libraries were prepared following a modified KAPA HyperPlus Library Preparation protocol described in the ResolveDNA EA Whole Genome Amplification protocol. In brief, the fragmentation step was skipped and end-repair and A-tailing were performed for 500 ng amplified DNA input. Adapter ligation was then performed using the SeqCap Adapter Kit (Roche, 07141548001). Ligated DNA was cleaned up using in-house beads and amplified through an on-bead PCR amplification

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step. Amplified libraries were selected for a size of 300–600 bp using double-size selection. Libraries were subjected to in-house quality control using a 5300 Fragment Analyzer Bioanalyzer for DNA fragment size distribution (Agilent Technologies). Successfully prepped samples were sent to Genuity Science for DNA sequencing, who further tested for quality using TapeStation (Agilent Technologies) before processing. Single-cell PTA-amplified genome libraries were sequenced on the Illumina NovaSeq 6000 platform (150 bp \times 2) at minimum 20 \times coverage (Supplementary Table 12). scWGS of some neurons was performed at Harvard for previous publications^{6,7} (Supplementary Table 12).

Bulk DNA isolation

Genomic DNA was isolated using the QIAGEN DNA Mini kit (QIAGEN 51304) according to the manufacturer's protocol for tissues. Approximately 25 mg of fresh-frozen tissue was minced on ice into small still-frozen pieces. Tissue was transferred to a dry-ice chilled sterile 1.5 ml microcentrifuge tubes with 180 μ l of buffer ATL. Then, 20 μ l of proteinase K (20 mg ml⁻¹) was added before 4 h of agitation at 56 °C on a thermomixer (1,400 rpm). DNA isolation proceeded as written in the protocol with the inclusion of the optional RNase A treatment step. A small sample was sent for fragment analysing and gDNA quality assessment.

Bulk DNA library preparation and sequencing

Bulk DNA was isolated as described above and libraries were prepared following the KAPA HyperPlus library preparation protocol. The KAPA fragmentation step was included in the bulk processed gDNA samples. Bulk gDNA sample libraries were sent to Genuity Science and sequenced on the Illumina NovaSeq 6000 platform (150 bp \times 2) at minimum 30 \times coverage and used as a reference genome against the case-match single-cell genomes. Bulk DNA for cases 1278, 4638, 4643, 5657 and 5817 was previously isolated and sequenced⁸ on an Illumina HiSeq X Ten platform by Macrogen Genomics or the New York Genome Center.

Analyses of snRNA-seq data

The snRNA-seq reads were aligned to the human genome and assigned to genes (GENCODE v.32) by Cell Ranger (v.6.0.2) with parameters --expect-cells=10000 --include-introns=true (ref. 61). The barcode and UMI solved counts were further processed with Seurat⁶² (v.4.3.0). The following filtering criteria were applied to each sample and cell: more than 100 cells in the sample; reads from mitochondrially encoded genes less than 5%; and more than 500 expressed genes in the cell. As discussed above, we further filtered samples '5817 200102', '5288 200128' and '5887 PFC 210601' owing to their batch-driven, not cell-type-driven, clustering, removing them from downstream analysis. To minimize false discovery and focus on universal changes in ageing, mitochondrially encoded genes and genes in sex chromosomes were removed in the downstream analysis. The filtered data were log-normalized with a factor of 10,000. The top 8,000 variable features were selected for principal component analysis (PCA), clustering and uniform manifold approximation and projection (UMAP) analysis. The top 30 principal components and 0.5 resolution were used for *k*-nearest neighbours (KNN)-graph based clustering, yielding 39 clusters.

Each of the cells in this study was anchored to the cells from Velmeshev et al.¹⁹ using the RPCA method with the top 30 principal components.^{19,62,63} For each of our 39 clusters, the percentages of cell types according to Velmeshev et al. were calculated, and the dominant cell types were used for each cluster. Those clusters with ambiguous cell types according to Velmeshev et al. were considered as artefacts and removed from the downstream analysis. We further defined marker genes for each cluster using the Seurat FindAllMarkers function by comparing each cluster with the remaining clusters, requiring expression in at least 25% of the cluster and a log₂-transformed fold change greater than 0.25. For analyses in which excitatory neuron layer or inhibitory neuron subtype are not specified, layer- and subtype-specific

clusters were combined and analysed as a group. Specifically, all neurons from the L2/3, L4, L5/6 and L5/6-CC clusters were combined into a non-layer-specific group of excitatory neurons, and neurons from the IN-SST, IN-SV2C, IN-PV and IN-VIP clusters were combined into a non-subtype-specific group of inhibitory neurons. Finally, we validated our cell-type assignment using the following marker genes (also shown in Supplementary Fig. 2): *CUX2* for L2/3 neurons; *RORB* for L4 neurons; *THEMIS* for L5/6-CC neurons; *TLE4* for L5/6 neurons; *VIP*, *PVALB*, *SST* and *SV2C* for inhibitory neuron subtypes; *OLIG1* for oligodendrocytes; *AQP4* for astrocytes; *PDGFRA* for OPCs; *PTPRC* for microglia; and *CLDN5* for endothelia.

We identified changes in expression during ageing using the Seurat FindAllMarkers function. In brief, a Wilcoxon rank-sum test followed by multiple test adjustment was applied to determine significantly differentially expressed genes ($q < 0.05$) between adult and elderly donors for each cell type. We further filtered genes expressed in less than 25% of elderly cells and adult cells, or with a log₂-transformed fold change less than 0.5. The same process was used to identify genes differentially expressed between infant cells and adult cells.

Continuous method to validate changes in expression during ageing

We used linear regression with sex as a covariate as an alternative method to determine continuous changes in expression during ageing. Average log-normalized expression levels and the age (in years) of each donor were used to build the linear model for each cell type. Genes with a slope less than -0.001 or greater than 0.001, a *P* value less than 0.05 and expressed in at least 25% of adult or elderly cells were considered as continuously changed genes during ageing. Both methods showed strong agreement on genes that go down during ageing across cell types, especially in excitatory neurons (Extended Data Fig. 5b and Supplementary Table 7). The linear model generally identified more genes that go up during ageing than the Wilcoxon test model, owing to the relatively strict log₂-transformed fold-change cut-off of 0.5.

Analysis of transcriptome change during ageing using three groups

We investigated the transcriptome changes during ageing in a more continuous way, by dividing our non-infant donors into three groups: young adult (5 donors; under 40 years old); adult (6 donors; 40–69 years old); and elderly (6 donors; 70 years old or over). As shown in Extended Data Figs. 5a, 7a,e and 10a,b, the results generally matched our conclusions using the two-group comparison (elderly versus adult).

Transcriptional variability during ageing

Transcription variability is calculated by the coefficient of variation (CV). Specifically, for each gene in a specific cell type and a specific donor, the normalized expression levels (CPM) of all cells are used to calculate the CV, defined by the ratio of standard variation to the mean. The average CV of all genes is defined as the CV for a specific cell type within a particular donor. Comparing elderly and adult donors using a Wilcoxon rank-sum test showed a significant increase in transcriptional variability for IN-SST neurons but not for any other cell type.

Infant-specific analysis

To identify infant-specific changes in gene expression, we performed differential expression testing using the Seurat FindAllMarkers function as described above, comparing the infant-specific clusters (L2/3-2 and Ast-3) with the other non-infant-specific clusters of the respective cell type. The infant-specific upregulated genes, those with higher expression in the infant-specific cluster relative to the other clusters, were used for GO analysis (described below).

To determine changes in cell-type proportion, we used a Wilcoxon rank-sum test comparing the proportion of each cell type in infants to the remaining samples (adult and elderly). Donor 1465 (a 17-year-old

male individual) was excluded from this analysis owing to the differences in nuclei preparation before snRNA-seq discussed above.

GO analysis

GO analysis of biological processes was performed on the differentially expressed genes for each cell type, both up and downregulated, using the R package gprofiler2 (v.0.2.3) with the correction method set to 'fdr' and source set to 'GO:BP' from the GO database. For each cell type, we used the active genes as the background gene set (indicated in the Supplementary Tables as control genes). Active genes were defined as those expressed in more than 25% of the cells to be consistent with the definition of a differentially expressed gene. Determination of the GO term categories shown in Figs. 2b and 3c was done manually (see Supplementary Tables 5 and 9 for mappings). To confirm the distinct GO enrichment profile in endothelial cells, we repeated the analysis after down-sampling. For each non-endothelial cell type, we chose the top 121 downregulated genes in elderly donors with the lowest FDR (121 matches the number of downregulated genes in the endothelial cells). There were fewer than 121 downregulated genes in oligodendrocytes, and thus down-sampling was not performed for this cell type. The GO down-sampling results are reported in Supplementary Table 10.

Random permutation test for shared downregulated genes in cell types from elderly donors

To test whether there are significantly more genes downregulated in at least one excitatory neuron, at least one inhibitory neuron and at least two glial cell types than expected, we performed a random permutation test. We randomly picked the same number of expressed genes to designate as downregulated for each cell type, using a minimum expression cut-off of 25% of the adult cells and 20% of the elderly cells, and recorded the number of shared genes as the expected value. A total of 1,000 permutations were performed, and all of the tests yielded fewer shared genes than observed in our data, generating a *P* value of less than 0.001.

Identification of sSNVs in neurons

To identify sSNVs, we used both scWGS and corresponding bulk WGS data. scWGS and bulk WGS data were first processed accordingly to the GATK (v.4.1.8.1) best practices⁶⁴. In brief, reads were aligned to the human genome using bwa-mem (v.0.7.12) with default parameters. PCR duplicates were then filtered using Picard, and the remaining reads were recalibrated with GATK BaseRecalibrator and ApplyBQSR. Genotypes were then identified with GATK HaplotypeCaller and GenotypeGVCFs. Finally, sSNVs were identified by comparing the scWGS data with corresponding WGS data from bulk tissues using SCAN2 with the following parameters: --snv-min-sc-dp 5 --snv-min-bulk-dp 10⁶. Common SNPs from dbSNP (v.20180418) and phasing information from the 1000 Genomes Project (v.3) were used as a reference panel while running the SCAN2 pipeline. We estimated the FDR for SCAN2 as 8.6% in a previous publication⁶.

Signature analysis of sSNVs

We performed signature analysis for sSNVs using the R package MutationalPatterns (v.3.16.0)⁶⁵. We first calculated the spectrum of sSNVs in the 96-trinucleotide contexts for each neuron from all donors. A non-negative matrix factorization (NMF) was applied to the spectrum of sSNVs and the signatures were identified. After applying various numbers of signatures in the practice, ranging from one to eight, we found that two signatures yielded the best performance with regard to stability and reconstruction errors (Supplementary Fig. 12). The signatures (A1 and A2) were then compared with the COSMIC v.3 signatures, and cosine similarities between signatures were calculated. To confirm the reproducibility of our signature analysis, a second method, SignatureAnalyzer, was used with default parameters. SignatureAnalyzer identified similar signatures to those identified by MutationalPatterns.

Enrichment and strand bias of sSNVs in genic features and chromatin states

To calculate the enrichment of sSNVs in genes and intergenic regions, we first simulated random controls with the same mutation spectrum as sSNVs restricted to suitable regions (that is, with enough depth) in our scWGS and bulk WGS dataset. The numbers of sSNVs and random controls at genes and intergenic regions were then calculated. NMF, using the R package MutationalPatterns, was further applied to sSNVs and random controls at genes and intergenic regions to trace the contribution of signatures A1 and A2. Genes were divided into five groups according to their transcriptional activity (CPM) in neurons and glia cells from our snRNA-seq data. The same enrichment analysis was also done over the 15 chromatin states in the human dorsolateral PFC from Roadmap⁶⁶. To test for strand bias in sSNVs, we used the UCSC table browser to identify all RefGene transcripts associated with single-neuron sSNVs. Only sSNVs that had known transcripts all going in the same direction were considered. Transcriptional directions for sSNVs that overlapped a transcript were tallied, and the numbers collapsed to report only one complement of each base pair (T>A, T>C, T>G, C>A, C>T and C>G).

DepMap analyses of the effects of upregulated and downregulated genes on cell viability

The requirement of each gene in overall cell viability was determined using the Cancer Dependency Map (DepMap; version Public 22Q4), which provides the cell viability effect of each gene knockout across 1,078 cancer cell lines of varying origin⁶⁷. Specifically, cell viability is determined by performing whole-genome pooled CRISPR screening across each cell line, and on the basis of the fold change in the abundance of cells containing Cas9 and guides against each specific gene. For example, if cells transduced with Cas9 and guides against a particular gene were depleted after the screen, this would indicate an essential gene. The overall effect of gene knockout for a given cell line is quantified using a cell population dynamics model called Chronos⁶⁸, which incorporates the efficacy of each guide and copy number correction (CRISPR toxicity unrelated to gene function can occur when high copy numbers are subjected to CRISPR-mediated strand breaks) to provide an overall 'gene effect score' that indicates the probability that a given cell line is dependent on the gene for survival⁶⁹. Notably, a value of -1.0 corresponds to the median gene effect score of all common essential genes, whereas a cell line is considered dependent if the gene effect score is ≤ -0.5 . Positive values would indicate increased cell viability or proliferation after loss of the gene.

Among the upregulated and downregulated 'hits' from the snRNA-seq, those encoding long non-coding RNAs, non-coding RNAs or pseudogenes are not covered in the DepMap essentiality analyses and thus were not analysed for effects on gene viability. Likewise, several coding genes (*CECR*, *NEFL*, *FTH1*, *COX4I1*, *SH3RF3*, *BMP2K*, *SHISA8*, *MYRFL* and *RPS3A*) did not have CRISPR screen data yet available, and were not analysed.

Defining housekeeping and neuron-specific genes

We first calculated the average logged CPMs for each gene in excitatory neurons, inhibitory neurons, microglia and endothelia. Then we defined housekeeping genes as genes with a difference of less than 0.1 between the four cell types that also had an average logged CPM greater than 0.1 in each cell type. The genes that fit these criteria also have an average logged CPM greater than 0.1 in oligodendrocytes, OPCs and astrocytes. The neuron-specific genes were defined as those genes with average logged CPMs higher than 0.2 in both neuron groups and lower than 0.1 in microglia and endothelia.

Determining what drives transcriptome change during ageing

To determine which feature is likely to drive expression change during ageing, we constructed a multiple linear regression model to estimate

the contribution of genetic and transcriptomic features to the expression change during ageing. To avoid the effect of non-expressed genes, we only assessed genes whose average logged CPM is at least 0.1 in excitatory neurons, inhibitory neurons, microglia, endothelia, oligodendrocytes, OPCs and astrocytes. Gene length, exon length, expression in each cell type and expression specificity for each cell type and neurons were used to build the regression model to predict the fold change of gene expression between elderly and adult. Expression specificity was calculated by the normalized expression in each cell type divided by the average normalized expression in the remaining cell types. Expression specificity for neurons was calculated by comparing the average expression in neurons and the average expression in glia cells. We also included broad cell specificity in the model, defined by the sum of the difference between maximum expressed cell type and other cell types, divided by (number of cell types used – 1). As the number of reads captured for each gene could be biased towards gene or exon length, we also included gene and exon length-corrected normalized expression levels in each cell types as input features. Bulk sequencing is also a quantitative way to measure absolute expression levels. Thus, we included the expression levels (TPM; transcripts per million mapped reads) in human frontal cortex from the GTEx portal⁷⁰. The squared correlation coefficient between the model prediction and observed fold change of expression, an indicator of model performance, ranged from 0.23 in microglia to 0.54 in excitatory neurons. Among all features assessed, gene length yielded the highest correlation coefficient, suggesting that it has a key role in determining expression change during ageing in neurons and glia cells.

Validation of snRNA-seq results using published data

No other single published study on human PFC spans the same age range as ours, so we looked to two different datasets for validation of our results. Herring et al.²⁵ includes PFC from 22 gestational weeks to 40 years old. To validate our infant-specific clusters, we obtained raw snRNA-seq reads from the Herring paper (publicly available at GSE168408) and processed them using Cell Ranger (v.7.0.1) with the following parameters: “--include-introns true --nosecondary”. We filtered and clustered the data in the same way as we did with our own (described above), using Velmeshev et al. as our reference for cell-type identification, confirming the presence of infant-specific astrocytes and excitatory neurons in a larger sample size. We compared the expression of the infant-specific differentially expressed genes from our own data (methods described above) with Herring data for infants (prenatal samples to 2 years) and adults (15–40 years), validating our findings of infant-specific clusters and their respective gene-expression profiles.

To validate the changes we described in the elderly brain, we used control PFC (BA46) data from Ling et al.²⁹, which includes donors aged 22–97 years. We downloaded their publicly available raw counts matrix for each cell type from NeMo (<https://assets.nemoarchive.org/dat-bmx7s1t>) and normalized the expression levels using the same strategy: to total number of reads for each cell with a factor of 10,000. We then compared the expression of genes of interest from our data in elderly and adult brains in the Ling data. Specifically, we assessed whether common genes are downregulated in elderly cells, and whether the decrease of expression during ageing is associated with gene length.

We also used the control PFC data from Mathys et al.³⁰ to validate our findings. We downloaded their publicly available raw counts matrix for each cell type from the Alzheimer’s disease and ageing brain atlas data repository (https://compbio.mit.edu/ad_ageing_brain) and normalized to the number of UMI reads per cell per 10,000 UMI reads. This dataset comprises 189 individuals, and includes only elderly donors (over 70 years old). We then compared the expression of downregulated genes, common genes and short and long genes in our adult donors, our elderly donors and the elderly donors from Mathys et al. The results were consistent with our own dataset: common genes and short genes showed decreased expression in neuron and glia cells from elderly

donors. We also compared the expression levels of genes in donors aged 70–79 years and 80 years and over from Mathys et al., and did not find a significant change.

MERFISH: sample preparation and imaging

Spatial transcriptomics was performed in two batches using two versions of the MERFISH platform. Data from each batch were analysed separately and not integrated into a single analysis. For batch 1, three adult donors and three elderly donors were selected for spatial transcriptomics on the basis of RNA integrity number, tissue availability and sex. Vizgen’s protocol for the sample preparation was followed with the following modifications. Brains were sectioned and mounted on Vizgen MERSCOPE slides. After adhering to the coverslip, samples were fixed in prewarmed 4% paraformaldehyde in 1× PBS for 30 min at 47 °C, followed by three washes in 1× PBS for 5 min each at room temperature. Samples were dried for one hour at room temperature. The samples were then incubated overnight in 70% ethanol at 4 °C to permeabilize the tissue. Samples were photobleached for 6 h at room temperature in the Vizgen Photobleacher. Next, the Vizgen sample preparation protocol for FFPE tissues was followed, beginning with anchoring pretreatment (step 3 in Vizgen protocol version 9160012 Rev D). After RNA anchoring, the tissue was embedded in gel embedding solution (containing 0.5% ammonium persulfate, 0.05% TEMED and Vizgen’s gel embedding premix) and incubated for 22 h with tissue clearing solution (Vizgen Clearing Premix and 1:100 proteinase K) at 47 °C. The probe library was applied to the sample and incubated for 48 h at 37 °C. Finally, the samples were washed, incubated with DAPI and polyT solution for 15 min at room temperature and washed with formamide wash buffer for 10 min at room temperature. For the imaging, the MERSCOPE 500 gene imaging kit was activated with 250 µl imaging buffer activator and 100 µl RNase inhibitor. Fifteen millilitres of mineral oil was added through the activation port, the instrument was primed and the imaging chamber was assembled according to the MERSCOPE user guide. A 10× low-resolution DAPI mosaic of the sample was acquired, and the imaging area was selected for data acquisition.

For MERFISH batch 2, an infant and three adult donors were selected on the basis of RNA integrity number, tissue availability and sex. All tissue processing steps were performed as described above, but imaging was performed on a MERSCOPE Ultra instrument. Owing to uncertainty in the back compatibility between instruments, these four samples were treated as their own set of data and never compared with the six-sample cohort processed on the older instrument.

MERFISH: post-imaging data processing and analysis

For batch 1, after the MERSCOPE run, the data were decoded using Vizgen’s analysis pipeline integrated within the MERSCOPE system. The Vizgen post-processing tool (VPT, Vizgen) was used to improve cell segmentation with a combination of pre-filtering with a Gaussian filter and the CellPose algorithm. For batch 2, the four samples imaged on the MERSCOPE Ultra were not subjected to additional processing using the VPT, because cell segmentation using CellPose was performed by the MERSCOPE Ultra instrument.

After cell segmentation, only cells with volumes greater than 200 µm³ were retained for downstream analysis. The cell × gene count matrix was then analysed with the Seurat R package (v.5.1.0) for cell-type assignment. Two datasets (one focusing on elderly versus adult, and one on infant versus adult) were analysed separated using the same pipeline. Specifically, PCA was performed using the count matrix, after filtering cells with fewer than 100 transcript counts, followed by logCPM transformation. We then performed UMAP and KNN clustering analysis using the top 30 principal components. The resolution of KNN clustering was set to 0.3, yielding 15 clusters in batch 1 and 16 clusters in batch 2. Each cluster was then assigned a specific cell type according to the expression of marker genes (Extended Data Fig. 6b and Supplementary Table 11).

In batch 1, clusters 12 and 14 showed mixed expression of marker genes, preventing cell-type assignment, and were removed from downstream analysis. Cluster 1 was composed of excitatory neurons that could not be assigned to a specific layer owing to mixed expression of layer-specific markers. To investigate the transcriptome change during ageing, we compared gene expression between adult and elderly donors for each cell type. We normalized gene expression first to cell volume (molecules per $2,000 \mu\text{m}^3$) and then to the average expression of a set of control genes that are stably expressed during ageing (Supplementary Table 11). The control genes were defined as genes with a \log_2 -transformed fold change of expression between elderly and adult donors >-0.3 and <0.3 in our snRNA-seq dataset.

In batch 2, clusters 9, 10, 12 and 15 showed mixed expression of marker genes and were removed from downstream analysis. Different layers of excitatory neurons and different subtypes of inhibitory neurons were analysed together because many of them could not be assigned to a specific layer owing to mixed expression of layer-specific markers. We investigated transcriptome change during brain development using the same strategy as we did in the elderly and adult dataset. Gene expression between infant and adult donors was compared, after normalizing to cell volume and a set of control genes stably expressed in infant and adult donors.

MERFISH gene panel selection

The gene panel used for MERFISH was composed to validate initial snRNA-seq findings generated from 13 donors, focusing on differences in the elderly and adult donors. It is composed of 70 marker genes (used to identify cell types), 33 short housekeeping genes, 33 long housekeeping genes, 24 short neuron-specific genes, 21 long neuron-specific genes, 9 ribosomal-protein genes, 10 nuclear-encoded mitochondrial genes, 11 DNA damage repair genes and 35 other genes of interest (Supplementary Table 11). All short housekeeping and neuron-specific genes came from the first length decile of their respective gene groups and all long housekeeping and neuron-specific genes came from the tenth length decile of their respective gene groups. After the addition of six donors to our snRNA-seq data, our housekeeping and neuron-specific gene lists changed slightly, although the method used to generate the list did not, and not all of the neuron-specific and housekeeping genes in the MERFISH panel met the criteria. The MERFISH gene tab of Supplementary Table 11 reports the decile according to the original housekeeping and neuron-specific lists used to generate the panel. If the gene is present on the current list, the corresponding decile is reported in parentheses.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Raw RNA-seq and scWGS sequencing data not previously published, and MERFISH data, are available at dbGaP (phs003445.v1.p1). Processed data are available at <https://publications.wenglab.org/SomaMut/>. An interactive genome browser of the snRNA-seq pseudo bulk expression data can be found at https://genome.ucsc.edu/s/yutianxiong/Weng_Lodato_Aging. Previously published single cells analysed in this study can be found at dbGaP (phs001485.v3.p1) and NIAGADS (NG00121). Previously published bulk DNA-sequencing data can be found at dbGaP (phs001485.v1.p1), the NCBI Sequence Read Archive (SRA; accession numbers SRP041470 and SRP061939) and NIAGADS (NG00121). The previously published Velmeshev et al.¹⁹ data used for

cell-type annotation were downloaded from SRA accession number PRJNA434002. The previously published Herring et al.²⁵ data were downloaded from the Gene Expression Omnibus (GEO) under accession number GSE168408. The previously published Ling et al.²⁹ data were downloaded from the Neuroscience Multi-omic Data Archive (NeMo) (<https://assets.nemoarchive.org/dat-bmx751t>). The previously published Mathys et al.³⁰ data were downloaded from the Alzheimer's disease and ageing brain atlas data repository (https://compbio.mit.edu/ad_ageing_brain). GTEx data were downloaded from the GTEx portal (https://www.gtexportal.org/home/downloads/adult-gtex/bulk_tissue_expression). GO biological process terms were sourced from <https://geneontology.org/>. DepMap data came from <https://dep-map.org/portal/> using version Public 22Q4.

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Author contributions A.M.J. and M.A.L. conceived and designed the study. A.M.J. performed snRNA-seq. T.Y. performed bioinformatic analysis. J.S.Z., C.B.S. and A.K.T. performed single-neuron sorting and sequencing. C.E.B. performed spatial transcriptomics. Y.K. and A.M.J. performed data analysis. A.M.J. and M.A.L. wrote the manuscript. M.A.L. and Z.W. supervised the study.

Competing interests The authors declare no competing interests.

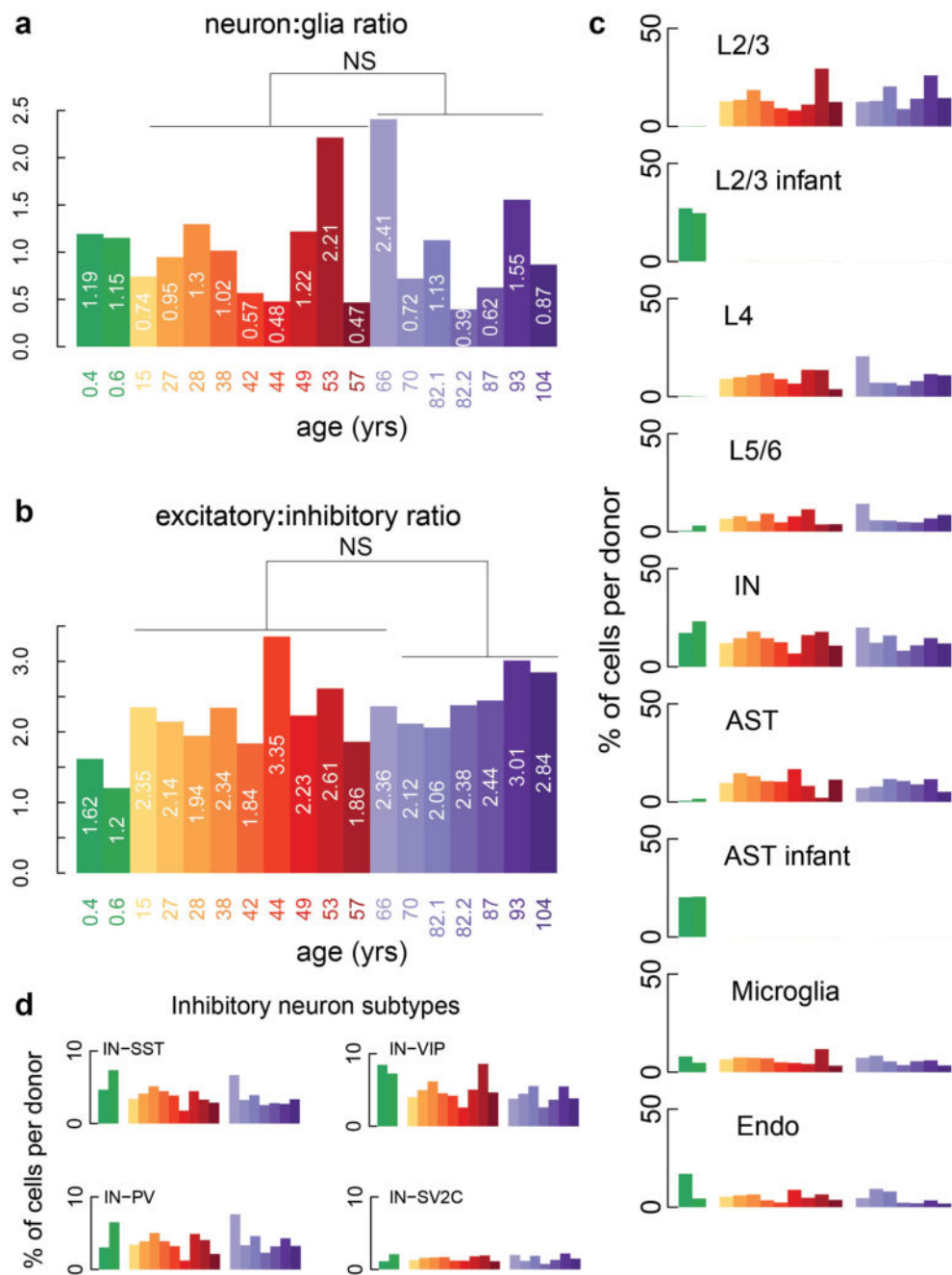
Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41586-025-09435-8>.

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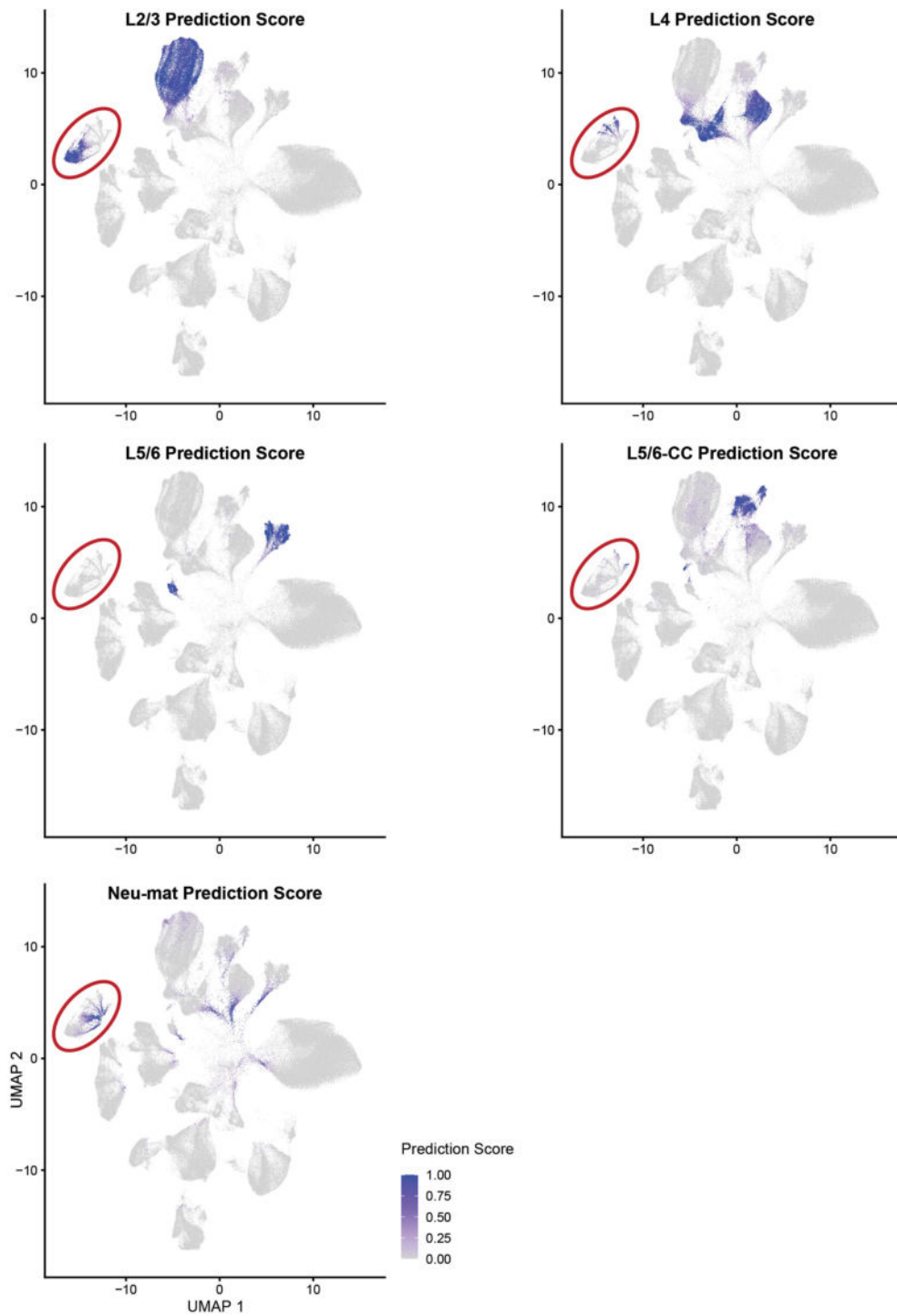
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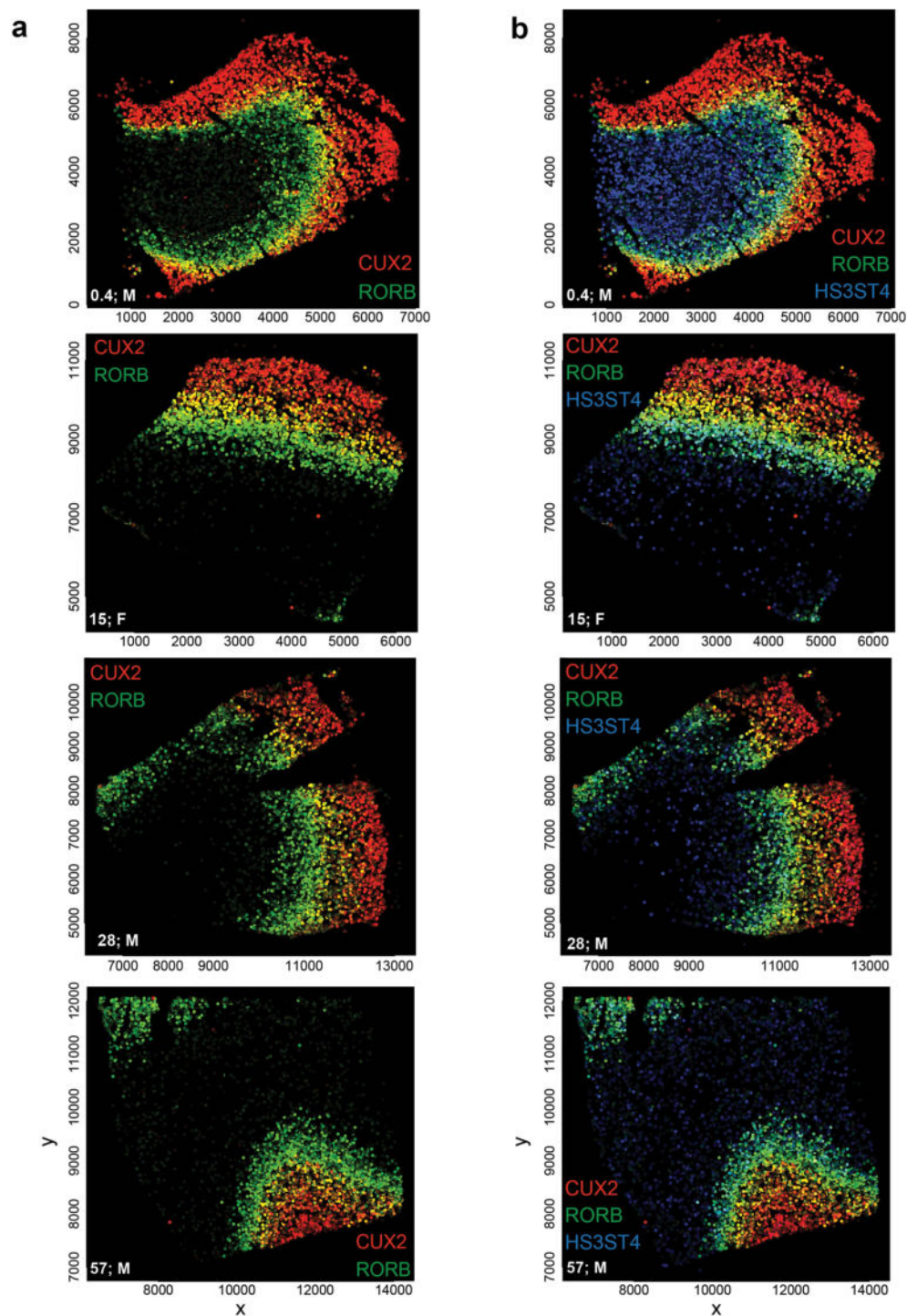
Extended Data Fig. 1 | Cell-type ratios do not change significantly with age. (a) Neuron to glia ratio shows no significant change with age. (b) Excitatory to inhibitory neuron ratio shows no significant change with age. (c) Contribution of each cell type to each donor, shown as a percentage of total cells. Columns in

this panel plus Fig. 2b sum to 100. (d) We see no significant differences between elderly and adult in the proportion of inhibitory neuron subtypes. (IN – inhibitory neurons, AST – astrocytes, Endo – endothelial cells).



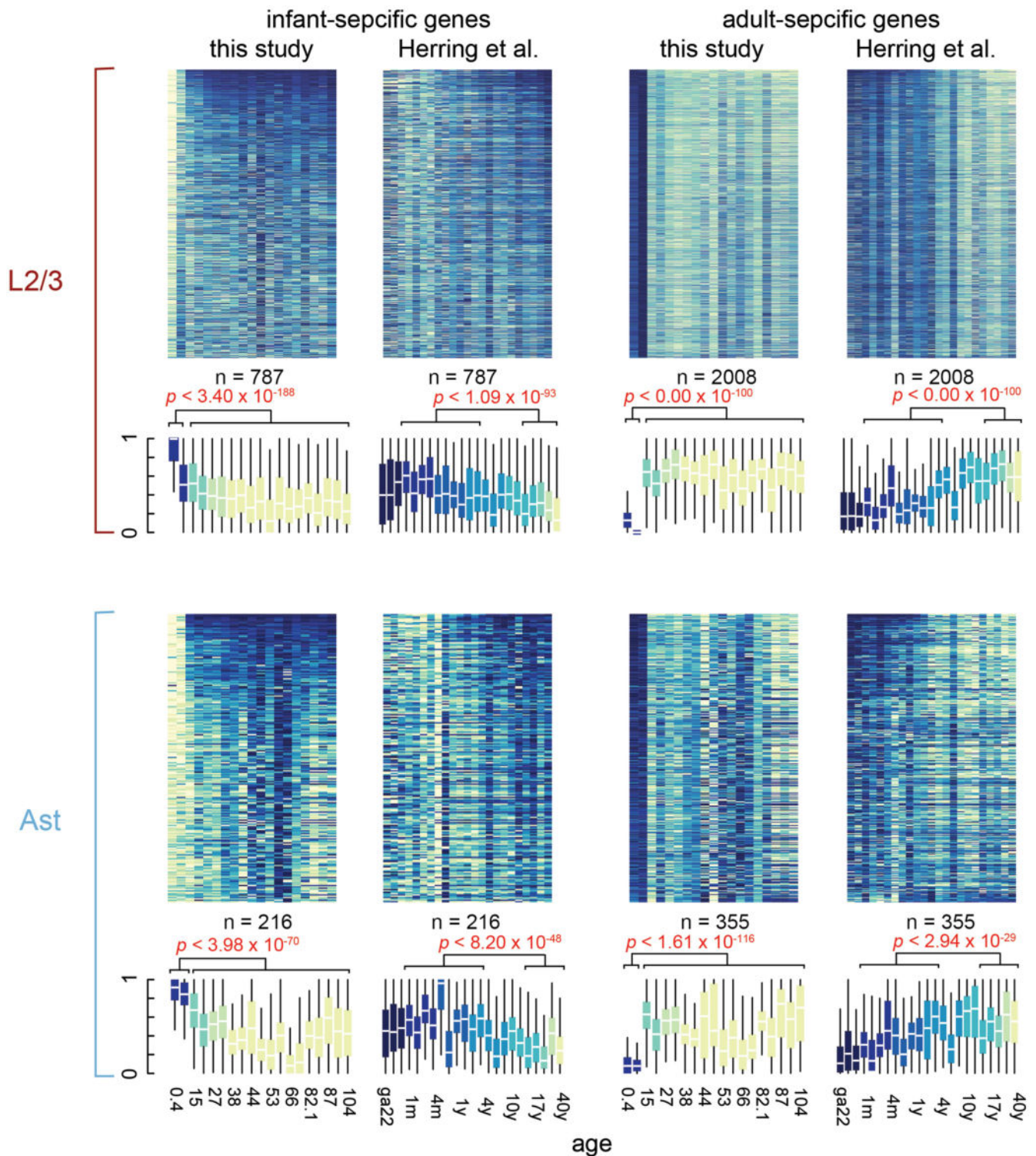
Extended Data Fig. 2 | Marker-gene expression in infant-specific neurons. UMAP shows cell-type prediction score for each cell on the plot. Prediction scores shown for L2/3 neurons, L4 neurons, L5/6 neurons, L5/6-CC neurons, and Neu-mat neurons, a cell type identified in our reference data thought to be

a mix of ambient RNA and maturing neurons. The infant-specific neuron cluster is circled in red and shows mixed cell-type composition based on prediction scores. Neuron prediction scores otherwise show cluster-specific patterning.



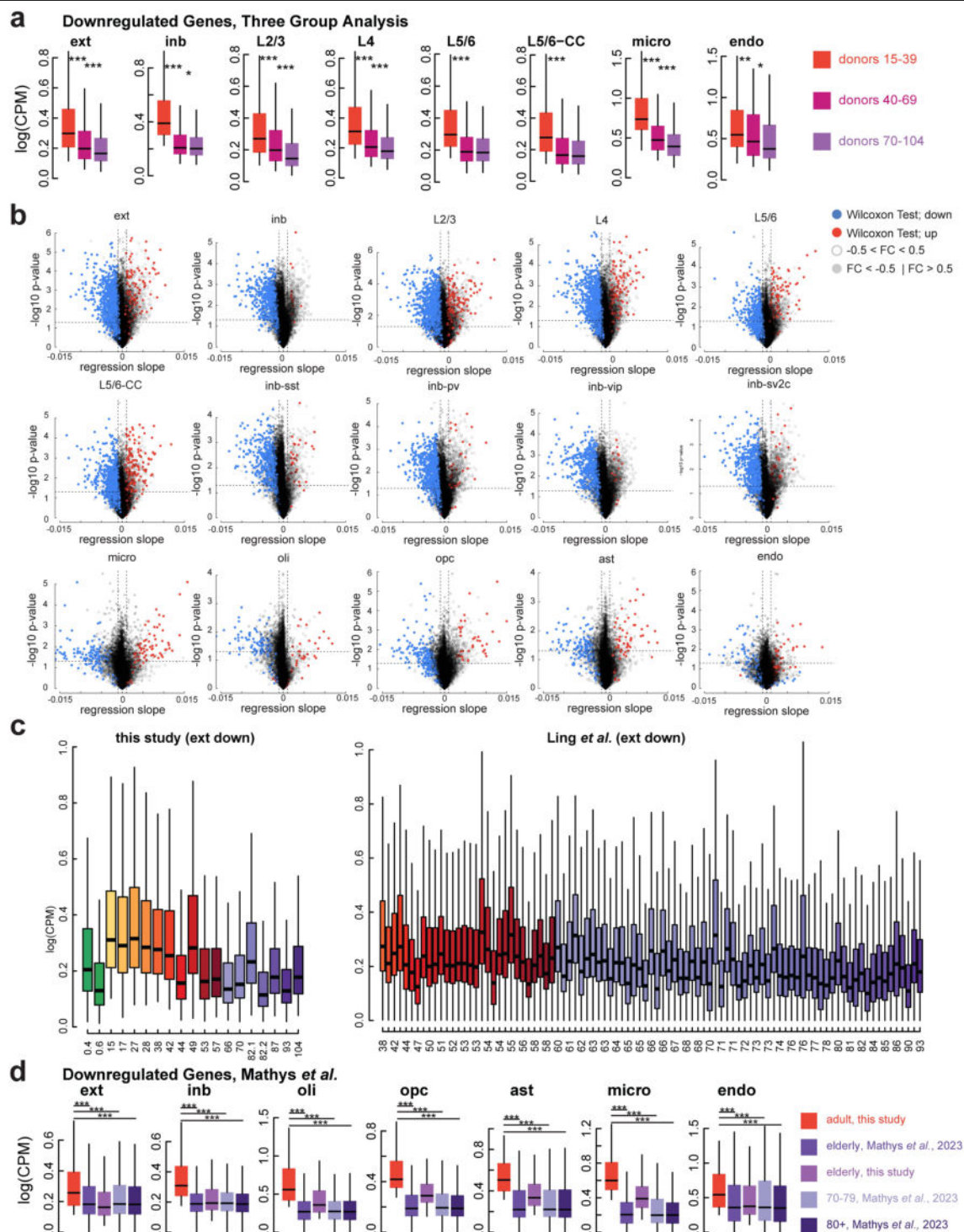
Extended Data Fig. 3 | Cortical layers in MERFISH data. MERFISH sections of infant male, 15-year-old female, 28-year-old male, and 57-year-old male individuals showing marker-gene expression for cortical layers. Circles represent excitatory

neurons coloured by gene expression. **(a,b)** Red: CUX2-L2/3; Green: RORB-L4; Yellow: CUX2 and RORB co-expression. **(b)** Blue: HS3ST4-L5/6; Teal: RORB and HS3ST4 co-expression. X- and Y-axis values reflect pixel positions.



Extended Data Fig. 4 | Infant-specific gene expression across ages in two independent datasets. Heat maps (top) plotting infant-specific gene expression ordered by age in this study and Herring et al.²⁵ showing higher expression in infant and gestational cases and lower expression in adults for L2/3 neurons and astrocytes. Box plots (bottom) showing mean expression of infant-specific genes and adult-specific genes in L2/3 neurons and astrocytes across ages in this study and Herring et al.²⁵ Expression of infant-specific genes is significantly higher in

donors 28 days to 301 days, the subset that most closely matches the ages of the two infants in this study, compared to donors ≥ 15 . Expression of adult-specific genes is significantly lower in the same group of infant donors compared to donors ≥ 15 . All box plots depict median, and first and third quartile. Whiskers show $1.5 \times$ IQR beyond the first and third quartiles. (Two-sided Wilcoxon rank-sum test).

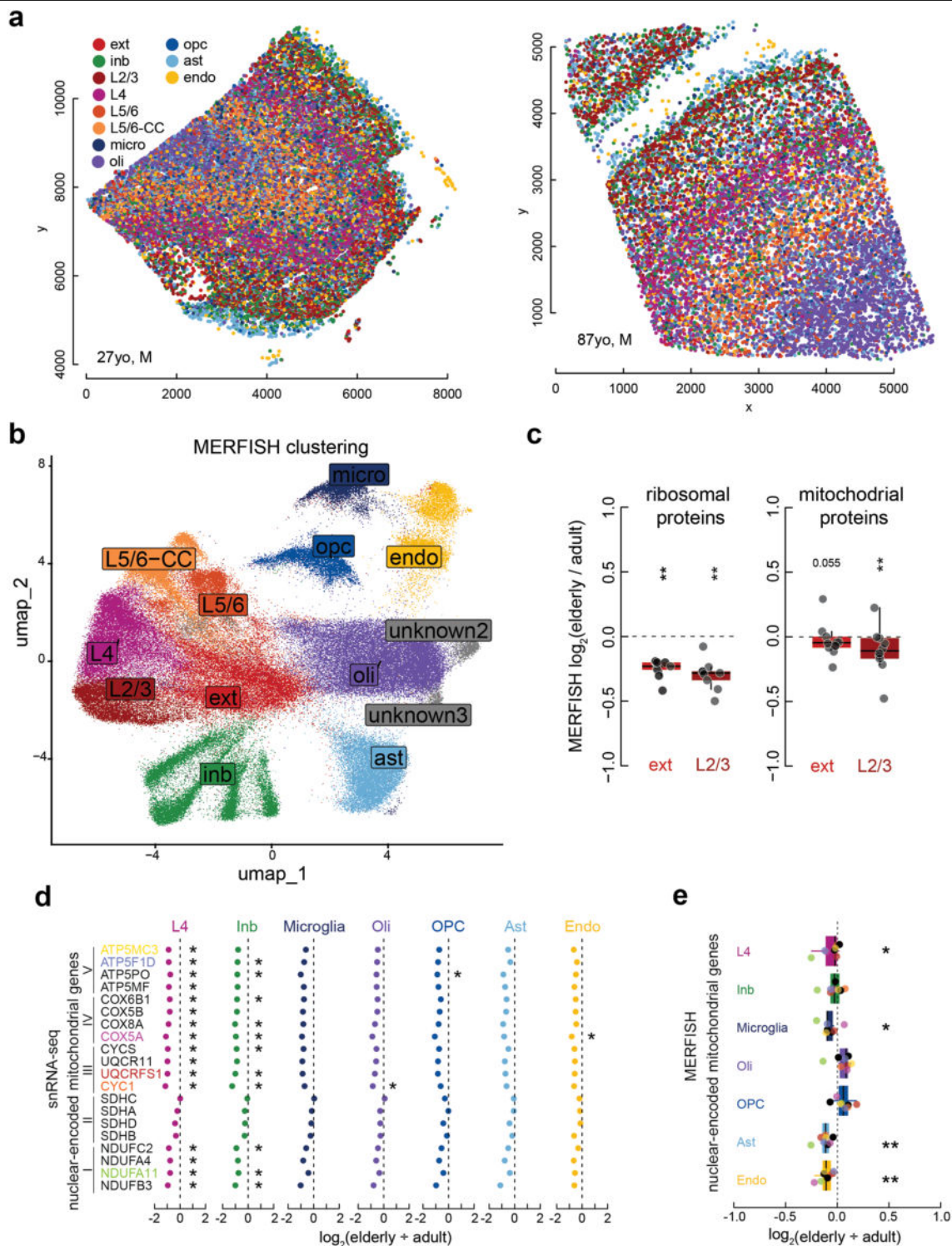


Extended Data Fig. 5 | See next page for caption.

Extended Data Fig. 5 | Validation of downregulation during ageing.

(a) Expression of genes downregulated in the primary analysis (elderly vs. adult) are also downregulated when data is broken down into three groups, donors 15–39 (red, N = 5), donors 40–69 (magenta, N = 6), and donors 70–104 (purple, N = 6). Some cell types exhibit continuous downregulation, showing significant decreases with each age group while others are significantly downregulated between the 15–39- and 40–69-year-old groups but expression does not change between the older adult and elderly groups (Two-sided Wilcoxon rank-sum test). (b) Volcano plots showing the results of expression changes during ageing, determined by linear regression. Regression slope is shown on the x axis and $-\log_{10}(\text{p-value})$ on the y axis. Dotted lines indicate slope and p-value thresholds, slope < -0.001 or > 0.001 and $p < 0.05$, used to determine significance. In addition, genes had to be expressed in at least 25% of the elderly or adult cells to be considered. Blue dots indicate genes that were also identified as significantly downregulated by pairwise comparison and Two-sided Wilcoxon rank-sum test. Red dots indicate genes that were identified

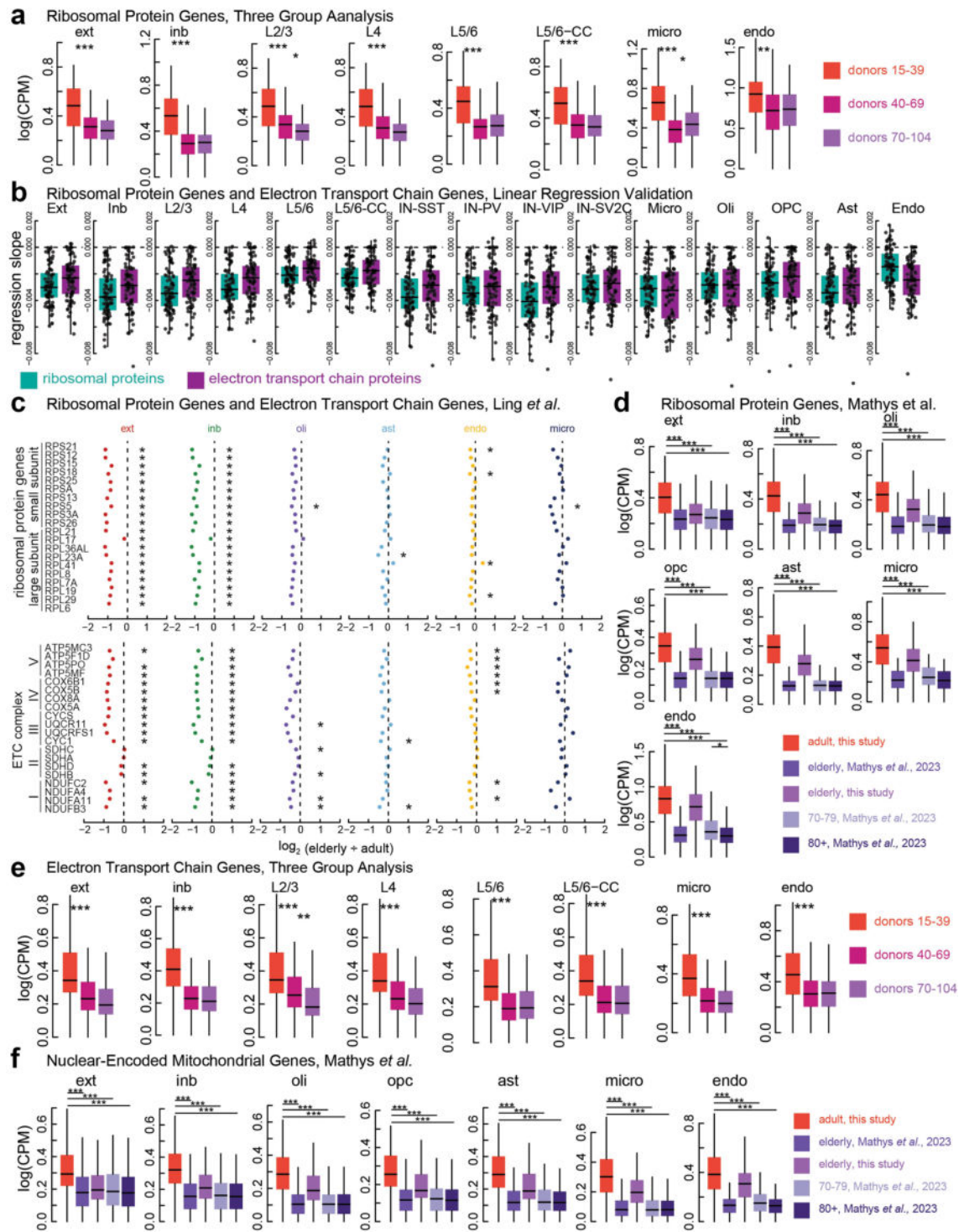
as significantly upregulated by pairwise comparison and Wilcoxon test. Open circles indicate genes that did not meet the pairwise comparison criteria for fold change and grey circles indicate genes that met the fold change criteria but did not have significant p-values in the pairwise comparison. (c) Box plots showing the expression, in log(CPM), of significantly downregulated genes in elderly excitatory neurons identified in this study in our donors (left) with the donors from Ling et al.²⁹ (right). (d) Box plots comparing expression of downregulated genes identified in this study in adults from this study (red, N = 9), all donors from Mathys et al.³⁰ (violet, N = 189), elderly donors from this study (lilac, N = 7), donors 70–79 from Mathys et al. (light purple, N = 34), and donors over 80 from Mathys et al. (dark purple, N = 155). Two-sided Wilcoxon rank-sum test comparing adults in this study to each of the Mathys groups are all significant. All box plots depict median, and first and third quartile. Whiskers show $1.5 \times \text{IQR}$ beyond the first and third quartiles. (*, $p > 0.05$; **, $p > 0.01$; ***, $p > 0.001$).



Extended Data Fig. 6 | Spatial transcriptomic validation of snRNA-seq data.

(a) Representative MERFISH sections, showing the assigned cell type from Seurat clustering. (b) UMAP clustering of MERFISH cells showing all identified cell types. Clusters of unknown cells were removed from downstream analysis. Ext indicates cells that expressed multiple excitatory markers and could not be assigned to a specific layer. X- and Y-axis values in a and b reflect pixel positions. (c) Fold change of elderly and adult MERFISH cells of 9 ribosomal proteins (left) and 10 nuclear-encoded mitochondrial proteins (right) in

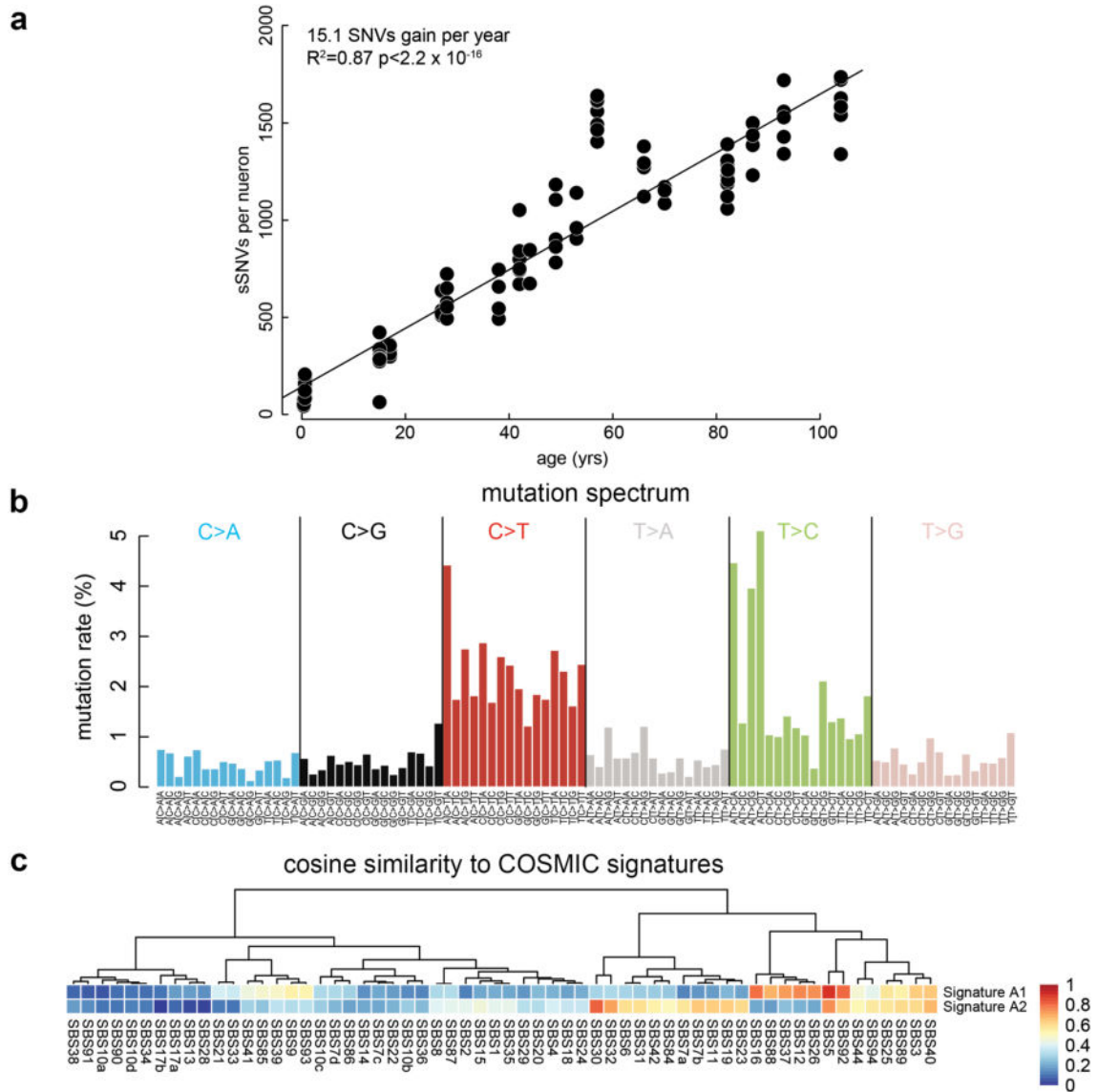
excitatory and L2/3 neurons (Two-sided Wilcoxon rank-sum test, elderly N = 3, adult N = 3). (d,e) \log_2 fold change of elderly vs. adult nuclear-encoded mitochondrial genes by snRNA-seq (Two-sided T-Test, elderly N = 7, adult N = 9) (d) and MERFISH (Two-sided Wilcoxon rank-sum test, elderly N = 3, adult N = 3) (e). Genes shown in both d and e are colour-coded. All box plots depict median, and first and third quartile. Whiskers show $1.5 \times \text{IQR}$ beyond the first and third quartiles. Points beyond whiskers are outliers. (*, $p < 0.05$; **, $p < 0.01$).



Extended Data Fig. 7 | See next page for caption.

Extended Data Fig. 7 | Validation of changes in ribosomal-protein genes and nuclear-encoded mitochondrial genes during ageing. (a) Expression of ribosomal-protein genes in three age groups, 15–39 (red, N = 5), 40–69 (magenta, N = 6), and 70–104 (purple, N = 6) always decreases significantly after age 39. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Two-sided Wilcoxon rank-sum test). (b) Box plots showing the regression slope of ribosomal-protein genes (teal, N = 81) and nuclear-encoded electron transport chain genes (purple, N = 82). Ribosomal genes shown are the same as shown in Extended Data Fig. 8 and mitochondrial genes shown are the same as shown in Extended Data Fig. 10. (c) Fold change of ribosomal proteins (top) and nuclear-encoded mitochondrial genes (bottom) in ageing in the Ling et al.²⁹ data for each cell type. The expression changes match those seen in this study. Ext, excitatory neurons; Inb, inhibitory neurons; Oli, oligodendrocytes; OPC, oligodendrocyte precursor cells; Ast, astrocytes; Micro, microglia; Endo, endothelial. (*, $p < 0.05$; two-sided T-test, elderly N = 116, adult N = 64). (d) Box plots comparing expression of ribosomal-protein genes in adults from this study (red, N = 9), all donors from Mathys et al.³⁰ (violet, N = 189),

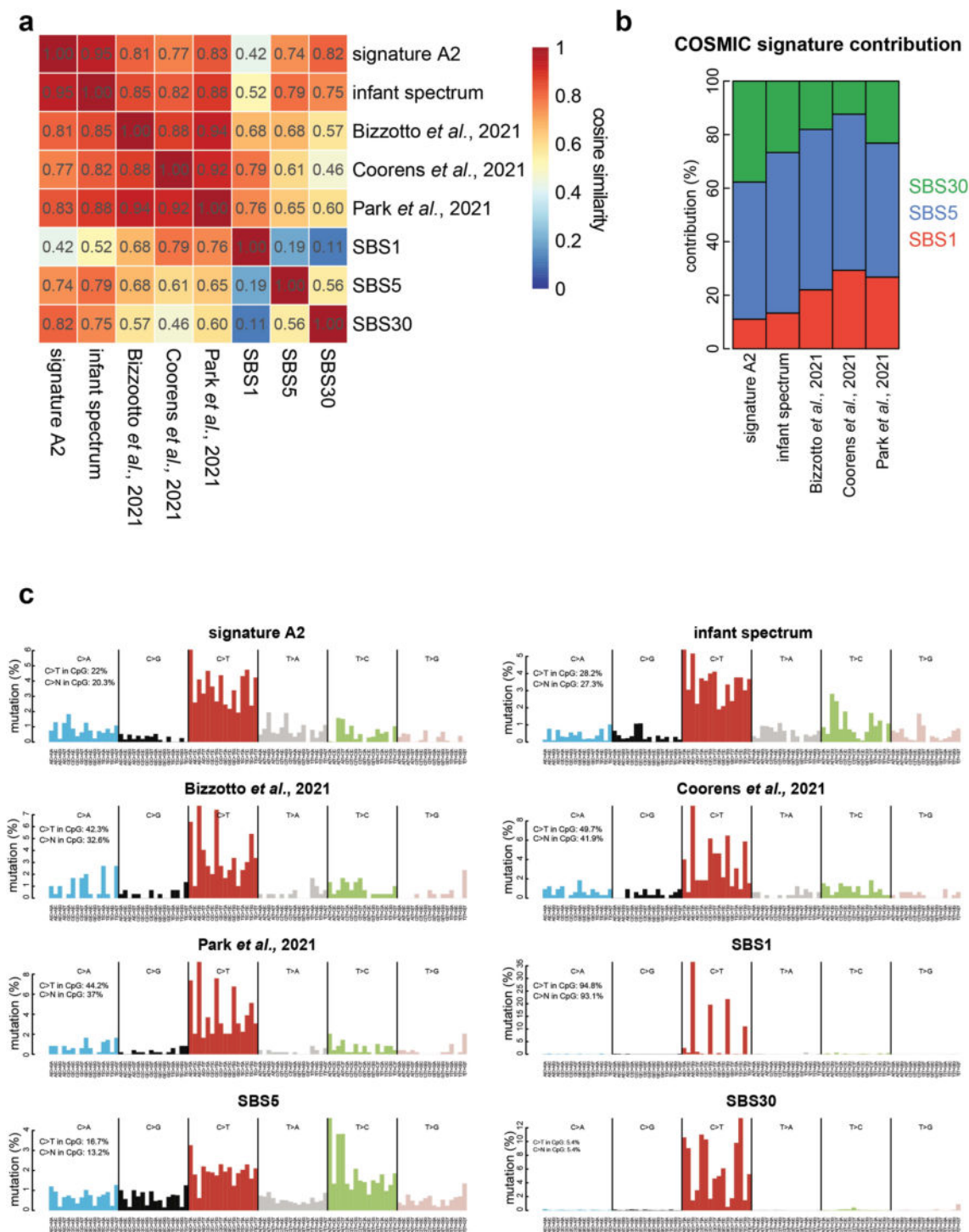
elderly donors from this study (lilac, N = 7), donors 70–79 from Mathys et al. (light purple, N = 35), and donors over 80 from Mathys et al. (dark purple, N = 155). Wilcoxon rank-sum test comparing adults in this study to each of the Mathys groups are all significant. (*, $p < 0.05$; ***, $p < 0.001$; Two-sided Wilcoxon rank-sum test). (e) Expression of nuclear-encoded mitochondrial genes of the electron transport chain in three age groups, 15–39 (red, N = 5), 40–69 (magenta, N = 6), and 70–104 (purple, N = 6) always decreases significantly after age 39. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Two-sided Wilcoxon rank-sum test). (f) Box plots comparing expression of nuclear-encoded mitochondrial genes of the electron transport chain in adults from this study (red, N = 9), all donors from Mathys et al. (violet, N = 189), elderly donors from this study (lilac, N = 7), donors 70–79 from Mathys et al. (light purple, N = 34), and donors over 80 from Mathys et al. (dark purple, N = 155). Wilcoxon rank-sum test comparing adults in this study to each of the Mathys groups are all significant. All box plots depict median, and first and third quartile. Whiskers show $1.5 \times \text{IQR}$ beyond the first and third quartiles. (***, $p < 0.001$; Two-sided Wilcoxon rank-sum test).



Extended Data Fig. 8 | Mutation spectrum of sSNVs in human neurons.

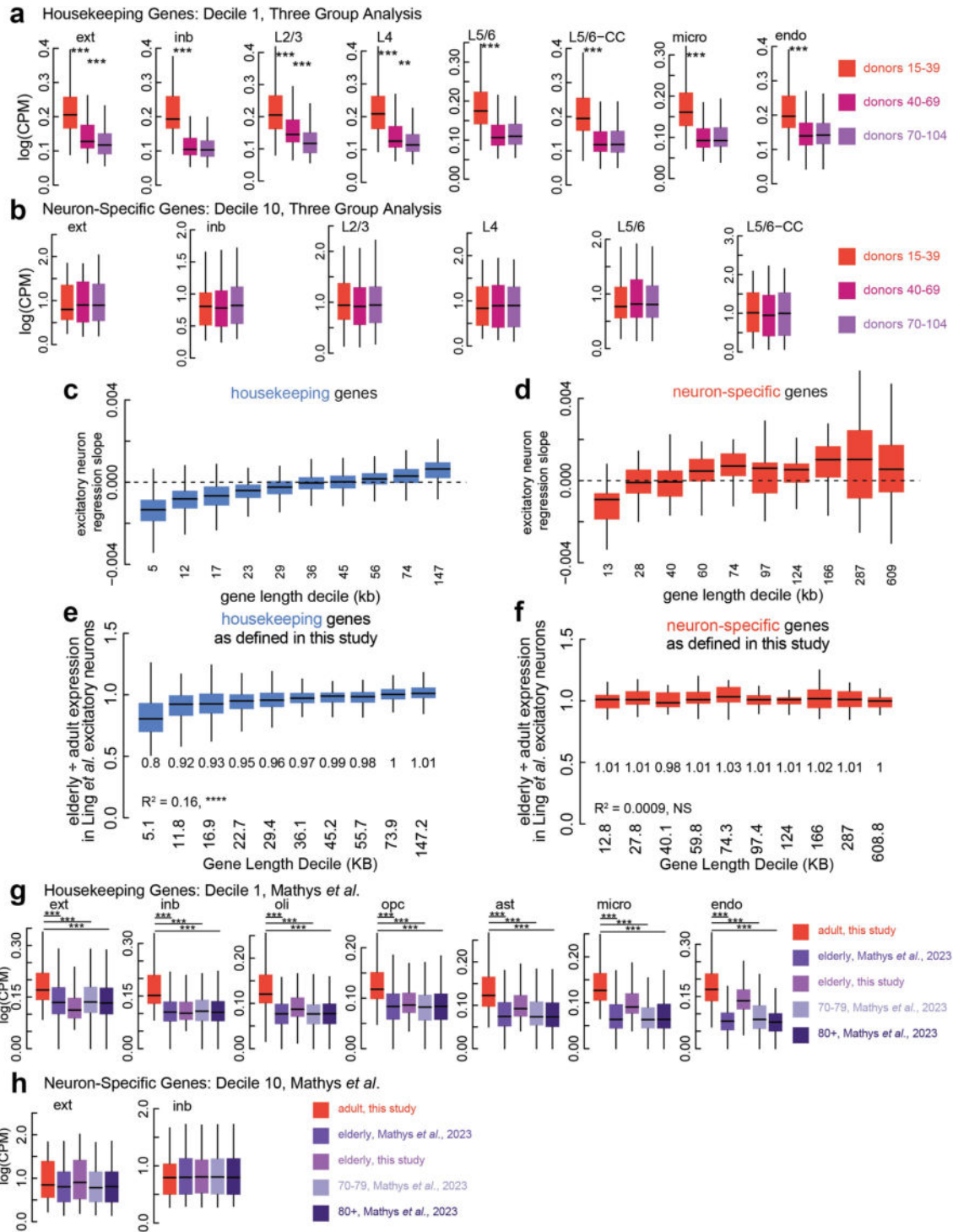
(a) Total mutation accumulation per neuron correlates significantly with age at a rate of 15.1 SNVs gained/year ($p = 2.2 \times 10^{-16}$, Pearson's correlation). (b) Mutation spectrum of sSNVs called in human neuron scWGS data. Each bar represents a

specific mutation in a different trinucleotide context. (c) Cosine similarity of the two signatures, A1 and A2, derived de novo from the total mutation spectrum to each single-base substitution signature in the COSMIC database. Signature A1 is most similar to SBS5. Signature A2 is most similar to SBS30.



Extended Data Fig. 9 | Comparison of signature A2 with COSMIC and known developmental signatures. (a) Heat map showing cosine similarity of Signature A2, the mutation spectrum of the infant cells included in this study, signatures identified in Bizzotto *et al.*³⁹, Coorens *et al.*⁴¹ and Park *et al.*⁴⁰, and COSMIC SBS1, SBS5, and SBS30. (b) COSMIC SBS1, SBS5, and SBS30 contribution to Signature A2, the infant mutation spectrum, and developmental signatures identified in Bizzotto *et al.*, Coorens *et al.* and Park *et al.* (c) Mutation plots of the signatures compared in a. Percentage of C>T mutations at CpG sites is higher than the percentage of C>N mutations at CpG sites for all signatures except SBS30. Our mutation calling algorithm, SCAN2, is biased against early developmental somatic mutations, because SCAN2 requires called somatic variants in single

cells to show no mutant reads in corresponding bulk tissue from the same donor. In practice, this means that many somatic mutations that occur very early in development, which are widely distributed across the body at a high mosaic fraction, are filtered out by our analysis, whereas late-occurring, lower allele fraction variants are likely to remain. Non-scWGS studies designed to study developmental mosaic mutations do not filter out early variants, probably contributing to differences in the overall patterns of mutations between A2 and clonal mosaics identified in other studies. Thus, Signature A2 may represent a mutational process that is prominent in late stages of development that persists in postnatal life.



Extended Data Fig. 10 | Validation of changes in gene length and expression during ageing. (a,b) Box plots show expression of housekeeping genes in decile 1 (a) and neuron-specific genes in decile 10 (b) in three age groups, 15–39 (red, N = 5), 40–69 (magenta, N = 6), and 70–104 (purple, N = 6). Housekeeping genes always decreases significantly after age 39, while neuron-specific genes show no significant changes. (**, $p < 0.01$; ***, $p < 0.001$; Two-sided Wilcoxon rank-sum test). (c,d) Linear regression slope of housekeeping (c) and neuron-specific (d) genes by length decile. (e,f) Comparison of elderly to adult expression of housekeeping (e) and neuron-specific genes (f), as determined in this study, by size decile in Ling *et al.*²⁹ ($R^2 = 0.16$, $p = 2.29 \times 10^{-67}$ and $R^2 = 0.0009$, N.S., respectively, elderly N = 116, adult N = 64). Housekeeping genes demonstrate the same length dependent expression changes seen in this study. Neuron-specific

genes show no significant relationship between length and expression change, matching the findings of this study. (g,h) Box plots show expression of housekeeping genes in decile 1 (g) and neuron-specific genes in decile 10 (h) in adults from this study (red, N = 9), all donors from Mathys *et al.*³⁰ (violet, N = 189), elderly donors from this study (lilac, N = 7), donors 70–79 from Mathys *et al.* (light purple, N = 34), and donors over 80 from Mathys *et al.* (dark purple, N = 155). Two-sided Wilcoxon rank-sum test comparing adults in this study to each of the Mathys groups are all significant in housekeeping genes, but neuron-specific genes show no significant changes. All box plots depict median, and first and third quartile. Whiskers show 1.5 \times IQR beyond the first and third quartiles. (***, $p < 0.001$; Wilcoxon rank-sum test).

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<input type="checkbox"/>	<input checked="" type="checkbox"/> A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
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<input type="checkbox"/>	<input checked="" type="checkbox"/> For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted <i>Give P values as exact values whenever suitable.</i>
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Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	No software was used in data collection.
Data analysis	The snRNA-seq reads were aligned to the human genome and assigned to genes (GENCODE v32) via Cell Ranger (v6.0.2 and v7.0.1). The barcode and UMI solved counts were further processed with Seurat (v4.3.0 and v5.1.0). scWGS and bulk WGS were first processed accordingly to GATK best practices. Reads were aligned to the human genome via bwa mem (v0.7.12) with default parameters. PCR duplicates were then filtered using picard, and the remaining reads were recalibrated via GATK (v4.1.8.1) "BaseRecalibrator" and "ApplyBQSR". Genotypes were then identified via GATK (v4.1.8.1) "HaplotypeCaller" and "GenotypeGVCFs". Finally, somatic SNVs were identified by comparing the scWGS to corresponding WGS from bulk tissues via SCAN2 (v1). Mutational signature analysis was performed using SignatureAnalyzer (version:GPU) and MutationalPatterns (v3.16.0). MERFISH data was decoded using Vizgen's analysis pipeline integrated within the MERSCOPE system. The Vizgen Post-processing tool (VPT) was used for cell segmentation and pre-filtering with a Gaussian filter and the CellPose algorithm. Gene Ontology analysis was performed in R using "gprofiler2" (v0.2.3) which sourced biological process terms from geneontology.org.

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All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
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Raw RNA-seq and scWGS sequencing data not previously published, and MERFISH data are available at dbGaP (phs003445.v1.p1). Processed data are available at <https://publications.wenglab.org/SomaMut/>. An interactive genome browser of the snRNA-seq pseudo bulk expression data can be found at: https://genome.ucsc.edu/s/yutianxiong/Weng_Lodato_Aging. Previously published single cells analyzed in this study can be found at dbGaP (phs001485.v3.p1) and NIAGADS (NG00121). Previously published bulk DNA sequencing can be found at dbGaP (phs001485.v1.p1), NCBI SRA (SRP041470 and SRP061939), and NIAGADS (NG00121). The previously published Velmeshev et al. data used for cell type annotation was downloaded from SRA accession number PRJNA434002. Previously published Herring et al. data was downloaded from GEO under accession number GEO: GSE168408. Previously published Ling et al. data was downloaded from the Neuroscience Multi-omic Data Archive (NeMo) (<https://assets.nemoarchive.org/dat-bmx7s1t>). Previously published Mathys et al. data was downloaded from the AD and aging brain atlas data repository (https://compbio.mit.edu/ad_aging_brain). GTEx data was downloaded from the GTEx portal (https://www.gtexportal.org/home/downloads/adult-gtex/bulk_tissue_expression). Gene Ontology Biological Process terms were sourced from <https://geneontology.org/>. DepMap data came from <https://depmap.org/portal/> using version Public 22Q4.

Research involving human participants, their data, or biological material

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Reporting on sex and gender

Our previous work identified no impact of sex on somatic mutation dynamics so sex was not included as a covariate in this analysis. To avoid sex bias in RNA-seq data interpretation we included males and females in our adult and elderly samples. Our infant samples were both male, but we confirmed our findings in infants using a previously published dataset (Herring et al.) comprising males and females. Additionally, we used a linear model as an alternative method to identify genes that change during aging. We performed this analysis with and without sex included as a covariate and found no difference in the results.

Reporting on race, ethnicity, or other socially relevant groupings

Race and ethnicity were not considered in our analysis.

Population characteristics

Donor tissue was selected to encompass as wide of a window of human lifespan as possible and a balance of male and female donors. The presence of neuropathology meeting the criteria for a neurodegenerative disease diagnosis or a neurological diagnosis prior death was exclusionary.

Recruitment

n/a

Ethics oversight

All tissue was provided by the National Institutes of Health NeuroBioBank and Banner Sun Health Research Institute Brain and Body Donation Program, which obtained written authorization and informed consent for all donors. Tissue collection and distribution for research purposes was conducted in accordance with protocols approved by the NIH NeuroBioBank (IRB Protocol Number: HM-HP-00042077 or the The Human Brain and Spinal Fluid Resource Center (managed by Sepulveda Research Corporation), IRB Protocol Number: PCC#: 2015-060672, VA Project #: 0002) and Banner Sun Health Research Institute Brain and Body Donation Program (WCG IRB Protocol #20120821). Tissue was collected from post-mortem, deidentified donors and is thus this work is not considered human subjects research by our Institutional Review Board.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

This study included 100 nuclei analyzed by scWGS from 19 different donors, 3-9 nuclei per donor. The number of donors and nuclei was informed by our previous works, Lodato et al. 2018 (PMID: 29217584) and Miller et al. 2022 (PMID: 35444284). The same 19 donors were used for the snRNA-seq studies to allow for gene expression and somatic mutation comparisons within each donor, determining the sample size. 8,710-36,921 nuclei were analyzed per donor for snRNA-seq, across 2-3 replicates per donor.

Data exclusions	The following filtering criteria were applied to each sample and cell: more than 100 cells in the sample, reads from mitochondrially-encoded genes less than 5%, and more than 500 expressed gene in the cell. We further filtered samples "5817 200102", "5288 200128", and "5887 PFC 210601" according to their poor consistency with other samples in the following clustering results. To minimize false discovery and focus on universal changes in aging, mitochondrially-encoded genes and genes in sex chromosomes were removed in the downstream analysis.
Replication	snRNA-seq library preparations were performed in duplicate or triplicate in multiple batches, with batches comprising donors from multiple age groups. After sequencing all cells across replicates were analyzed together in one group.
Randomization	Samples were processed in batches comprising young, adult, and elderly samples to control for batch effects.
Blinding	Investigators were not blinded. In order to appropriately control for batch effects and perform analyses to draw conclusions about changes across age, the investigators needed to know the age of each donor included in the study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

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<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology and archaeology
<input checked="" type="checkbox"/>	<input type="checkbox"/> Animals and other organisms
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Methods

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input type="checkbox"/>	<input checked="" type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Antibodies

Antibodies used	MAB377X, mouse anti-NeuN antibody 488 conjugated from Millipore, Donkey anti-Rabbit IgG 647 conjugated (Fisher).
Validation	MAB377X was validated as neuron-specific using single-cell transcriptomics, published in our previous manuscript doi: 10.1038/s41586-022-04640-1. Secondary antibody is a commonly used reagent and was not validated.

Plants

Seed stocks	n/a
Novel plant genotypes	n/a
Authentication	n/a

Flow Cytometry

Plots

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Methodology

Sample preparation

Fresh frozen samples were processed using a 7ml glass dounce homogenizer with approximately 20µg tissue in 5 ml of filter-sterilized tissue lysis buffer (0.32M sucrose, 5mM CaCl₂, 3mM MgAc₂, 0.1mM EDTA, 10mM Tris-HCl (pH 8), 0.1% Triton X-100, 1mM fresh DTT). The homogenized solution was loaded on top of a filter-sterilized sucrose cushion (1.8M sucrose, 3mM MgAc₂, 10mM Tris-HCl (pH 8), 1mM DTT) and spun in an ultracentrifuge in an SW28 rotor (13,300 RPM, 2hrs, 4°C) to separate nuclei. Supernatant was removed and nuclear pellets were resuspended in ice-cold resuspension buffer (8.5mL 1xPBS/3mM MgCl₂ + 1mL 1xPBS/3mM MgCl₂/1% BSA + 500µL Sucrose Cushion), filtered with a 40µm cell strainer, then stained with anti-NeuN antibody (directly conjugated to Alexa Fluor 488; Millipore cat. No. MAB377X, clone A60; 1:1,250) and an anti-rabbit IgG Alexa Fluor 647 antibody as a negative control for 30min. Using a BD biosciences FACSaria Fusion machine and BD FACSDiva Software, forward scatter A (FSC-A) was first used to isolate large non-replicating cells. This non-replicating high-FSC-A plus high-NeuN population was confirmed to comprise an excitatory neuron population, comprising 2–5% of the total population of nuclei in each sample.

Instrument

BD biosciences FACSaria Fusion

Software

BD FACSDiva™ Software

Cell population abundance

Our targeted large, excitatory neuron population comprised 2-5% of the total sample

Gating strategy

Nuclei were gated away from debris using side scatter (SSC) and forward scatter (FSC). Doublets were gated out using FSC-area vs. FSC-height and SSC-area vs. SSC_height. NeuN staining produced a bimodal signal distribution, distinguishing NeuN+ and NeuN- nuclei. Large neuronal nuclei, representing excitatory pyramidal neurons, were further identified by collecting the nuclei with highest NeuN signal among the NeuN+ neuronal fraction, and gating for the population with the highest FSC-A signal and excluding Alexa-Fluor-647-high events.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.