Overexpression of schizophrenia susceptibility factor human complement C4A promotes excessive synaptic loss and behavioral changes in mice

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The complement component 4 (C4) gene is linked to schizophrenia and synaptic refinement. In humans, greater expression of C4A in the brain is associated with an increased risk of schizophrenia. To investigate this genetic finding and address how C4A shapes brain circuits in vivo, here, we generated a mouse model with primate-lineage-specific isoforms of C4, human C4A and/or C4B. Human C4A bound synapses more efficiently than C4B. C4A (but not C4B) rescued the visual system synaptic refinement deficits of C4 knockout mice. Intriguingly, mice without C4 had normal numbers of cortical synapses, which suggests that complement is not required for normal developmental synaptic pruning. However, overexpressing C4A in mice reduced cortical synapse density, increased microglial engulfment of synapses and altered mouse behavior. These results suggest that increased C4A-mediated synaptic elimination results in abnormal brain circuits and behavior. Understanding pathological over-pruning mechanisms has important therapeutic implications in disease conditions such as schizophrenia.

Schizophrenia is a heritable neuropsychiatric disorder of unknown etiology and affects 1% of the adult population. Schizophrenia is associated with impaired cognition, perception and motivation, which become clinically apparent in late adolescence and early adulthood. The concurrent excessive loss of gray matter and dendritic spines during this period suggests that the reduction in synaptic connections might contribute to the behavioral and cognitive deficits. However, it is unclear when synaptic deficits in the cerebral cortex arise and how reduced synaptic connections relate to genes and alleles and the proteins they encode.

The human genome’s largest population-level influence on the risk of schizophrenia arises in substantial part from the C4 genes. The C4A and C4B genes, which reside in the major histocompatibility complex (MHC) locus, encode two highly conserved C4 isoforms, C4A and C4B. Although the peptide sequences of C4A and C4B differ by only four amino acids, the isoforms exhibit differential binding to unique chemical targets. Thus, their molecular structures could dictate the corresponding binding sites in tissues. In addition, both genes exhibit common variations in copy number, with one to four total C4 genes per haplotype, and vary in length due to insertion of an endogenous retroviral element in an intron, most commonly present in the C4A isoform. Alleles that increase C4A expression correlate with increased schizophrenia risk, but ones that increase C4B expression do not. In this study, we sought to explain these findings by distinguishing the roles of C4A and C4B in the brain and exploring whether C4A overexpression affects brain development and synaptic pruning.

Synaptic pruning is a process by which supernumerary neuronal connections are eliminated, which allows appropriate connections to strengthen and mature. Previous studies have identified some key players of the pruning processes, including microglia through CD47 and tropocytosis, astrocytes by MERTK and MEGF10 pathways, and many immune system molecules such as MHC I, pentraxins, paired immunoglobulin-like receptor B and complement. The classical complement cascade, including C1q, C3 and C4, is critical to pruning, as mice deficient in these components show defects in synaptic refinement in the retinogeniculate system. C4 can localize to subsets of synapses; however, functional and behavioral consequences of increased C4 expression and its downstream pathways are unknown and challenging to characterize. Moreover, although synaptic refinement in the visual system is well characterized, the time line of synaptic pruning, the critical periods of plasticity and the role of complement in regions relevant to schizophrenia remain to be identified.

Here, we constructed humanized mouse models to examine the functional differences between human C4A and C4B in the brain and to determine whether C4A overexpression can pathologically affect brain development. In this model, variable copy numbers of C4A and C4B genes were introduced into the mouse genome to investigate their physiological effects in vivo. We found distinct roles for C4A and C4B in the developing brain, and further discovered that C4A overexpression affected microglia engulfment, synaptic pruning and mouse behavior. These findings highlight the importance of C4A in synaptic pruning and brain development, and draw attention to the parallels observed between C4A overexpression and schizophrenia pathology.

Results

C4A is more efficient than C4B in synaptic pruning. Genetic analyses of patients with schizophrenia made the surprising prediction that C4A and C4B differ in their effects on brain development. Although within primates there exists two isoforms of C4,
which differ in four amino acid residues in the TED domain of activated C4 protein, mice have only one corresponding C4 gene (mC4), from which primate C4A and C4B evolved (Fig. 1b). mC4 is made up of a chimeric C4A/C4B sequence in the key isotypic residues: positions 1101–1102 are identical to C4A residues (proline and cysteine), whereas positions 1105–1106 resemble residues in C4B (isoleucine and histidine). The mouse Slp gene, which is a mC4-like gene, is a sequence outlier and not functional in the classical complement pathway as its C1s cleavage site is mutated and cannot be activated. Given the limitations of the mC4 lacking isotypes, we introduced human C4A (hC4A) or C4B (hC4B) genes into the mouse genome using bacterial artificial chromosome (BAC) DNA transgenesis. The use of BACs enabled the delivery of human genes with their introns and cis-regulatory elements. The two resulting mouse strains were backcrossed onto a mC4-deficient background, thus creating hC4A+ and hC4B− mice that express C4 only from the human transgene (Fig. 1a). The number of gene copies for each mouse strain was determined using droplet digital PCR (ddPCR), and this showed that hC4A− mice had four copies of C4A long (C4A-L) and hC4B− mice had two copies of C4B short (C4B-S) (Fig. 1c). Whole-genome sequencing analysis to locate the integration site of the BACs showed tandem insertion in chromosome 2 and chromosome X in hC4A− and hC4B− mice, respectively (Extended Data Fig. 1a–d). Of note, the C4A BAC DNA insertion resulted in the duplication of the endogenous gene Sema6d; however, its expression was unchanged between hC4A+ and wild-type (WT) mice (Extended Data Fig. 1f).

Both hC4A− and hC4B− strains had peripheral C4 mRNA expression and serum protein levels consistent with their relative gene copy numbers (Fig. 1d and Extended Data Fig. 2a). In the periphery, C4 functions to opsonize pathogens and cellular debris as a part of the classical complement cascade. C4, together with C2a, forms the C3 convertase complex to cleave and activate the downstream protein C3. To investigate whether the known function of C4 was maintained in this model, C3 serum levels were measured. Similar levels of C3 were observed in hC4B/S and hC4B−/− mice compared with WT mice (Fig. 1e). Strikingly, hC4A− mice showed a significantly lower percentage of overlapping territories compared with C4−/− littermates and similar levels to WT mice (Fig. 2f). Conversely, hC4B− mice had a similar percentage of overlap as C4−/− littermates, but significantly higher levels than WT mice (Fig. 2f). The difference is unlikely explained by the C4 gene copy number differences between hC4A−/− and hC4B−/− mice, since a single copy of the C4A transgene in the hC4AB− mouse model (Extended Data Fig. 4a) was sufficient to rescue the defect observed in C4−/− mice (Fig. 2g). These results suggest that C4A can rescue the synaptic refinement deficits of C4−/− mice and that C4B, unlike C4A, is functionally less active in synaptic pruning. Overall, these in vitro binding assays and early development experiments highlight the unique part that C4A plays in synaptic pruning compared with C4B. These findings, taken together with the established connection of C4A to schizophrenia, made C4A and its overexpression the focus of the remainder of our study and led us to question whether increased C4A expression alters synaptic refinement.

To visualize hC4 deposition and investigate synaptic refinement processes in hC4A−/− and hC4B−/− mice in vivo, the retinogeniculate system was utilized. The visual system in the developing mouse brain has been extensively used as a model to not only delineate synaptic elimination pathways but also to examine the involvement of the complement system in synaptic pruning. During early postnatal mice, retinal ganglion cells (RGCs) from both eyes form synapses onto the same relay neurons within the dorsolateral geniculate nucleus (dLGN). During a key subsequent critical period (postnatal day (P) 3–9), many of these synaptic connections are refined due to spontaneous activity, which results in RGCs from either eye to synapse onto different relay neurons and create nonoverlapping, eye-specific territories in the dLGN. P5 was determined as the most robust period of pruning, when most C3 deposition has been observed. At this time point, differential deposition of C4A and C4B onto the RGC synaptic terminals was investigated. dLGN sections were stained for hC4 and vesicular glutamate transporter 2 (Vglut2; Fig. 2e), and a colocalization analysis was performed. We observed greater numbers of C4+ puncta colocalizing with Vglut2+ synapses in hC4A−/− mice compared with hC4B−/− mice (Fig. 2d) after normalizing for C4 mRNA expression in the LGN (Extended Data Fig. 4c). As expected, C4+ mice showed only background levels of staining (Fig. 2e,d). These results demonstrate that C4A and C4B have differing binding capacities in the brain, with C4A binding to synapses at a higher level than C4B.

Furthermore, eye-specific territories of the dLGN were used to determine the functionality of C4A and C4B in synaptic pruning. The segregation of RGC projections was traced by fluorescently tagged cholera toxin subunit B (CTB) molecules, which were intravitreally injected (Fig. 2e). Previous studies using global knockout mice showed that C1q-, C4- and C3-deficient mice fail to fully segregate the axonal projections of RGCs from each eye into nonoverlapping territories, thereby indicating defects in synaptic refinement. Strikingly, hC4A− mice showed a significantly lower percentage of overlapping territories compared with C4−/− littermates and similar levels to WT mice (Fig. 2f). Conversely, hC4B− mice had a similar percentage of overlap as C4−/− littermates, but significantly higher levels than WT mice (Fig. 2f,g). The difference is unlikely explained by the C4 gene copy number differences between hC4A−/− and hC4B−/− mice, since a single copy of the C4A transgene in the hC4AB− mouse model (Extended Data Fig. 4a) was sufficient to rescue the defect observed in C4−/− mice (Fig. 2g). These results suggest that C4A can rescue the synaptic refinement deficits of C4−/− mice and that C4B, unlike C4A, is functionally less active in synaptic pruning. Overall, these in vitro binding assays and early development experiments highlight the unique part that C4A plays in synaptic pruning compared with C4B. These findings, taken together with the established connection of C4A to schizophrenia, made C4A and its overexpression the focus of the remainder of our study and led us to question whether increased C4A expression alters synaptic refinement.

C4A overexpression increases synaptic refinement via microglia in the dLGN. An increased C4A gene copy number is associated with schizophrenia, which is characterized by an excessive reduction in cortical gray matter and dendritic spine density on cortical neurons. To understand the underlying biology of this genetic association and to study the effects of C4A copy number on synaptic pruning, the hC4A mouse model was used to generate hC4A-overexpressing mice. This overexpression model (hC4A/A) was obtained by crossing hC4A−/− mice together, which resulted in eight C4A copies per strain (Fig. 3a). It was confirmed that hC4A/A mice had approximately twice the level of serum C4 protein than C4−/− mice (Extended Data Fig. 5a). In the retinogeniculate system, hC4A/A mice expressed more C4A mRNA in the retina, and, although not significant, in the LGN (Fig. 3b).
Fig. 1 | Human C4A and C4B are functionally active in a mouse model. **a,** Schematic of hC4A−/− and hC4B−/− transgenic mice. **b,** A three-dimensional (3D) structure of activated hC4 protein showing the TED domain and the amino-acid sequence differences between C4A and C4B. The mC4 protein is a hybrid of C4A and C4B, gaining positions 1,101–1,102 from C4A and 1,105–1,106 from C4B. **c,** Gene copy numbers for C4A, C4B, C4A-L and C4B-S were determined by ddPCR using Rpp30 as a reference gene (n = 5 representatives per group). **d,** C4 serum levels were measured by ELISA in adult (P60) WT (n = 9), C4−/− (n = 10), hC4A−/− (n = 7) and hC4B−/− (n = 6) mice. **e,** C3 serum levels were measured by ELISA in adult (P60) WT (n = 8), C4−/− (n = 8), hC4A−/− (n = 8) and hC4B−/− (n = 2) mice. **f,** Representative confocal images of spleen showing C4 deposition on CD21+ FDCs in follicles delineated by CD169+ macrophages in WT, C4−/−, hC4A−/− and hC4B−/− mice (red, C4; cyan, CD21; green, CD169). White arrows indicate C4 puncta deposited onto FDCs. Scale bar, 50 µm. **g,** C4 deposition was quantified by fluorescence intensity per FDC area (in each FOV) for WT (n = 3), C4−/− (n = 11), hC4A−/− (n = 4) and hC4B−/− (n = 3) mice. Kruskal–Wallis test with Dunn’s multiple comparison test. Bar graphs show the mean ± s.e.m. NS, not significant (P > 0.05).
**Fig. 2** Human C4A is more efficient than C4B in synaptic pruning. a, At the synapse, complement-dependent pruning is carried out by the classical complement cascade. After C1q tagging, C4 binds the synapse and C3 is then activated for microglia recognition by the receptor CR3. Microglia engulf the complement-bound synapses for refinement. b, Synaptosomes from C4−/− mice were isolated and incubated with serum containing the same amount of C4 from hC4A−/− (n = 10) or hC4B−/− (n = 9) mice. C4 deposition on synaptosomes was detected and quantified by flow cytometry (serum from three independent experiments; Mann–Whitney test, two-tailed, ****P < 0.0001). c,d, C4 deposition onto Vglut2+ synaptic terminals in the dLGN was identified by immunofluorescence staining at P5 in C4−/− (n = 4), hC4A−/− (n = 6) and hC4B−/− (n = 4) mice. c, Representative images of C4 (red) and Vglut2 (green) staining in the dLGN showing C4−/−/Vglut2+ synapses (white circles). Scale bar, 5 μm. d, Quantification of C4+Vglut2+ synapse density in P5 hC4 transgenic mice (three independent experiments; results were normalized to hC4 mRNA expression in the LGN (Mann–Whitney test, two-tailed; **Pc4+/− vs hC4A−/− = 0.0095, *Pc4+/− vs hC4B−/− = 0.0286, *Pc4+/− vs hC4AB−/− = 0.0190). e, To investigate complement-dependent synaptic pruning in the retinogeniculate system, the eye-specific segregation assay was performed. Mice were injected with fluorescently tagged CTB (CTB-488 and CTB-594) in each eye at P9 and brains were collected at P10. f, Representative images of WT, C4−/−, hC4A−/− and hC4B−/− mice at P10 are shown. Inputs from contralateral (Contra; red) and ipsilateral (Ipsi; green) eyes and overlapping area (yellow) are shown. Scale bar, 100 μm. g, The percentage overlap of the contralateral and ipsilateral areas were calculated for P10 WT (n = 4), C4−/− (n = 18), hC4A−/− (n = 7), hC4B−/− (n = 6) and hC4AB−/− (n = 3) mice (2 independent experiments; all results were calculated for P10 WT (n = 4), C4−/− (n = 18), hC4A−/− (n = 7), hC4B−/− (n = 6) and hC4AB−/− (n = 3) mice (2 independent experiments; all results were normalized to the C4−/− group to retain littermate controls; one-way ANOVA with Tukey’s multiple comparisons test). Bar graphs show the mean ± s.e.m.

Considering the increase in copy number and mRNA expression in hC4A/A mice, higher levels of synaptic pruning were expected. In the dLGN, the level of synaptic pruning was measured by quantifying the RGC inputs31. RGC synapses (Vglut2+) are morphologically distinct from other terminals in the dLGN as they form clusters and appear larger32. By measuring the area of Vglut2+...
inputs, we found that the RGC axon terminal surface in hC4A/A mice was significantly reduced compared with hC4A−/− mice at P10 (Fig. 3c,d), which indicated higher levels of synaptic pruning. These results were supported by the reduced overlapping territories in the dLGN in hC4A/A mice compared with hC4A−/− littermates (Fig. 3e,f). To control for morphological changes in retinal arborization, projections from each eye were examined (Extended Data Fig. 6a). Notably, the ipsilateral area was smaller in hC4A/A mice than in hC4A−/− mice, thus confirming that ipsilateral projections were excessively pruned in hC4A-overexpressing mice (Extended Data Fig. 6b–d). To test whether C4A overexpression leads to abnormal developmental processes before the critical period, the eye-specific segregation assay was performed at P5. At this time point, we found that projections from each eye almost completely overlapped in C4−/−, hC4A+ and hC4A/A mice (Extended Data Fig. 6f), which suggests that C4A overexpression does not affect synaptic inputs before the critical pruning period. This result could not be attributed to differing numbers of cells, as cells stained positive for 4,6-diamidino-2-phenylindole (DAPI+) in the dLGN and Brn3a+ RGCs in the retina were equivalent in number (Extended Data Fig. 6g,h). Observing the morphological and functional effects of C4A expression on synaptic inputs led us to investigate the role of microglia in the complement-dependent synaptic pruning process.

Microglia are crucial for development of the brain, and in the retinogeniculate system, they participate in synapse elimination by phagocytosing complement-tagged synapses via complement receptor 3 (CR3; also known as CD11b)25. In the dLGN, P5 is the time point at which microglia are most phagocytic26. To determine whether excessive synaptic loss is accompanied by increased microglial engulfment, mice were injected with fluorescent CTB, and microglial phagocytosis of synaptic material in the dLGN of P5 mice was measured (Fig. 3g). Importantly, microglia from hC4A/A mice engulfed more inputs than those in hC4A−/− mice (Fig. 3h,i), while having similar densities in each group (Extended Data Fig. 6e). This suggests that increased C4A expression induces a greater degree of synaptic engulfment per microglia and excessive synaptic refinement in the dLGN. Overall, utilizing the robust retinogeniculate system gave insight into the isoform differences of hC4 and the role of C4A in the mechanism of complement-mediated synaptic pruning and its copy number effects on synapse elimination via microglia. However, to better examine the schizophrenia-related effects of C4A, the focus was shifted to the medial prefrontal cortex (mPFC), a region highly associated with schizophrenia pathology.

C4A overexpression affects microglia-mediated synaptic pruning in the mPFC. The complex organization and circuitry of the frontal cortex (FC) make it a difficult region to study synaptic pruning processes. Due to the limited knowledge of the role of C4 in the FC and how it is regulated, C4 protein levels were measured at different time points from development to adulthood. Forebrains from hC4A/A mice had significantly higher levels of C4 compared with hC4A−/− mice at P10 (development) and P40 (adolescence; Fig. 4b). Although not significant, a trend of an increased C4 level was observed in hC4A/A mice at P60 (adulthood).

In humans, schizophrenia symptoms arise during adolescence, the period in which the FC matures27. Given this, the complement profile both in the periphery and the FC was investigated at the analogous time point in mice, P40. In serum, levels of C4, C3 and Clq were similar between WT and hC4A−/− mice (Extended Data Fig. 5a–c). In the FC, RNA expression levels of mC4, hC4A, C3 and Clqβ were also similar between the two groups (Extended Data Fig. 5d–f). Thus, the hC4A−/− model presents a context whereby the effect of C4A overexpression can be assessed in the brain.

As microglia play a crucial role in complement-dependent synaptic pruning28,34, their engulfment levels were investigated in the mPFC of hC4A−/− and hC4A/A mice (Fig. 4a). Microglia that are highly phagocytic show increased levels of CD68, a monocytic cytoplasmic biomarker found on the lysosomal membrane29,30. Microglial engulfment levels were measured in the mPFC by immunostaining for the markers Iba1 and CD68 (Fig. 4d). Cell Profiler was used to perform morphology analyses and to quantify the CD68-stained area in each microglia (Extended Data Fig. 7a). Similar to C4 protein expression, the CD68+ area was significantly higher in the hC4A/A mice at P10 and P40, when the peak difference was observed, but not at P60 (Fig. 4e). Microglia density and morphology were unchanged (Extended Data Fig. 7b–h). Although these results suggest that microglia are more phagocytic under C4A overexpression during adolescence, they do not indicate engulfment of synaptic material. To overcome this issue, we developed a fluorescence-activated cell sorting (FACS)-based assay to detect intracellular synaptic proteins in microglia. Purified microglia from the FC of P40 mice were permeabilized, stained for SV2 and quantified by FACS (Fig. 4e). Microglia isolated from hC4A/A mice contained more synaptic material than microglia from hC4A−/− mice (Fig. 4f), thereby strengthening our previous findings that C4A overexpression induces increased microglial engulfment of synaptic material in the mPFC.

The pronounced phagocytic state of microglia in hC4A/A mice raised the question of whether these microglia engage in transcriptional programs for regulating synapse plasticity under C4A overexpression. To investigate whether C4A overexpression affected gene expression during adolescence, FC microglia from mice were sequenced. Bulk-sequencing analysis showed an unchanged RNA profile in C4−/−, hC4B−, hC4A− and hC4A/A mice compared with WT mice (Extended Data Fig. 8a). Moreover, the normalized counts of phagocytic pathway genes showed similar levels among all groups (Extended Data Fig. 8b,c). These results suggest that the increased synapse engulfment observed in the hC4A/A microglia is not due to a change in the microglial transcriptional profile, but instead may be guided by complement tagging of synapses.

To test whether C4A overexpression and increased microglial engulfment resulted in synapse loss in the mPFC, the synapse density was quantified in WT, C4−/−, hC4A− and hC4A/A mice by immunostaining for pre- and post-synaptic markers (SV2 and Homer-1, respectively) at P10, P40 and P60 (Fig. 5a). hC4A/A mice did not exhibit a decrease in synapse density at P10 and P40 (Fig. 5c,d); however, a striking reduction in synapse density in hC4A/A mice was observed at P60 (Fig. 5b,e). Additional analyses revealed that expression of the synaptic proteins was not altered in P60 hC4A/A mice (Extended Data Fig. 9a–e), but rather their colocalization was reduced (Extended Data Fig. 9f). Surprisingly, synapse density did not differ between WT and C4−/− mice in this region across all time points, which suggests that complement might not be the only mechanism involved in synaptic pruning in the mPFC.

To investigate whether the reduced synapse density observed at P60 in C4A-overexpressing mice was sustained through later stages of adulthood, a dendritic spine density analysis was performed using the Golgi–Cox staining method in the mPFC. Pyramidal neurons from layer 2/3 were analyzed from 6-month-old (P180) mice (Extended Data Fig. 9i). Dendritic spine density was significantly reduced in hC4A/A mice compared with hC4A−/− mice (Fig. 5f), whereas the total cell number, the neuron number and the neuronal dendrite size analyzed were unchanged (Extended Data Fig. 9g,h,j). Altogether, these results show that increased C4A expression leads to excessive synaptic loss in the adult mPFC via increased, cumulative microglia engulfment.

C4A overexpression alters mouse behaviors. Given the microglial engulfment and synapse density phenotypes, we focused on investigating whether the molecular and cellular effects of C4A overexpression on the murine brain were accompanied by behavioral phenotypes analogous to schizophrenia symptoms. Patients with
Fig. 3 | Increased C4A copy number induces excessive synaptic pruning via microglia engulfment. 

a, Schematic of hC4A−/− and hC4A/A transgenic mice. 
b, C4A mRNA expression was measured at P5 in the retina and the LGN from hC4A−/− (A/−; n = 4) and hC4A/A (A/A; n = 3) mice by ddPCR ( littermates from two independent cohorts; two-way ANOVA with Sidak’s multiple comparisons test, **P = 0.0039). c, d, The presynaptic marker Vglut2 was stained by immunofluorescence at P10 for hC4A−/− (n = 10) and hC4A/A (n = 10) mice. c, Representative images of Vglut2 immunostaining are shown. White circles indicate examples of Vglut2 synapses. Scale bar, 10 µm. 
d, Percentage area of Vglut2 staining was measured ( littermates from three independent cohorts; unpaired, two-tailed t-test, ***P = 0.0009). e, f, The eye-specific segregation assay was performed for hC4A−/− (n = 6) and hC4A/A (n = 6) mice. e, The percentage overlap over multiple thresholds was used for analysis in P10 hC4A−/− and hC4A/A mice (n = 6 in each group, littermates from 3 independent experiments; two-way ANOVA with Sidak’s multiple comparisons test, 0.0026 ≤ **P ≤ 0.0054). Box-and-whisker plot displays the median (center line), the 25th to 75th percentile (box), and the minimum and maximum values (whiskers). f, Representative images of hC4A−/− and hC4A/A littermates at P10 from three independent experiments showing inputs from contralateral (red) and ipsilateral (green) eyes and the overlapping area (yellow). Scale bar, 100 µm. g, Schematic of the experiment. Mice received an intravitreal injection of fluorescently conjugated CTB (CTB-555) at P4 and brains were collected at P5, when microglia engulfment is at its peak. Microglia were stained with Iba1 for confocal imaging and 3D reconstruction. 
h, Representative surface-rendered microglia (green) and engulfed retinogeniculate inputs (red) from the dLGN. Grid line increments are 5 µm. i, Quantification of the percentage of CTB engulfment within individual microglia volume for hC4A−/− (n = 3) and hC4A/A (n = 4) mice (three independent experiments; unpaired, two-tailed t-test, **P = 0.0060). Bar graphs show the mean ± s.e.m.
C4A was measured in hC4A/mice at P10 (hC4A/s.e.m. the mean ±****0.0001). Representative images of microglia with the CD68 signature. White arrowheads indicate CD68−de, mice, however, had altered social behavior by not showing a preference toward interacting with the mouse as opposed to the object. To measure anxiety levels, mice were placed in the open-field test (Fig. 6c). The hC4A/A mice displayed anxiety-like behavior compared with WT, C4−/− and hC4A−/− mice, as measured by the number of rearing events in the center zone (Fig. 6d). Similarly, hC4A/A mice exhibited an anxiety phenotype in the light–dark box test compared with WT and C4−/− mice, although the difference between hC4A−/− and hC4A/A mice did not reach significance (Extended Data Fig. 10c). Furthermore, when exposed to the novel Y-maze (Fig. 6e), hC4A/A mice displayed spatial working memory deficits compared with WT, C4−/− and hC4A−/− mice by showing no preference toward the novel arm of the maze (Fig. 6f). By contrast, prepulse inhibition (PPI), motor defects, coordination defects or depression-like phenotypes were not detected in hC4A/A mice (Extended Data Fig. 10d–h). These findings suggest that C4A overexpression and its downstream effects can be associated with abnormal mouse behaviors in social, emotional and cognitive contexts, which closely resemble the negative symptoms experienced by patients with schizophrenia.
Discussion

To investigate the genetic findings that link the human C4 gene locus to schizophrenia risk, we constructed two mouse models that express human C4A or C4B, and one that overexpresses C4A. We found that C4A and C4B result in different outcomes in synaptic pruning and that the C4A overexpression not only induces increased microglia engulfment, resulting in a reduced synapse density, but also alters mouse behavior. Thus, our findings establish functional differences between C4A and C4B and identifies the impacts of C4A overexpression on the brain.

C4A and C4B have different covalent binding properties, which are driven by the amino acid residue differences in the isotypic region. C4A preferentially forms amine bonds with proteins, whereas hC4B binds hydroxyl groups of carbohydrates\textsuperscript{24,39}, as observed in the hemolytic assays. Although there have been various studies showing C4A and C4B differences in vitro, the number of investigations in vivo is limited\textsuperscript{40}. The hC4A and hC4B mouse models serve as important tools as they allow the isotype differences to be tested in the context of synaptic pruning within the brain.
Synaptic pruning and complement components have previously been challenging to study in the mouse cortex. However, the narrow synaptic refinement period of the retinogeniculate circuits in dLGN make it a desirable brain region to investigate complement-dependent synaptic pruning. For this, our initial investigations focused on the dLGN to characterize the functional differences of C4A and C4B and the effects of C4A overexpression. By utilizing these circuits, we found that C4A is functionally more efficient in synaptic pruning than C4B, as it binds to synapses at a higher level. This increased hC4A deposition could be due to its binding properties arising from the hydrophilic amino acid sequence at positions 1101–1102, which bears the exact sequence to its binding properties arising from the hydrophilic amino acid sequence at positions 1101–1102, which bears the exact sequence to
an increased copy number of C4A leads to excessive synaptic pruning by microglia engulfment in the dLGN, which is enhanced most likely by increased opsonization of synapses by C3. Importantly, the discovery of an excessive pruning phenotype due to C4A overexpression provides an impetus to explore C4A in regions with higher brain functions.

Previous findings highly associate schizophrenia phenotypes to processes in the mPFC, which makes it an appealing region for investigating synaptic pruning in the context of C4A overexpression. Unlike the retinogeniculate system, the critical refinement period in the mouse FC is likely to span over several weeks, as it occurs over several years during adolescence and early adulthood in humans15. The limited knowledge in synaptic pruning time lines and complement expression in the mPFC necessitates testing multiple time points across different stages of the life of the mouse. We found that the order of events (C4 deposition, microglia engulfment, and synapse elimination) in the dLGN is similar in the FC, although the pace and range of the developmental time line are markedly different. The highest levels of C4 protein expression in the forebrain of C4A-overexpressing mice was observed during development (P10). Microglia engulfment in response to hC4 protein expression and deposition in the mPFC peaked during adolescence (P40) in the hC4A-overexpressing mice. Reduced synapse density was observed in hC4A-overexpressing mice only after adolescence (at the P60 time point). Taking into consideration the trends of hC4 protein expression and microglia engulfment, it is likely that the effects of C4A overexpression on synaptic pruning are not instantaneous, but rather cumulative in the mPFC. In the C4A-overexpressing mice, there was an increased C4 protein level, and likely deposition onto synapses, at a level that exceeds a threshold for pathology during development and adolescence. Consequently, there is a continuous increased engulfment of synapses over a period from P10 to P40, thereby resulting in the reduced synapse density pathology observed after adolescence and retained during later stages of adulthood. This study not only demonstrates an ‘excessive pruning’ phenotype in the mPFC, a novel concept in the field, but also establishes the time line of onset and relative order of events regarding synaptic pruning in C4A-overexpressing mice.

The hC4A transgenic mice also helped us identify mouse behavior associated with C4A overexpression. Abnormal social behavior, spatial working memory defects and anxiety phenotypes were observed in mice that overexpress C4A; strikingly, these behaviors resemble negative symptoms of schizophrenia in humans. The observed behaviors are associated with circuits in the mPFC; however, they can also be linked to other various brain regions such as the parietal cortex, the basal ganglia, the hippocampus and the amygdala41–43. Our results showing reduced synapse density in the mPFC can only start to explain the behavior phenotypes observed in C4A-overexpressing mice, and future electrophysiological and behavioral studies are needed to explore specific mPFC circuitry and other brain regions in relation to the aforementioned behaviors.

In contrast to overexpression, the loss of C4 did not affect cortical synapse density, which indicates that C4 is not required for circuit maturation in the mPFC and that other mechanisms may be involved. Moreover, C4−/− mice did not exhibit behavioral defects. Thus, our data suggest that the excessive elimination of synapses after C4A overexpression has pathological consequences on brain development, whereas C4 is not necessary, or has compensatory pathways, for normal brain development in the mPFC. Therefore, the use of therapeutic strategies targeting C4 downregulation can prevent and address the abnormal physiology resulting from C4A overexpression, while not distorting the endogenous synaptic refinement processes in the brain.

The effects of C4A overexpression manifest in molecular, cellular and behavioral phenotypes from development into adolescence and early adulthood. The strongest effects of human C4A became apparent only at substantial numbers of C4A gene copies, which suggests that there is a threshold for pathology. An intriguing possibility proposed by these results is that C4A-related pathology might arise primarily in the context of unknown biological events that provoke greater-than-usual C4 expression and result in C4 levels above the threshold. Individuals with more C4A gene copies could exhibit particularly strong increases in C4 expression in response to such events, and therefore increased vulnerability in particular contexts, but without manifesting brain phenotypes under normal circumstances.

The pathophysiology of schizophrenia is poorly understood, and animal models that recapitulate key molecular events have long been needed. Our findings from creating C4-humanized mice show that C4A is more effective in synapse refinement compared with C4B. We also demonstrated that C4A overexpression is linked to increased microglial engulfment, reduced cortical synapses and altered mouse behaviors that resemble the negative symptoms of schizophrenia. Our findings on the time line of onset, impaired synaptic pruning processes and anomalous behavior in hC4A-overexpressing mice draws important parallels between our overexpression mouse model and schizophrenia pathology. Using mouse models such as described here will be important to further understand the impact of C4A overexpression on other brain regions and ways in which excessive synapse loss affects brain circuits in the context of disease.

Online content

Any methods, additional references, Nature Research 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/s41593-020-00763-8.

Received: 24 August 2019; Accepted: 20 November 2020; Published online: 22 December 2020.

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Methods

Mice. All animal experiments were carried out in agreement with the institutional guidelines provided by the Institutional Animal Care and Use Committee at Harvard Medical School and Boston Children’s Hospital (protocol numbers 00000748, 00000111, 000004762 and 00002660), under applicable laws and regulations and in accordance with the guidelines of the Laboratory Animal Center at the NIH for the humane treatment of animals. C57BL/6J mice (WT; The Jackson Laboratory, 009664), C4−/− mice (The Jackson Laboratory, 003643) and four new transgenic mice strains, hC4A−/−, hC4A/A, hC4B−/− and hC4AB−/−, were bred and maintained under specific pathogen-free conditions. Mice were kept at an ambient temperature of 21 °C under a 12-h light–dark cycle with a standard chow diet at the AAALAC-accredited animal facility at Harvard Medical School. C4−/− mice were generated by our laboratory and previously described1. C4−/− heterozygous parent mice were used to generate litters with WT and C4−/− littersmates could be compared. Mice from both sexes were analyzed in all of the experiments. For experiments involving the dclGN, mice at the ages of P5 and P10 were used. For experiments involving the mPFC, mice at the ages of P10, P40, P60, P90 and P180 were used. Further details of the ages can be found in the figure legends. Mice that showed a state of hydrocephalus were excluded from the study. No data points were excluded from any analyses.

Generation of hC4 transgenic mice. Standard microinjection procedures were used to produce the transgenic mice1. Fertilized mouse eggs were flushed from the oviducts of superovulated 3-week-old C57BL/6J DBA/2 J mice (The Jackson Laboratory), and male pronuclei were injected with BAC DNA (2 ng/µl) that had been extracted from a chloroform/chloroform/chloroform-isolated BAC DNA (New England Biolabs) at its eugene restriction site located in the pBACE4-3.6 vector. BAC DNA containing hC4A, hC4B or both sequences from a common MHc haplotype were selected (Supplementary Table 1) and purchased from CHORI (https://bacpacresources.org). BAC DNA size and linearization were validated by pulse-field gel electrophoresis (Extended Data Fig. 1e). The injected eggs that developed into the two-cell stage were implanted in the oviducts of pseudopregnant recipient females. At 3 weeks of age, the animals were tested for the presence of the hC4A and hC4B genes by PCR analysis of their genomic DNA (see primer sequences in Supplementary Table 2). hC4A−/−, hC4B−/− and hC4AB−/− strains were generated from one founder for each transgene. Both strains were backcrossed into the C4−/− strain for at least ten generations before use in experiments to ensure a C57BL/6J genetic background. All animals used for experiments were tested for the presence of the transgene by ddPCR (see below).

Determining gene copy number variation by ddPCR. hC4A, hC4B, C4A-L and C4B-S gene copy numbers in the transgenic mice were determined by ddPCR as previously described for human samples1. Tail specimens were incubated with Proteinase K (Bio-Rad) for 1 min, and digested with AluI (New England Biolabs) for 1 h at 37 °C. We mixed 30 ng of digested DNA with 2 µl of ddPCR Supermix for Probes (no dUTP, Bio-Rad) and 20x primer–probe mix for hC4A or hC4B and the mouse gene Rpp30 as a reference (18 µM of forward and reverse primers each; 5 µM of fluorescent probe). The sequences for primers and probes are listed in Supplementary Table 2. For each reaction mixture, this reaction mixture was then emulsified into approximately 20,000 droplets in an oil/ aqueous emulsion using a microfluidic droplet generator (Bio-Rad). The droplets containing this reaction mixture were subjected to PCR by 20,000 droplets in an oil/aqueous emulsion using a microfluidic droplet generator (Bio-Rad) and 20x primer–probe mix for hC4A or hC4B and the mouse gene Rpp30 as a reference (18 µM of forward and reverse primers each; 5 µM of fluorescent probe). The sequences for primers and probes are listed in Supplementary Table 2. For each reaction, the fluorescence (both colors) in each microfluid was measured by Poisson-correcting the fraction of droplets that each whole-genome sequencing sample using Genome STRIP (v101690) and generated read depth profiles (command: ComputeDepthProfiles) using a custom uniqueness mask generated for each transgenic genome reference (command: ComputeGenomeMask) that masked non-uniquely aligned reads to the reference and then plotted read depth profiles near the candidate insertion sites and across the inserted BAC sequence.

RNA expression analysis by ddPCR and PCR with reverse transcription. hC4A, hC4B, C3 and C1qb RNA levels were determined by reverse transcription followed by ddPCR as previously described in human samples6. Briefly, RNA was isolated using the TRIzol protocol, and for each sample, 1 µg of RNA was reverse transcribed using an iScript kit (Bio-Rad). A similar ddPCR mix and PCR program as for gene copy number was used. Primers and probes for expression are listed in Supplementary Table 3. Mouse C4b2a1 for brain samples and C1qb for spleen and liver samples was used as a normalization control to calculate the ratio of hC4A (or hC4AB) to Hs2s1 (or Eif4f6) expression. PCR with reverse transcription reactions were assembled for genes of interest (S02a, Senad3 and Padu95) using the Taq universal SYBR Green supermix system (Bio-Rad) and run on a CFX96 machine (Bio-Rad). Gene expression was normalized to Gapdh and compared using the ΔΔCT method. All primer sequences are listed in Supplementary Table 2.

Serum ELISA. C4 protein levels were detected in serum from WT (C57BL/6J) and hC4 transgenic mice. Details of all the antibodies used are provided in Supplementary Table 3. High binding microplates were coated overnight with rat anti-C4c (16D2, in-house) or goat anti-C4 antibody (Quidel) for hC4 at 4 °C. All washes were done with 0.05% Tween 20 in PBS, repeated three times. Plates were then blocked with 1% BSA in PBS for 1 h at 37 °C. Samples were applied in a twofold dilution series, starting at 1:2,000 dilution in PBS for hC4 transgenic mouse serum, 1:1,000 for C57BL/6J mouse serum, and incubated for 2 h at 37 °C. Purified hC4 (Complement Technology) or mC4 (provided by G. R. Andersen, Aarhus, Denmark) was used as standards starting at 100 ng ml−1 and 500 ng ml−1, respectively, followed by twofold dilutions. C4 in samples was detected with rabbit anti-C4-c antibody (Dako), followed by goat anti-rabbit horseradish peroxidase, both incubated at 1 h at 37 °C. The signal was developed by the addition of TMB substrate, and the reaction was stopped with 2 M H2SO4. Absorbance at 450 nm was measured, and absolute quantification was measured based on a standard curve.

To detect C3 levels, plates were coated with goat anti-C3 and detected by rabbit anti-C3 (Abcam). As standard, purified mouse C3 (Complement Technology, M113) was used, starting at 50 ng ml−1 followed by twofold dilutions. C1q levels were measured by coating with rat anti-C1q (Abcam) and detecting by rabbit anti-C1q (Abcam). As standard, purified mouse C1q (Complement Technology, M099) was used, starting at 50 ng ml−1 followed by twofold dilutions. For both proteins, ELISA was performed with the same protocol described above.

Tissue ELISA. To measure C4 protein levels in the CNS, hC4 transgenic mice were transcardially perfused with PBS and brains were collected as fresh-frozen. The forebrain was cryosectioned as 100-µm sections and collected. Total protein from tissue was extracted using N-PER neuronal extraction buffer (Thermo Fisher) containing 1x protease inhibitor cocktail (Pierce, A32965) according to the manufacturer’s protocol. Total protein (200 µg determined using a BCA kit) was loaded as the starting volume and followed by twofold dilutions. C4 amount was detected as described above. Results are plotted as C4 amount (ng) in 100 µg total protein.

Immunochemistry. Brains were collected from mice after transcardial perfusion with PBS followed by 4% paraformaldehyde (PFA). Tissue was then immersed in 4% PFA overnight following perfusion, cryoprotected in 30% sucrose, embedded in OCT and frozen. Tissue was cryosectioned (14 µm), sections were dried, washed three times in PBS and blocked with 5% BSA and 0.2% Triton X-100 in PBS for 1 h. Primary antibodies were diluted in the antibody buffer (containing 5% BSA and 0.2% Triton X-100 and 5% PBS). Details of all the antibodies used are provided in Supplementary Table 3. The antibodies were incubated overnight at 4 °C, and secondary Alexa-conjugated antibodies (Invitrogen) were added to 1:200 in the antibody buffer for 2 h at room temperature. Slides were mounted in Vectashield (containing DAPI). Spleen sections (14 µm) were fixed with acetone, blocked with 10% goat serum and 0.1% Triton X-100 in PBS for 1 h. Details of all the antibodies used are provided in Supplementary Table 3. Primary antibodies were diluted in the antibody buffer (containing 10% goat serum and 0.1% Triton X-100). The antibodies were incubated for 2 h at room temperature, and secondary Alexa-conjugated antibodies (Invitrogen) were added to 1:200 in the antibody buffer for 2 h at room temperature. Slides were mounted in Vectashield (containing DAPI).

All sections were imaged with an Olympus IX 81 microscope with a Fluoview FV1000 confocal system at Boston Children’s Hospital (Program in Cellular and Molecular Medicine Microscopy Core). All images from confocal microscopy were processed using Fiji (ImageJ, NIH) unless otherwise specified.
Eye-specific segregation analysis. Visualization and analysis of RGC synaptic inputs in the mouse dLGN was performed as previously described23, CTB conjugated to Alexa 488 (green label) and CTB conjugated to Alexa 594 (red label; Sigma-Aldrich; Cat. No. A21032) were intraretinally injected into the right eye, respectively, of P9 mice, which were killed the following day. Images were acquired using a Zeiss Axioimager and quantified blinded to experimental conditions and compared to age-matched littermate controls. The percentage of overlap was measured as previously described19,29.

Synapse density analysis. FC sections were stained with pre- and post-synaptic markers (SV2 and Homer-1, respectively). Details of all the antibodies used are provided in Supplementary Table 3. Sections were imaged using an Olympus FV 1000 confocal microscope at x60 magnification and x2 optical zoom. Z-stacks were taken 0.5-μm above and below the midplane with 0.2-μm increments. Four fields of FOV were imaged per animal from two separate tissue sections. Images were analyzed using Fiji software. Projection images were subjected to background subtraction with a rolling ball radius of 10 pixels. To identify SV2+ and Homer-1+ puncta, each image was thresholded according to the software’s auto function, followed by the Analyze particles function. Colocalized puncta were identified by the image calculator “AND” function for the SV2+ and Homer-1+ images, with a lower limit of 15 pixels for size.

To compare WT and transgenic mice groups, the common genotype from each litter (Ct+ group) was used as the baseline. C4-deficient animals from both WT and hC4A litters were used to normalize data points for their respective litters, allowing us to represent our data as relative changes in synapse density in WT; hC4A- and hC4A/A groups with respect to their Ct− littersmates.

Synaptic C4 deposition quantification. dLGN sections from P5 mice were stained for the pre-synaptic marker Vglut2 and for hC4. Details for all the antibodies used were taken 0.5-μm above and below the midplane with 1-μm increments. Ten FOVs were taken 0.5-μm above and below the midplane with 0.2-μm intervals. The sections were imaged using the same method as for synapse density analysis. Vglut2-stained areas were recorded for further analysis. The groups were normalized to the number of hC4 gene copies: four copies for hC4A− and two copies for hC4B−.

Spleenic C4 and C3 deposition quantification. Spleen sections were stained for CD169, CD21 and C4 or C3. Sections were imaged using an Olympus FV 1000 confocal microscope at x20 magnification. Z-stacks were taken 5-μm above and below the midplane with 1-μm increments. Four FOVs were imaged per animal from two separate spleen sections. Z-projection images were analyzed using Cell Profiler (Broad Institute of Harvard and MIT, v4.0.2.2). The follicular dendritic cell (FDC) networks in each FOV were identified and used as a mask to identify C4 or C3 puncta. The fluorescence intensity measurements were normalized by the FDC area.

Golgii–Cox staining and dendritic spine density analysis. Brains were collected from P180 transgenic mice and processed according to the protocol provided with the FD Rapid Golgi Stain kit (FD Neurotechnologies) for the Golgii–Cox staining method. The brains were then sectioned (100 μm per slice) using a cryostat. Sections were collected on gelatin-coated microscope slides, dehydrated in absolute alcohol, cleared in xylene and cover slipped. The stained pyramidal neurons were captured using cellSens software (Olympus). Images at ×4 magnification were used for mapping the region of interest and ×20 images were used for selecting neurons (Extended Data Fig. 9). To be selected for analysis, Golgi-impregnated neurons had to meet the following characteristics: (1) dark and consistent impregnation throughout the extent of all of the dendrites; (2) relative isolation from neighboring impregnated cells that could interfere with analysis; and (3) a cell body in the middle third of the tissue section to avoid analysis of impregnated neurons that extend largely into other sections. Images at ×100 magnification were used for dendritic spine and dendritic arbor evaluated by the number of primary dendrites and the number of dendritic spines per 10 μm of dendrite length. Dendritic spines were manually counted by an experimenter blinded to the mouse genotype, regardless of shape. The density of dendritic spines was calculated as the number of spines per 10 μm of dendrite. All image processing was carried out using Fiji (Image J, NIH).

Microglia isolation. All procedures were done at 4 °C using precooled reagents. Brains were removed from the cranium into HEPES-buffered sucrose (0.32 M sucrose, 5 mM HEPES (pH 7.4) containing protease inhibitor cocktail (Roche mini complete tablets) and homogenized in a glass dounce. The homogenate was centrifuged at 1,200 × g at 4 °C for 10 min and the resulting supernatant was spun at 15,000 × g for 15 min to yield the crude synaptosomal pellet. The pellet was resuspended in 1 ml of HEPES-buffered sucrose and layered on top of a discontinuous gradient containing 5 mM HEPES and 0.8 to 1.0 to 1.2 M sucrose (top to bottom, which equals 27%/34%/41%) in an ultracentrifuge tube then centrifuged at −150,000 × g for 2 h in a swinging bucket rotor. Synaptosomes were recovered in the layer between 1.0 and 1.2 M sucrose.

Complement binding assay. Synaptosomes were incubated with 10% fresh serum from C4−/−, hC4B−/− or hC4A−/− mice (diluted in half with C4−/− serum) for 30 min at 37 °C in gelatin veronal buffer (Complement Technology) then washed three times in PBS. Synaptosomes were fixed and permeabilized (fixation and permeabilization buffers from BioLegend) before antibody staining with anti-sv (DSHB/Ulwowa). For C4 detection, synaptosomes were stained with polyclonal rabbit anti-C4 (Dako) and acquired using a FACS Canto (BD Biosciences), and data were analyzed using FlowJo (Tree Star, v10.4) software.

Hemolytic assay. EA sheep red blood cells (Complement Technology) were washed with gelatin veronal buffer (Complement Technology) and counted to a spectrophotometer at 540 nm. To obtain 106 cells per ml, the following formula was used: final volume = initial volume × OD540/0.62. Cells were then diluted to 106 cells per ml, incubated with 2% C4-deficient guinea pig serum mixed with serial dilutions of fresh serum from hC4A−/− and hC4B−/− mice for 30 min at 37 °C. Absorbance was read at 415 nm. The percentage of hemolysis was measured based on 100% lysis control. In parallel, C4 concentration was measured by ELISA for each sample, and hemolytic activity per g of C4 was calculated and normalized to hC4A samples.

Western blotting. Equal amounts of proteins (20 μg as determined using a BCA protein assay) were diluted 1:1 with 2× Laemmli buffer with 1 mM dithiothreitol. Samples were heated at 95 °C for 5 min, except for the samples used for SV2 detection. Protein electrophoresis was performed using a Mini-Trans-blot cell (Bio-Rad). Proteins were then transferred onto polyvinylidene difluoride membranes (Immun-BlotPVDF, Bio-Rad) for western blot analysis. Membranes were blocked in a 5% milk solution in TBS-T, 0.1% Tween 20 and then incubated with anti-SV2 (DSHB/Ulwowa), anti-Homer-1 (Synaptic Systems) and anti-GAPDH (BioLegend) primary antibody overnight at 4 °C. Following washes in TBS-T, secondary antibody conjugated with horseradish peroxidase (Southern Biotech) was hybridized for 1 h at room temperature. Membranes were washed in TBS-T again and then reactivity was revealed via a chemiluminescence reaction performed with ECL detection reagents (Pierce) and film exposure.

Microglia engulfment analysis in the mPFC. We immunostained 40-μm sagittal sections containing the regions of interest of the brain for Iba1 (1:400, Wako) and CD68 (1:300, BioLegend). A confocal microscope was used to collect four ×40 FOVs per mouse with 1-μm interval Z-steps. The analysis was performed on maximum-intensity projections of the 40-μm section using the software Cell Profiler (Broad Institute of Harvard and MIT, v4.0.2.2). The microglial soma and processes were mapped out based on Iba1 signature, and the microglia identified were used as a mask for identifying intracellular CD68 puncta. The engulfment processes were mapped out based on Iba1 signature, and the microglia identified were used as a mask for identifying intracellular CD68 puncta. The engulfment processes were mapped out based on Iba1 signature, and the microglia identified were used as a mask for identifying intracellular CD68 puncta. The engulfment processes were mapped out based on Iba1 signature, and the microglia identified were used as a mask for identifying intracellular CD68 puncta.

Microglia engulfment analysis in the dLGN. The in vivo microglia phagocytosis assay and analyses were performed as previously described in detail24,25. Briefly, mice received an intraocular injection of fluorescent-conjugated anterograde tracer CTB (Supplementary Table 4) at P4. Animals were killed at P5 and brains were fixed overnight in 4% PFA at 4 °C, followed by cryoprotection with 30% sucrose. We prepared 40-μm sections from only those brains with sufficient dye fill. For each condition, three to four adjacent dLGN sections were imaged for reconstruction of RGC inputs, Iba1 staining and subsequent engulfment analysis. Images were acquired using a confocal microscope at x60 magnification with 0.2-μm interval Z-steps. Three to five FOVs were collected from each section (10−15 FOVs per animal). Images were processed using ImageJ (NIH) and analyzed using Imaris software (Bitplane).
Bulk RNA-sequencing analysis. Microglia were first sorted from FC homogenates using a FACS Aria. For each mouse, 1,000 microglia were sorted based on CD11b$^+$ and CD45$^+$ expression into 20 μl guanidine thiocyanate buffer (Qagen) supplemented with 1% Triton-X and 0.5% sodium dodecyl sulfate (SDS). RNA was extracted using the Qiagen Trizol kit. Libraries were prepared using the TruSeq Library Prep kit (Illumina). Libraries were quality checked using a Bioanalyzer 2100 (Agilent) using an Agilent High Sensitivity DNA kit. Library quantitation was performed using a Qubit fluorimeter. Paired-end sequencing (101 bp, 2x) was performed using the Illumina HiSeq 2500 system.

Behavior analyses. All behavioral analyses were performed in a Mouse Behavior Core of Harvard Medical School and Brigham and Women's Hospital. Before each test, mice were allowed to acclimate in their housing cages in a separate room for 30 min.

Three-chamber social-interaction test. The three-chamber sociability test included two consecutive 10-min sessions and was performed in a rectangular arena constructed of clear Plexiglas that was 63 cm long × 40 cm wide × 22 cm high. The test arena contained three equally sized chambers (40 cm long × 20 cm wide × 22-cm high), and the outer left and right chambers each contained an inverted wire cup. During the habituation session, test mice were initially placed into the center chamber and allowed to freely explore the three-chambered arena. For the social-interaction session, test mice were returned to the center chamber and an unfamiliar target mouse was placed under one of the two wire cups, an object was put under the second cup, and test mice were again allowed to move freely among the three chambers. The position of the first target mouse was alternated and equally distributed between the left and right cups for both transgenic and wild-type mice. The test session began with a habituation block of six presentations of the target stimulus alone, followed by ten PPI blocks of six different types of trials. The following trial types were used: null (no stimulus), startle (120 dB), startle plus prepulse (variable dB over a background noise of 65, 75, or 85 dB) and prepulse alone. Each trial began with a 50-ms null period during which baseline movements were recorded. There was a subsequent 20-ms period during which prepulse stimuli were presented and responses to the prepulse measured. After a further 100 ms, the prepulse was turned off and the full startle stimulus was applied for 100 ms. Responses were sampled every ms. The inter-trial interval varied, with an average of 15 s (range from 10 to 20 s). The percentage PPI = (1− (prepulse trials/startle-only trials)) × 100.

Rotated test. The purpose of the rotated test is to assess the sensorimotor coordination and/or fatigue resistance of the mouse. The test is sensitive to damage of the basal ganglia and cerebellum, and to drugs that affect motor functions. The rotated apparatus consisted of a gridded or textured plastic roller (Ugo Basile) flanked by large round plates on each end to prevent the animal from escaping. The plastic rod sat at a height of approximately 20 cm above individual electronic sensing platforms. The platform started with the platform removed, and mice were not allowed to experience any discomfort from falling from that height. Before actual testing, mice were habituated to the rotated apparatus by placing them onto the rod as it revolved at a very low rotation speed (4 r.p.m. for 5 min) (the number of times a mouse falls was recorded and the mouse was placed back on the rotated if it falls). Following the habituation session, mice were tested in the rotated arena (4–40 r.p.m. in 3 min). The latency until the mouse falls from the rod onto the sensing platform below was recorded automatically. This test was repeated after a 15–min break.

Tail-suspension test. This procedure is an alternative to the forced-swim test and is used to induce a despair-like state and to test the effects of antidepressants in mice. Antidepressants will reduce the amount of time the mouse spends immobile in this test. At the beginning of a trial, mice were suspended by the tail (taped onto a suspension hook so that the animal hangs with its tail in a straight line) above a flat surface covered with soft padding material. During testing (10-min trial), the duration of immobility was automatically scored (Med Associates) or by an experimenter.

Forced-swim test. This procedure is used to induce a despair-like state and to test the effects of antidepressants in mice. Antidepressants will reduce the amount of time the mouse spends immobile in this test. Mice were placed for 6 min in a glass cylinder (height, 35 cm; diameter, 17 cm) filled with water (25 ± 2°C) to a depth of 25 cm. The water depth was adjusted so that the animals must swim or float without their hind limbs or tail touching the bottom. During testing, the duration of immobility (the time during which the subject makes only the small movements necessary to keep their heads above water) was scored. The experimenter monitored each mouse continuously during the entire session. The mouse was immediately taken out of the water and excluded from the test if it failed to swim or keep its head above water. After completion of the trial, the subject was dried using a paper towel and placed back in its home cage. After every trial, the water was changed, and the cylinder rinsed with clean water.

Statistical analysis and study design. All data in dot plots are presented as the mean ± s.e.m. All box-and-whisker plots display the median (center line), the 25th to 75th percentile (box), and the minimum to maximum values (whiskers). To determine the distribution of datasets, a D’Agostino–Pearson normality test was performed in all analyses. For data that passed the normality test, a two-tailed parametric unpaired t-test was used; for the datasets that were not normally distributed, a non-parametric Mann–Whitney test was used. For multiple tests, a Bonferroni correction was used (for Kruskal–Wallis test for non-normally spread data followed by Dunn’s multiple comparisons tests) and two-way analysis of variance (ANOVA) tests were followed by Tukey’s multiple comparisons and Sidak’s multiple comparisons tests, respectively. Details of statistical analyses are provided in Supplementary Table 5. All statistical analyses were carried out using GraphPad Prism 8 and Microsoft Excel.
Experiments were repeated with two to three independent cohorts, and the obtained results were replicable. Details of the number of independent experiments are provided in the figure legends. Investigators involved in conducting experiments, collecting data and performing analyses were blinded to the mice genotypes. No statistical method was used to predetermine sample sizes. However, our sample sizes were similar to those reported in previous publications. All mice in the study were randomly allocated in experimental groups. Randomization protocols in behavior experiments are further explained in their respective subsections. Additional specifics regarding study design and methods are provided in the Nature Research Reporting Summary.

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

**Data availability**
The bulk RNA-sequencing data have been uploaded to the NCBI GEO under accession code GSE161557. Source data for Extended Data Figs. 1a and 9c are provided in Supplementary Fig. 1. The datasets generated and analyzed during the current study, along with the detailed protocols and codes for the experiments, are available upon request from the corresponding author.

**Code availability**
The study utilized simple codes commonly used for bulk RNA sequencing. All scripts and codes are available upon request from the corresponding author.

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**Acknowledgements**
We thank members of the Carroll, Stevens and McCarroll laboratories for helpful comments on experimental design and feedback on the manuscript; E. M. Carroll for invaluable assistance; G. R. Andersen for providing the purified mouse C4 protein; X. Chen, D. Ghitza and L. Prince-Wright for technical support; B. Caldarone at the Mouse Behavior Core of Harvard Medical School and Brigham and Women's Hospital; H. Leung at the Boston Children's Hospital/PCMM microscopy core; the Boston Children's Hospital/PCMM flow cytometry core; K. Holton at the HMS research computing core; and C. Usher and D. E. Potts for help with editing the manuscript. Parts of Figs. 2a and Fig. 3 were drawn by using pictures from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/). Parts of Figs. 1a, 3a, 4a, 5a and 6c were created with BioRender (https://biorender.com/). This research was supported by funding from NIH P50MH112491-01 (to B.S., S.A.M. and M.C.C.) and AR072965 (to M.C.C.).

**Author contributions**
M.Y., E.Y., J.P. and M.C.C. designed all the experiments. J.P. performed the BAC microinjections and created the human transgenic mice. M.Y. and J.P. conducted the splenic complement deposition, eye-specific segregation, microglia engulfment and behavior experiments and analyzed the corresponding data. M.Y. performed and analyzed the dendritic spine density analysis and the RNA sequencing. E.Y. performed and analyzed the synapse density analyses, the microglia reconstruction/engulfment assays and the C1q, C3 and C4 ELISAs for serum and tissue. E.A. assisted with the Cell Profiler analysis, the RNA-sequencing analysis and editing the manuscript. M.M. assisted in the generation of the human transgenic mice and the serum and tissue ELISAs. C.W. performed the whole-genome sequencing analysis. M.Y., E.Y. and J.P. wrote the manuscript. B.S. and S.A.M. provided expertise and comments on the experiments and the manuscript. M.C.C. conceived the development of the human transgenic mice and advised on the design of experiments, participated in the analysis of the results and in writing the manuscript.

**Competing interests**
B.S. serves on the scientific advisory board of Annexon LLC and is a minor shareholder of Annexon LLC; however, this is unrelated to the submitted work. All other authors declare no competing interests.

**Additional information**
Extended data is available for this paper at https://doi.org/10.1038/s41593-020-00763-8. Supplementary information is available for this paper at https://doi.org/10.1038/s41593-020-00763-8.

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**Peer review information** *Nature Neuroscience* thanks Marie-Eve Tremblay, Mischuake Yuzaki, and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Extended Data Fig. 1 | Characteristics of C4 BAC DNA integration. 

a–d. Insertion sites and associated rearrangements are shown for each mouse strain. 
a–c, The BAC DNA insertion site is shown for each mouse strain. Normalized read depth in 2-kb genomic windows at the insertion sites of the BACs. Dashed vertical lines indicate the insertion site. The hC4A insertion was associated with a duplication, and the hC4B insertion was associated with a small deletion. 
b–d, The BAC DNA-associated rearrangements are shown for each mouse strain. Normalized read depth in 1-kb genomic windows shows the copy number of the inserted constructs. Gene models are shown beneath the read depth plots. Human C4A was likely inserted into hC4A transgenic mice at a normalized read depth between 5 and 9. Human C4B was likely inserted into hC4B transgenic mice at a copy number between 2 and 5. The inserted hC4B BAC constructs contain additional internal copy number variants. 
e, Pulse-field gel showing linearized hC4B and hC4A BAC DNA used for microinjection into zygotes (representative of 3 independent experiments). Unprocessed versions of the gels can be found in Supplementary Figure 1a. 
f, Sema6D mRNA expression level in cortex was not changed by the region duplication caused by hC4A BAC DNA insertion. Gapdh was used as a control housekeeping gene (n = 4 mice per group; ns = P > 0.05, Kruskal–Wallis test with Dunn’s multiple comparisons test). Bar graph shows mean ± s.e.m.
Extended Data Fig. 2 | Peripheral expression and function of C4 in hC4 transgenic mice. a, C4A- and C4B-specific mRNA level was measured by ddPCR in the spleen (left) and liver (right) of hC4A/- and hC4B/- mice. eiF4H was used as the control housekeeping gene (n = 4 mice per group; * P_{spleen} = 0.0286, * P_{liver} = 0.0286; two-tailed Mann-Whitney test). b, C4 hemolytic activity was measured with equal amounts of hC4A or hC4B protein using sensitized sheep red blood cells. Data were normalized to hC4A samples (n = 8 hC4A and n = 15 hC4B combined from 3 independent experiments; **** P < 0.0001, Unpaired, two-tailed t test). Bar graphs show mean ± s.e.m.
Extended Data Fig. 3 | Complement activation and binding to synaptosomes. a, Representative dot plots showing the FSC-A / SSC-A of 1-µm beads, 6-µm beads, and synaptosomes. Further analysis will be gated on 1-µm synaptosomes. b, Synaptosomes were permeabilized and stained with anti-SV2 antibody (+ SV2 Ab) or no antibody (FMO CT). More than 85% of the particles analyzed contain SV2 protein. c, Representative histogram plot of C4 staining on synaptosomes isolated from C4-/- mice and incubated with serum from C4-/- (red), hC4A (orange), and hC4B (blue) mice. d, C1q deposition is shown and quantified using serum from hC4A mice (orange; n = 2), hC4B mice (blue; n = 2), or no serum CT (red). e, C4 (left) and C1q (right) deposition was detected on synaptosomes using fresh (red) or heat-inactivated (blue) serum. Bar graph shows mean ± s.e.m.
Extended Data Fig. 4 | Human C4 in the retinogeniculate system. a, hC4AB/- mice: gene copy numbers for C4A, C4B, C4A-L (C4L), and C4B-S (C4S) were determined by ddPCR using Rpp30 as a reference gene. It showed an insertion of two C4A-L genes and one C4B-L gene (n = 5 representative mice). Whole-genome sequencing revealed the hC4AB BAC DNA was inserted in chromosome 3 and that one C4A copy was truncated resulting in only one C4A coding copy (data not shown). b–c, Absolute quantification by ddPCR was used to measure C4A- and C4B-specific mRNA level in the retina and LGN from P5 hC4A/- (n = 3), hC4B/- (n = 3), and hC4AB/- mice (n = 2). Bar graphs show mean ± s.e.m.
Extended Data Fig. 5 | Complement profile in hC4 transgenic mice during adolescence (P40). a–c, Level of classical complement cascade proteins were measured in serum by ELISA in adolescent (P40) WT, hC4A/-, and hC4A/A mice. a, C4 serum level was measured in WT (n = 8), hC4A/- (n = 10), and hC4A/A mice (n = 6). b, C3 serum level was measured in WT (n = 5), hC4A/- (n = 10), and hC4A/A mice (n = 6). c, C1q serum level was measured in WT (n = 5), hC4A/- (n = 9), and hC4A/A mice (n = 8). d–f, RNA expression of classical complement cascade components in the FC were measured by ddPCR in adolescent (P40) WT, hC4A/-, and hC4A/A mice. All RNA measurements were normalized to Hs2st1 expression level. d, C4 mRNA expression level in FC was measured in WT (n = 4), hC4A/- (n = 4), and hC4A/A mice (n = 2). e, C3 mRNA expression level in FC was measured in WT (n = 4), hC4A/- (n = 3), and hC4A/A mice (n = 2). f, C1qb mRNA expression level in FC was measured in WT (n = 4), hC4A/- (n = 4), and hC4A/A mice (n = 2). Bar graphs show mean ± s.e.m.
Extended Data Fig. 6 | C4A overexpression doesn’t change LGN cellularity or morphology. a, Representative images of contralateral (top), ipsilateral (middle), and overlapping regions (bottom; scale = 20 µm). b–d, dLGN size (b), contralateral area (c), and ipsilateral area (d) were measured in P10 hC4A/- and hC4A/ mice (n = 6 hC4A/- and n = 6 hC4A/ littermates from 3 independent cohorts; two-tailed Mann-Whitney test; ns = P > 0.05, * P = 0.0411; scale = 20 µm). e, Iba1 staining was used to count the total number of microglia in the dLGN FOV (n = 4 mice per group; two-tailed Mann-Whitney test; ns = P > 0.05). f, Mice were injected with fluorescent-conjugated cholera toxin subunit-B (CTB) in each eye at P4 and brains were harvested at P5. Representative pseudo-colored images of P5 dLGN from C4-/-, hC4A/-, and hC4A/ littermates of contralateral (red), ipsilateral territories (green), and overlap (yellow) between the two territories (scale = 20 µm). Percent of overlap in ipsilateral region was compared in C4-/- (n = 3), hC4A/- (n = 4), and hC4A/ littermates (n = 2; littermates). g, DAPI was used to count the total number of cells in the dLGN field of view (FOV) (n = 6 hC4A/- and n = 5 hC4A/ littermates from 3 independent cohorts; two-tailed Mann-Whitney test; ns = P > 0.05). h, The number of Brn3a+ RGCs was compared between C4-/-, hC4A/-, and hC4A/ mice (n = 5 mice per group; Kruskal-Wallis test with Dunn’s multiple comparisons test; ns = P > 0.05; scale = 20 µm). Bar graphs show mean ± s.e.m.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Cell Profiler microglia morphological analysis. 

a, Cell Profiler software was used to analyze microglia morphology and lysosomal activity in the frontal cortex of P40 hC4A/- and hC4A/A mice. Microglial soma and processes are identified by Iba1 signature by using the image-based watershed method (top row). Microglia are used as a mask to select and quantify intracellular CD68 puncta (bottom row; scale = 50 μm).

b-h, Morphological parameters for mPFC microglia (b-f) and their soma (g-h) from hC4A/- (n = 4) and hC4A/A (n = 5) mice were calculated by Cell Profiler software at P40 timepoint (ns = P > 0.05, two-tailed Mann-Whitney test). Bar graphs show mean ± s.e.m.
Extended Data Fig. 8 | Microglial RNA sequencing analysis reveals no transcriptomic alterations in hC4 transgenic mice. **a**, Bulk RNA sequencing analysis of microglia isolated from the frontal cortex of adolescent (P40) mice from WT (n = 4), C4-/- (n = 5), and hC4A/- (n = 5), hC4B/- (n = 4), and hC4A/A (n = 2) groups. Heatmap representation of differentially expressed genes between all experimental groups shows no significant transcriptional profile difference between any two groups. **b-c**, Normalized gene counts for the TREM2/DAP12 signaling pathway (b) and TAM receptor genes (c) were calculated for WT (n = 4), C4-/- (n = 5), hC4A/- (n = 5), hC4B/- (n = 4), and hC4A/A (n = 2; ns = P > 0.05, Kruskal-Wallis test with Dunn’s test). Bar graphs show mean ± s.e.m.
Extended Data Fig. 9 | Synaptic protein expression is not affected by C4A overexpression in mice. a–b, rt-PCR was used to measure mRNA expression of Sv2a (a) and Pds95 (b) in the FC in adult mice (C4-/- n = 2, hC4A/- n = 3, and hC4A/A littermates n = 7). RNA expression was normalized to Gapdh expression. c, SV2 and PSD95 protein level were analyzed by western blot. GAPDH was used as a loading control protein (C4-/- n = 4, hC4A/- n = 4, and hC4A/A n = 4 littermates from one experiment; Kruskal-Wallis test with Dunn’s multiple comparisons test; ns = P > 0.05). Unprocessed versions of the western blots can be found in Supplementary Figure 1b. d, Total SV2 area per FOV in the mPFC was calculated from immunofluorescence staining between WT (n = 3), C4-/- (n = 11), hC4A/- (n = 13), and hC4A/A (n = 6) groups (Kruskal-Wallis test with Dunn’s multiple comparisons test). e–f, Total Homer1 puncta and percentage of colocalized Homer1 puncta from the mPFC in adult mice were calculated for WT (n = 3), C4-/- (n = 11), hC4A/- (n = 13), and hC4A/A (n = 6) groups (Kruskal-Wallis test with Dunn’s multiple comparisons test; ns = P > 0.05, * PWT vs A/A = 0.0139, ** PWT vs A/A = 0.0139). g–h, Brain sections were stained with DAPI and NeuN, and cellularity was measured in frontal cortex at P180. Total cells (g) and neurons (h) per FOV in frontal cortex is represented for hC4A/- (n = 3) and hC4A/A mice (n = 4; two-tailed Mann-Whitney test; ns = P > 0.05). i, Method used for the manual counting of dendritic spines in mPFC of hC4 mice. j, Length of dendrites that have been used for spine density analysis for hC4A/- (80 dendrites) and hC4A/A (96 dendrites; two-tailed Mann-Whitney test; ns = P > 0.05). Bar graphs show mean ± s.e.m.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Human-C4A overexpression alters mouse behavior. a–h, WT, C4-/-, hC4A/-, and hC4A/A mice were subjected to a battery of behavioral tests. a–b, Weight of male and female mice were compared between WT (n = 10), C4-/- (n = 4), hC4A/- (n = 9), and hC4A/A mice (n = 8; Kruskal-Wallis test with Dunn’s multiple comparisons test; ns = P > 0.05). c, Anxiety levels were measured in the light-dark box test by time spent in the light-zone between WT (n = 8), C4-/- (n = 22), hC4A/- (n = 20), and hC4A/A (n = 12) groups (two-tailed, unpaired t test; ** P = 0.0085; two-tailed Mann-Whitney test, *** P = 0.0009). Results were normalized to the C4-/- group to retain littermate controls. d, In the rotarod test, latency to fall was measured in seconds for WT (n = 10), C4-/- (n = 7), hC4A/- (n = 8), and hC4A/A (n = 7) groups (Kruskal-Wallis test with Dunn’s multiple comparisons test, ns = P > 0.05). e–f, Immobility time in seconds was compared in the tail suspension test (e) and the forced swim test (f) for WT (n = 10), C4-/- (n = 7), hC4A/- (n = 8), and hC4A/A (n = 7) groups (Kruskal-Wallis test with Dunn’s multiple comparisons test, ns = P > 0.05). g, Prepulse inhibition was measured between WT (n = 10), C4-/- (n = 7), hC4A/- (n = 8), and hC4A/A (n = 7) groups (two-way ANOVA with Tukey’s multiple comparisons test; ns = P > 0.05). h, Percent of correct decisions were recorded in the water t maze and the reversal water t maze for WT (n = 10), C4-/- (n = 7), hC4A/- (n = 8), and hC4A/A (n = 7) groups (two-way ANOVA with Tukey’s multiple comparisons test; ns = P > 0.05). Bar graphs show mean ± s.e.m. Box-and-whisker plots display the median (center line), 25th to 75th percentile (box), and minimum to maximum values (whiskers).
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Software and code

Policy information about availability of computer code

Data collection

Droplet digital PCR data were collected using QuantaSoft software (Bio-Rad, version 1.7.4.0917). Confocal microscopy and bright-field microscopy images were collected using Fluoview (Olympus, FV1000) and cellSens (Olympus, version 1.7).

Flow cytometry data and cell sorting were collected using FACSDiva (BD Biosciences, version 8.0).

RNA sequencing data were collected using Illumina sequencing software (Illumina, version 2.6).

Mouse behavior data were collected using TopScan software (CleverSys, Inc.), EthoVision XT software (Noldus, version 14), and Activity Monitor software (Med Associates Inc., version 7).

Data analysis

Droplet digital PCR data were analyzed using QuantaSoft software (Bio-Rad, version 1.7.4.0917).

Confocal microscopy and bright-field microscopy images were analyzed using Fiji (Image J, NIH, version 2.0.0-rc-69), Imaris software (Bitplane, version 9.5.1) or Cell Profiler (Broad Institute of Harvard and MIT, version 4.0.2).

Flow cytometry data were analyzed using FlowJo (Tree Star, Inc., version 10.4).

Whole genome sequencing data were analyzed using BWA MEM (version 0.7.12; arXiv:1303.3997 [q-bio.GN]), Picard Mark Duplicates (http://broadinstitute.github.io/picard/), DELLY (version 0.7.6, default parameters), Manta (version 1.0.3, default parameters), and Genome STRIP (version 1.0.9).

RNA sequencing data were analyzed using STAR aligner (Dobin et al, Bioinformatics, 2013; version 2.7.0), EdgeR (Bioconductor, version 3.12), R and RStudio (The R Foundation, version number 4.0.3, and 1.3.1609).

Behavior studies data were analyzed using TopScan software (CleverSys, Inc.), EthoVision XT software (Noldus, version 14), and Activity Monitor software (Med Associates Inc., version 7).

Statistical data analysis was performed with Microsoft Excel (version 16.41) and GraphPad Prism 8 (version 8.4.2).

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Source data for Ext. Figs. 1a and 9c have been provided in Supplementary Figure 1. The bulk RNA-sequencing data has been uploaded to NCBI GEO under the accession code GSE161557. The datasets generated and analyzed during the current study, along with the detailed protocols and codes for the experiments are available upon request from the corresponding author.

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

- **Sample size**
  - No statistical method was used to pre-determine sample sizes. However, our sample sizes are similar to those reported in previous publications (Sekar et al, Nature (2016); Stevens et al, Cell (2007); Schafer et al, Neuron (2012); Koyama et al, Front Neuroanat (2015).

- **Data exclusions**
  - Mice that showed a state of hydrocephalus were excluded from the study. No data points were excluded from any analyses.

- **Replication**
  - Experiments were repeated with 2-3 independent cohorts and the obtained results were replicable.

- **Randomization**
  - All mice from this study were randomly allocated in experimental groups.

- **Blinding**
  - Investigators involved in conducting experiments, collecting data, and performing analyses were blinded to the mice genotypes.

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### Materials & experimental systems

- **n/a** Involved in the study

- **Antibodies**
  - Involved in the study

- **Eukaryotic cell lines**

- **Palaeontology and archaeology**

- **Animals and other organisms**

- **Human research participants**

- **Clinical data**

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### Methods

- **n/a** Involved in the study

- **ChIP-seq**

- **Flow cytometry**

- **MRI-based neuroimaging**

### Antibodies

Antibodies used in the study are provided blow in the format (Host species α- target protein; [Clone - when applicable]; (Conjugation - when applicable); Manufacturer; Catalog #: Lot #: Use in study (dilution)):

- Rabbit α-hC4c; Dako; F0169; 20030271; IHC (1:500), ELISA (1:1000), FACS (1:200)
- Goat antiserum to hC4; Quidel; A305; 136829; ELISA (1:1000)
- Goat α-mC3; MP Biomedicals; 0855463; 07503; ELISA (1:1000)
- Rabbit α-mC3; Abcam; Ab11887; GR3333817-4; ELISA (1:1000)
- Rabbit α-hC3d; Dako; A0063; 00005390; IHC (1:500)
- Rat α-C1q; [7H8]; Abcam; Ab11861; GR3284291; ELISA (1:500), FACS (1:200)
- Mouse α-NeuN; [A60]; Millipore; MAB377; 2967854; IHC (1:300)
- Mouse α-SV2; Developmental Studies Hybridoma Bank, U of Iowa; AB2315387; IHC (1:1000), WB (1:1000), FACS (1:1000)
Validation

All antibodies used in the study was validated by previously published studies, the manufacturers, or in this paper by the use of appropriate positive and negative controls with genetically modified mice.

Rabbit α-hC4c; Dako; This paper (Figs 1f and 2c).

Goat antiserum to hC4; Quidel; By manufacturer: https://www.quidel.com/research/polyclonal-antisera/polyclonal-antiserum-to-human-c4-protein

Goat α-mC3; MP Biomedicals; By manufacturer: https://www.mpbio.com/us/0855463-goat-igg-fraction-to-mouse-complement-c3

Rabbit α-C1q; Abcam; By manufacturer: https://www.abcam.com/c1q-antibody-7h8-ab11861.html

Mouse α-NeuN; [A60]; Millipore; Lehrman, E. K. et al. CD47 Protects Synapses from Excess Microglia-Mediated Pruning during Development. Neuron 100, 120-134.e126, doi:10.1016/j.neuron.2018.09.017 (2018).

Mouse α-SV2; Developmental Studies Hybridoma Bank, U of Iowa; Feany, M. B., Lee, S., Edwards, R. H. & Buckley, K. M. The synaptic vesicle protein SV2 is a novel type of transmembrane transporter. Cell 70, 861–867, doi:10.1016/0092-8674(92)90319-8 (1992).

Rabbit α-Homer1; Synaptic Systems; Lehrman, E. K. et al. CD47 Protects Synapses from Excess Microglia-Mediated Pruning during Development. Neuron 100, 120-134.e126, doi:10.1016/j.neuron.2018.09.017 (2018).

Rabbit α-Iba1; Wako; Lehrman, E. K. et al. CD47 Protects Synapses from Excess Microglia-Mediated Pruning during Development. Neuron 100, 120-134.e126, doi:10.1016/j.neuron.2018.09.017 (2018).

Rat α-CD68; (AF488 Conj); BioLegend; By manufacturer: https://www.biolegend.com/en-us/products/alexa-fluor-488-anti-mouse-cd68-antibody-6619

Guinea pig α- VGlut2; Millipore; Lehrman, E. K. et al. CD47 Protects Synapses from Excess Microglia-Mediated Pruning during Development. Neuron 100, 120-134.e126, doi:10.1016/j.neuron.2018.09.017 (2018).

Rabbit α-GAPDH; BioLegend; Joas, S. et al. Nef-Mediated CD3-TCR Downmodulation Dampens Acute Inflammation and Promotes SIV Immune Evasion. Cell Rep 30, 2261-2274.e7, doi:10.1016/j.celrep.2020.01.069 (2020).

Rat α-mC4; [16D2]; Simoni, L. et al. Complement C4A Regulates Autoreactive B Cells in Murine Lupus. Cell Rep 33, 108330, doi:10.1016/j.celrep.2020.108330 (2020).

Mouse α-Brn3a; [14A6]; By manufacturer: https://www.scbt.com/p/brn-3a-antibody-14a6

Rat α-CD21/35; [7E9]; By manufacturer: https://www.biolegend.com/co/it/products/pacific-blue-anti-mouse-cd21-cd35-cr2-cr1-antibody-4336

Rat α-CD169; By manufacturer: https://www.biolegend.com/de-de/products/fitc-anti-mouse-cd169-siglec-1-antibody-9324

Rat α-CD11b; [M1/70]; By manufacturer: https://www.biolegend.com/en-us/products/pe-anti-mouse-human-cd11b-antibody-349

Rat α-CD45; [30-F11]; By manufacturer: https://www.biolegend.com/en-us/products/pe-cyanine7-anti-mouse-cd45-antibody-1903

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

CS7Bl/6J (WT; Jackson Laboratory #000664), C4-/- (Jackson Laboratory #003643), and four new transgenic mice strains, hC4A/-, hC4A/A, hC4B/-, and hC4AB/- mice were bred and maintained under specific pathogen-free conditions. Mice were kept at an ambient temperature of 21°C, under a 12-hour light/dark cycle with standard chow diet at the AAALAC-accredited animal facility at Harvard Medical School. C4-/- were generated by our laboratory and previously described.44 C4+/- heterozygous parents were used to generate litters where WT and C4-/- littersmates could be compared. Mice from both sexes were analyzed in all of the experiments.
For experiments concerning the dLGN, mice at the age of P5 and P10 were used. For experiments concerning the mPFC, mice at the ages of P10, P40, P60, P90, and P180 were used. Mice that showed a state of hydrocephalus were excluded from the study.

**Wild animals**
No wild animals were used in our study.

**Field-collected samples**
No field-collected samples were used in our study.

**Ethics oversight**
All animal experiments were carried out in agreement with the institutional guidelines provided by the Institutional Animal Care and Use Committee at Harvard Medical School and Boston Children's Hospital (protocol numbers IS00000748, IS00000111, IS00004762, and IS00002660), per applicable laws and regulations and in accordance with the guidelines of the Laboratory Animal Center at NIH for the humane treatment of animals.

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

## Flow Cytometry

**Plots**
Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

**Methodology**

**Sample preparation**
To prepare single-cell suspensions, the frontal cortex was dissected and the tissue was digested in 330U papain in DPBS at 35°C for 30 min. Papain was neutralized in an ovomucoid solution and cells were dissociated by gentle trituration and filtered through a 70-μm nylon mesh to remove clumps. Microglia were then purified by magnetic purification using anti-CD45 microbeads (Miltenyi). Cells were treated with antibody clone 2.4G2 to block Fc receptors before staining, and staining was performed in PBS solution containing 5% FCS and 2 mM EDTA. For intracellular staining, microglia were fixed and permeabilized (fixation and permeabilization buffers from Biolegend) before antibody staining. Anti-SV2 (DSHB/UIowa) was prepared and conjugated to fluorochromes in-house. Monoclonal antibodies against CD45, CD11b, and CD68 were purchased from BioLegend, viability dye (Ebioscience #65-0865-14) was used to exclude dead cells.

**Instrument**
Stained cells were acquired using BD FACSCanto II

**Software**
Data were collected with BD FACSConnect software and analyzed using FlowJo.

**Cell population abundance**
When microglia were purified using CD45 microbeads, purity was determined by flow cytometry. Microglia represented 80-90% of the population.
When microglia were FACS sorted for RNA sequencing analysis, microglia represented 5% of the entire cell suspension and their purity after cell sorting was determined by the RNA sequencing results.

**Gating strategy**
For all experiments an initial gating strategy was used to define cells (SSC-A low and FSC-A low), followed by two double exclusion gates (FSC-W/FSC-H and SSC-W/SSC-H) then live cells. Subsequent gates are defined in the figures/figure legends.

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