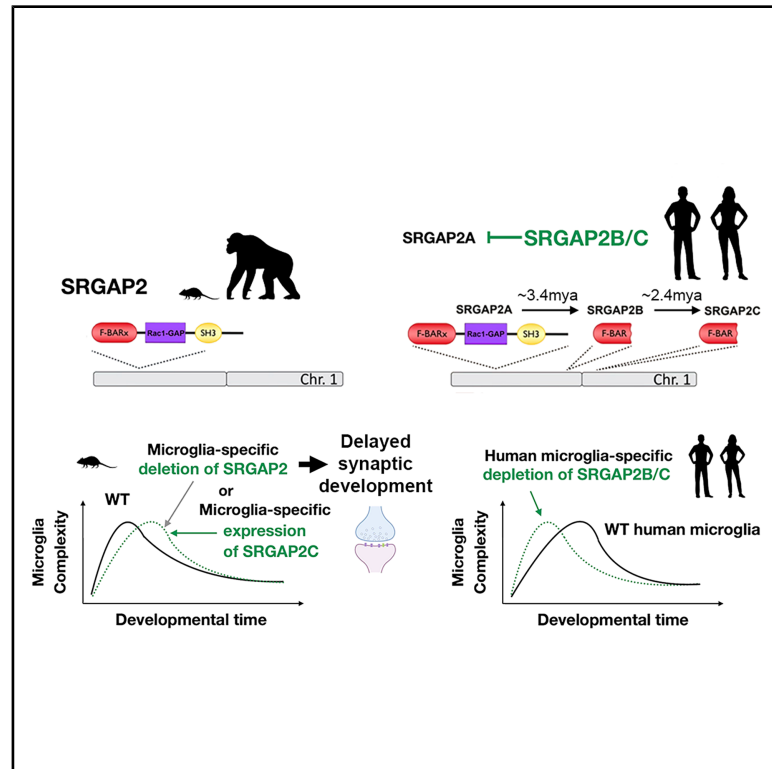


Human-specific SRGAP2 paralogs synchronize neotenic microglial maturation and synaptic development

Graphical abstract



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In brief

Using a combination of microglia-specific conditional mouse knockout and knockin approaches and xenotransplantation of human iPSC-derived microglia, Diaz-Salazar et al. demonstrate that the human-specific genes SRGAP2B/C, which downregulate the ancestral SRGAP2A, are necessary and sufficient to induce neotenic features of microglial structural maturation, leading to delayed timing of synaptic development in cortical pyramidal neurons.

Highlights

- Human cortical microglia display neotenic structural maturation compared with mouse microglia
- SRGAP2 promotes microglial structural maturation during cortical development
- Human-specific SRGAP2B/C decrease SRGAP2A and induce neotenic microglial maturation
- Microglia-specific downregulation of SRGAP2 delays the timing of synaptic development

Article

Human-specific SRGAP2 paralogs synchronize neotenic microglial maturation and synaptic development

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<https://doi.org/10.1016/j.neuron.2026.07.007>

SUMMARY

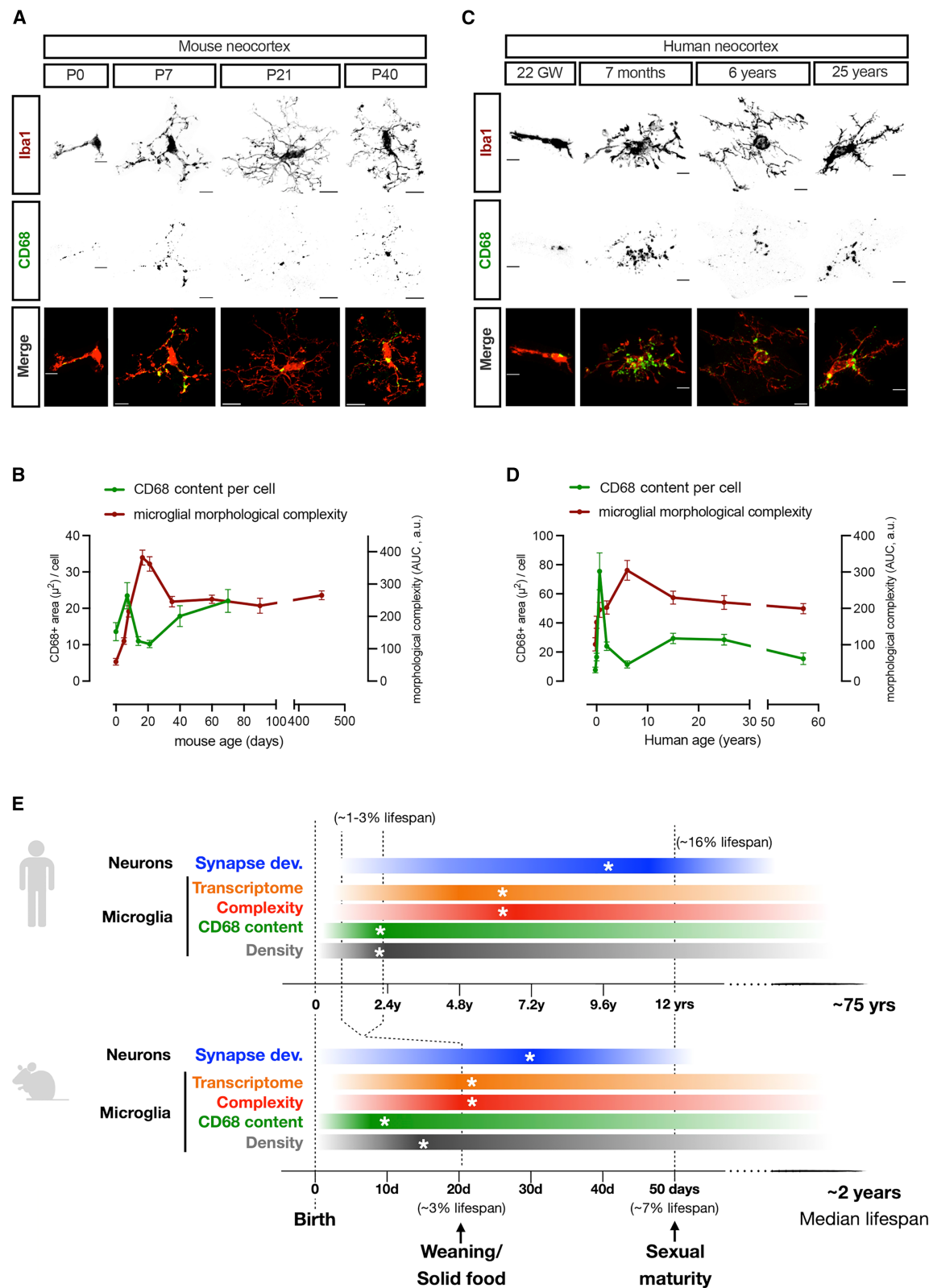
The human brain is distinguished by unusually prolonged developmental timing, yet the genetic mechanisms coordinating this neoteny across cell types remain incompletely understood. Here, we show that human cortical microglia undergo neotenic structural and transcriptional maturation relative to mouse microglia. We identify *SRGAP2B/C*, human-specific paralogs of the ancestral *SRGAP2A*, as the only human-specific gene duplications expressed in human microglia. Using xenotransplantation of human induced pluripotent stem cell (hiPSC)-derived microglia and mouse genetic models, we demonstrate that human-specific *SRGAP2B/C*, previously shown to reduce *SRGAP2A* protein abundance, are both necessary and sufficient to induce neotenic structural microglial maturation. Our results reveal that neotenic microglial maturation modifies the timing of synaptic development, linking microglial developmental programs to the timing of circuit formation. Together with previous evidence for neuronal *SRGAP2A* function, our results suggest that the human-specific *SRGAP2B/C* paralogs coordinated the emergence of neotenic synaptic development by acting in both neurons and microglia during human brain evolution.

INTRODUCTION

Microglia play key roles during brain development, neural circuit homeostasis, and pathology.^{1–3} As the resident immune cells of the central nervous system (CNS) parenchyma, they provide essential support for proper neuronal development, synaptic maturation, and function, both embryonically and postnatally.^{1,2} In mammals, from rodents to non-human primates and humans, adult neuronal connectivity is established during postnatal development through a combination of synapse formation, maturation, and elimination.^{4–6} Microglia are critical for shaping and maintaining synaptic connectivity in various circuits by regulating synapse development, maintenance, and function.^{1,2,7–9} In developing and adult circuits, microglial processes display high levels of dynamic behavior that respond to and modulate neuronal activity at the synaptic level.^{10–12} Additionally, as tissue-resident macrophages, they continually survey their environment, searching for and quickly responding to injury cues and phagocytosing any debris, pathogens, or injured cells they may encounter.^{9,13}

Microglia originate from the embryonic yolk sac and migrate to the brain early during development (around embryonic day [E]8.5 in mice and gestational week [GW]5 in humans), where they mature *in situ*.^{14–16} However, like neuronal development, microglial development continues postnatally.^{1,2} In mice, microglia transition from an amoeboid shape into a highly complex and branched morphology while acquiring expression of a set of genes allowing them to sense their environment and perform surveillance and phagocytic functions.^{17–19} Importantly, in mice, the timing of postnatal microglial structural and functional maturation largely coincides with that of postnatal synaptic maturation, highlighting the importance of synchronizing both cellular processes at the molecular level.^{1,6}

Although the dynamics and timing of neuronal and microglial maturation have been well characterized in the brains of rodents^{1,2,6,16,20–23} and, to some extent, in non-human primates,^{24–26} less is known about the timing and features of microglial maturation in the human brain.^{4,16,27} In brain structures such as the neocortex, one of the most salient and striking features characterizing human neuronal development is the slow



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pace of morphological and functional maturation. Specifically, synaptic maturation in the neocortex takes weeks to months in most mammals, from rodents to non-human primates, whereas in humans it extends for approximately a decade after birth, a form of heterochrony termed neoteny, which corresponds to the retention of immature, juvenile features for longer periods during development.^{28–31} This strikingly prolonged period of synaptic development and maturation is thought to extend critical periods of circuit remodeling and plasticity, enabling longer periods of postnatal development during which circuits can be modified by experience-dependent plasticity.³²

Because microglia are well known to play multiple important roles in synaptic development, we hypothesized that the timing of microglial maturation might be prolonged to match the strikingly neotenic period of synaptic maturation characterizing human cortical neurons compared with other mammals. Surprisingly, very little is known about whether the developmental timing of microglial maturation exhibits human-specific features, including neoteny. Although recent attempts to characterize the transcriptional states of fetal and postnatal human microglia have pointed to clear differences between these two stages, the precise timing and magnitude of such changes are still poorly explored, impairing our ability to identify candidate molecular effectors of microglial structural and functional maturation during development and to determine whether some of these effectors act as species-specific genetic modifiers of microglial development and function.^{26,33–36}

Current efforts to establish native-like models for studying human microglial function, such as those using microglia derived from human pluripotent stem cells (hPSCs), have been largely focused on proof-of-concept modeling and applicability to disease models in which microglia play an important role, thereby highlighting, for example, certain human-specific features of microglial responses to injury or pathological states such as neurodegeneration.^{37,38}

Here, we provide evidence that, in the developing cortex, human microglia exhibit a strikingly slower, neotenic structural maturation trajectory compared with mouse microglia. We previously identified the role of the gene *SRGAP2A*, shared among all mammals, and its human-specific paralogs, *SRGAP2B/C*, as critical human-specific genetic modifiers necessary and sufficient for the induction of the neotenic timing of synaptic maturation and increased synaptic connectivity in cortical pyramidal neurons

(CPNs).^{39–43} However, *SRGAP2A* and its human-specific paralogs, *SRGAP2B/C*, are also expressed in developing and adult human microglia, and in the present study, using gain- and loss-of-function approaches in mouse cortical microglia, we demonstrate that *SRGAP2* regulates the timing of microglial morphological maturation, which, in turn, prolongs the timing of synaptic development and increases synaptic connectivity in CPNs in a cell-non-autonomous manner. We also show that the human-specific paralogs *SRGAP2B/C*, through inhibition of *SRGAP2A*, induce human-specific neotenic features of microglial maturation.

RESULTS

Comparative analysis of the timing of maturation of mouse and human cortical microglia *in vivo*

Few studies have reported the developmental maturation of microglia using morphological and functional marker analysis in the human cortex.²⁷ To assess human cortical microglial maturation *in vivo*, we employed a set of human cortical tissue samples covering multiple fetal, postnatal, and adult time points during human cortical development. We first compared the timing of endogenous microglial maturation in the mouse and human neocortex in terms of cell density, morphological complexity using Iba1 as a marker, and CD68 content, a phagolysosomal protein related to the degradation potential of myeloid cells, including microglia.^{44,45} Consistent with previous reports, the density of Iba1+ microglia in the mouse cortex rapidly increases during the first 2 weeks after birth, then declines and stabilizes around the fourth postnatal week (Figures S1A and S1B).⁴⁶ On the other hand, we found that the number of Iba1+ microglia in the human cortex rapidly increases embryonically, peaks at around 2 years of age, then declines and stabilizes at adult-like levels around 15 years of age (Figures S1C and S1D), consistent with a previous report.²⁷

We then quantified microglial morphological complexity using Sholl analysis of Iba1 immunofluorescence and high-resolution confocal microscopy (Figures S1E–S1G), and, consistent with the literature,¹⁷ we observed that microglial branching complexity increases linearly during the first 3–4 postnatal weeks of development (Figures 1A and 1B). Interestingly, we found that following this period of hyper-ramification, microglial complexity is reduced and reaches adult levels approximately 1 month after birth (Figures 1A and 1B). We found that human

Figure 1. Comparative analysis of the timing of human and mouse cortical microglial morphological and functional maturation *in vivo*

(A and C) Representative images of human (A) and mouse (C) cortical microglia throughout brain development, displaying their morphology (Iba1 staining) and phagolysosomal content (CD68 staining). Scale bar, 10 μ m. (B and D) Quantification of human (B) and mouse (D) microglial morphological complexity over time (red, as revealed by Iba1 staining), computed as the area under the curve (AUC) of each microglia's Sholl traces (see Figures S1E–S1G), and microglial degradation potential (green, revealed by CD68 staining), expressed as the cumulated area (μ m²) of all CD68+ puncta per microglia. Data from one human cortical sample (B) per time point, 17–77 microglial cells per sample, and 3–8 mice (D) per time point, 9–20 microglia per mouse. Curve data show the group mean of each time point, and error bars indicate the 95% confidence interval. (E) Comparison of the timing of microglial maturation in human (top) and mouse (bottom) cortical microglia represented relative to sexual maturity (~12 years in human corresponding to ~17% of lifespan; ~P50 in mouse corresponding to ~7% of lifespan^{47,48}). The peaks of microglial complexity (red), degradation potential (CD68 expression, green) and microglial density (see Figures S1A–S1D; gray) are indicated by white stars. In blue, the temporal profile of synaptic development is illustrated using data from the human cortex, with a peak of dendritic spine density at ~10–15 years after birth^{30,49} whereas the peak of spine density (white star) is reached around P30 in mouse CPNs.^{28,50,51} Finally, we also indicate the peak (white star) of expression of a gene module (module 160 identified in Figure 3) by comparative single-cell transcriptomics, which corresponds to genes well characterized for their role in the differentiation of the structural and functional properties of microglia whose expression increases monotonically during microglial development (peak at ~P20 in mouse and ~6 years in human microglia).

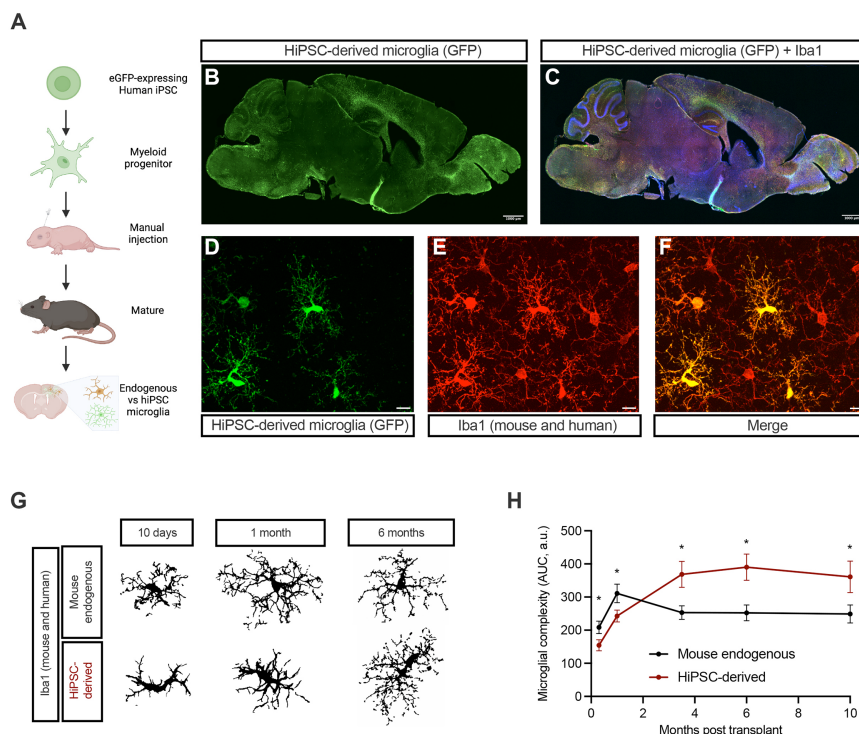


Figure 2. hMGs exhibit increased morphological complexity and delayed morphological maturation compared with mouse microglia

(A) Schematic representation of the experimental approach used to comparatively assess human and mouse microglial morphology *in vivo*. Human induced pluripotent stem cells (hiPSCs) stably expressing GFP were differentiated into myeloid precursors, xenotransplanted into the brain of immunodeficient neonatal (P1–3) mice, and enabled to differentiate into microglial cells until 10 months post-transplantation (MPT).

(B–F) Representative low-magnification (B and C) and high-magnification (D–F) images of human (green in D) and mouse (Iba1+ only, red in E) cortical microglial morphology at 6 MPT. Scale bars in (B) and (C): 1 mm; (D)–(F): 10 μ m.

(G) Representative traces of Iba1+ mouse (endogenous) and human (hiPSC-derived) microglia following xenotransplantation in the cortices of mice aged 10 days, 1 month, and 6 months.

(H) Quantification of microglial morphological complexity of mouse and human microglia in the cortices of mice aged 10 days, 1 month, and 6 months, computed as the AUC of the Sholl analysis for each microglial cell. Data from three to six mice per time point, with 7–22 Iba1+ microglial cells per mouse. Curve data show the group mean of each time point, and error bars indicate the 95% confidence interval. Statistical differences were calculated using a two-way ANOVA (Sidak multiple-comparison test); * $p \leq 0.05$.

cortical microglia follow a similar developmental trajectory but at a much slower pace: microglial morphological complexity increased until 6 years of age and then normalized at around 15–20 years of age (Figures 1C and 1D). We note that at the peak of morphological complexity (postnatal day [P]21 in mice and 6 years of age in humans) or in young adults (P35 in mice versus 25 years of age in humans), human microglial cells are not morphologically more complex than mouse cortical microglia (Figures S1H and S1I).

Strikingly, in both mouse and human, we observed that microglial CD68⁺ content, which is classically thought to reflect the degradation potential of microglia,^{44,45} sharply increased postnatally, but on significantly different timescales in the mouse and human cortex: the peak of CD68 content per cell is reached at ~P7 in the mouse versus ~2 years of age in the human cortex (Figures 1B and 1D). These results demonstrate that microglial developmental trajectories are shared between mice and humans, albeit at strikingly different timescales: the time difference between the peak of CD68 content and the peak of morphological complexity is ~2 weeks in mice versus ~6 years in human cortical microglia (Figures 1B and 1D).

Beyond striking differences in the absolute timing of these features of microglial maturation between human and mouse, to demonstrate that microglial maturation is truly neonatal in human, i.e., that human microglia display immature features for a longer period of development compared with mouse microglia, we normalized the developmental timing of microglial complexity (morphological complexity measured by Sholl analysis of Iba1),

degradation potential (CD68 content), and microglial density (Figures S1A–S1D) relative to a developmental landmark, sexual maturity: ~P50 in mice, corresponding to ~7% of lifespan, and ~12 years after birth in humans, corresponding to ~17% of lifespan (see Figure 1E). This analysis shows that microglial density and CD68 content occur at relatively similar developmental stages in both species but that both the peak of microglial complexity and synaptic maturation (dendritic spine density^{30,49}) are strikingly neonatal in the human compared with the mouse cortex when normalized either to other developmental landmarks, such as sexual maturity (Figure 1E), or to median lifespan (Figure S2).

Delayed morphological maturation of human microglia is a cell-intrinsic property

To assess which features of microglial development are cell intrinsic and which were driven by changes in the microglial environment, we performed xenotransplantation of human induced pluripotent stem cell (hiPSC)-derived microglia (hMGs) into mouse neonatal brains and compared their morphological maturation to that of the endogenous mouse microglia (Figure 2A). Using this paradigm, hPSCs are differentiated into myeloid precursors, which, upon xenotransplantation into neonatal mouse brains from immunodeficient Rag2 knockout mice, engraft extensively, differentiate into microglia, and closely recapitulate the transcriptional signature of primary human microglia at diverse developmental stages (Figures 2B–2F), as previously described.^{33,38,52–54} At 6 months post-transplantation (MPT),

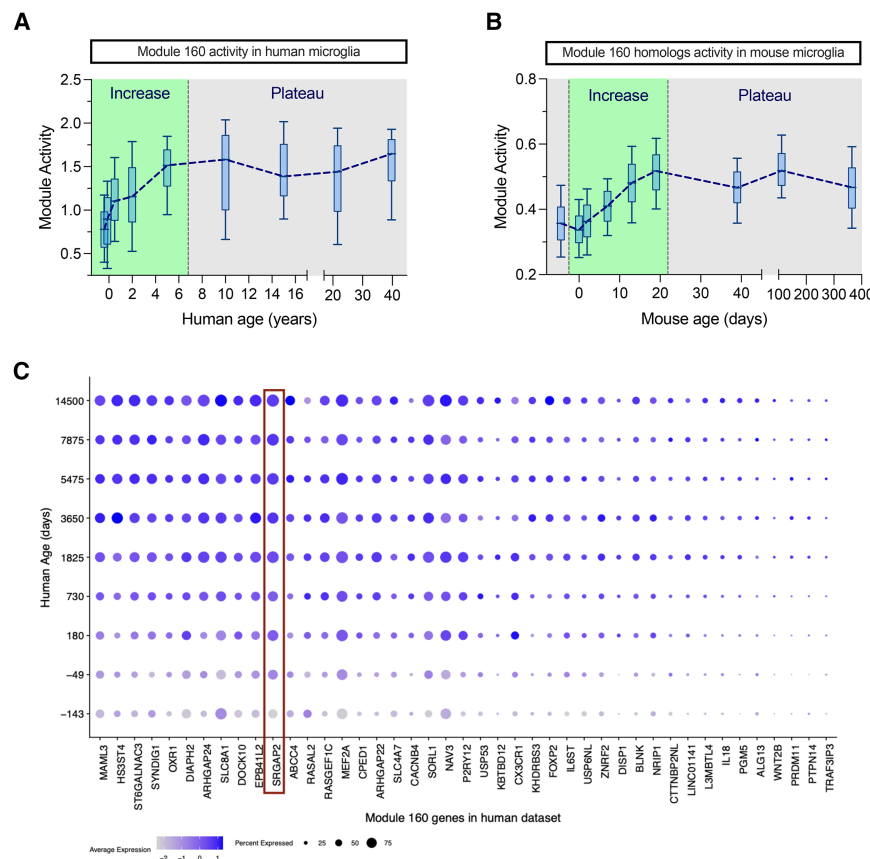


Figure 3. Human microglia display a transcriptomic signature of neonatal development

(A) Average activity throughout human development of the genes contained in human module 160.

(B) Average activity throughout mouse development of the mouse homologs of the genes contained in human module 160. Dashed line connects the median activity for each time point. Box encompasses the 25th–75th percentile, and whiskers extend to the 10th–90th percentile. Module activity increase (green shaded area) is defined as a significant, directional change between at least four consecutive time points. Plateau (gray shaded area) is defined as no significant directional change between four consecutive time points. Statistical differences between each consecutive time point were calculated with Welch's two-sample *t* test.

(C) Dot plot heatmap representing the developmental expression of the 43 human genes contained in module 160. *Srgap2* is boxed in red.

xenotransplanted hMGs were significantly more ramified than their endogenous mouse counterparts (Figures S3A–S3C). We then assessed hMG morphological maturation longitudinally and found a significant delay in reaching peak morphological complexity compared with their endogenous mouse counterparts and the absence of a time point with a “simplified” morphology, as observed *in vivo*, even at 10 MPT (Figures 2G and 2H). These results are consistent with our characterization of the timing of human cortical microglial maturation *in vivo*, showing that the peak of microglial morphological complexity is reached at ~6 years after birth (see Figures 1C and 1D).

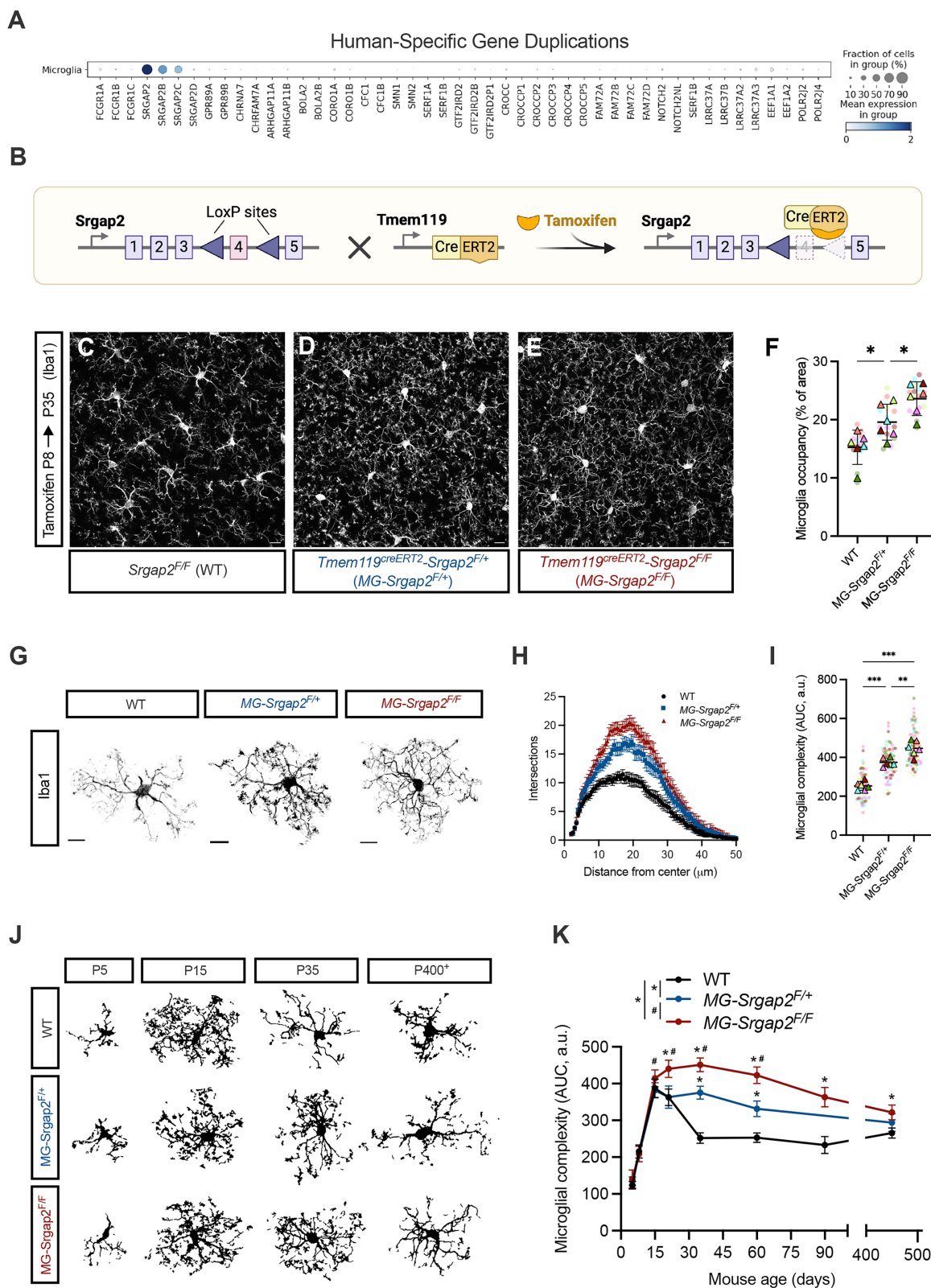
Interestingly, CD68⁺ content in hMGs was constant throughout maturation following xenotransplantation (Figures S2D and S2E). These data suggest that the timing of morphological maturation is driven by largely cell-intrinsic mechanisms, whereas the expression of markers pertaining to microglial functional maturation, such as CD68, may depend, at least in part, on the environmental cues microglia encounter throughout development.

Transcriptional signatures of microglial development reveal strikingly delayed maturation of human compared with mouse microglia

Like other cell types in the developing brain, microglia exhibit transcriptional signatures that can be used to infer their maturation.^{27,33,55–58} In the mouse, it is widely accepted that microglia adopt a mature transcriptional signature at 2 weeks after birth.^{20,23} However, despite studies highlighting the differences

between fetal and postnatal microglia, a comprehensive transcriptional signature of human microglial maturation is not yet available.^{33,34} To generate a transcriptional atlas of human microglial maturation, we used a publicly available single-nucleus RNA sequencing (snRNA-seq) dataset containing primary human microglia and extracted their transcriptional trajectories from the second gestational trimester to adulthood.⁵⁷ Of the ~15,000 unique transcripts expressed throughout human microglial development,⁵⁷ weighted gene correlation network analysis (WGCNA) identified 166 modules of developmentally co-expressed genes, which we then grouped into 5 highly correlated clusters of modules using a *k*-means unsupervised clustering algorithm (Figures S4A and S4B). These modules do not reflect the signature of any microglial subset (disease-associated, resting, etc.) but rather sets of co-regulated genes sharing similar developmental trajectories.

Most of the identified gene expression modules showed limited developmental expression dynamics and clustered tightly together, suggesting that most of the genes belonging to such modules display stable expression patterns during microglial maturation (Figures S4A and S4B). However, some modules showed marked changes in module activity throughout early human development and did not plateau until approximately 5 years of age (arrow in Figure 3A). To confirm that this approach can infer developmental trajectories over absolute time, we applied the same methodology to a publicly available single-cell RNA-seq (scRNA-seq) dataset of mouse microglia⁵⁸ and identified similar temporal trajectories to those observed in human microglia (Figures S4C and S4D). Mainly, the activity of most modules remained flat during development, and those that showed a developmental change in activity reached an activity plateau at 2 weeks after birth, consistent with previous



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reports²³ and our inferred timelines for microglial morphological maturation (see [Figures 1C and 1D](#)). These data further indicate that mouse and human microglia have similar developmental trajectories but that the absolute timing of human microglial maturation is delayed compared with mouse microglia.

Among all the temporally regulated gene modules in human microglia, module 160 showed the steepest increase in gene expression module activity throughout human development, reaching peak activity at around 5 years of age (arrow in [Figure 3A](#)). In comparison, the activity of the mouse orthologs of the genes contained in module 160 peaked at ~3 postnatal weeks (arrow in [Figure 3B](#)), highlighting that human and mouse microglia share a common developmental profile, albeit at very different timescales, with a clear signature of transcriptional neonity for human cortical microglia compared with those of the mouse.

Interestingly, module 160 contains genes essential for microglial homeostasis, such as *cx3cr1* and *p2ry12*, transcription factors and coactivators, and genes putatively involved in regulating cytoskeletal dynamics by modulating the activation state of small Rho-GTPases ([Figure 3C](#)).

SRGAP2 is a critical regulator of microglial maturation

To identify potential candidate genes acting as human-specific modifiers of microglial morphological and/or functional maturation, we examined human-specific gene duplications (HSGDs). We and others recently demonstrated that HSGDs represent a potent source of human-specific genomic modifiers that have enabled the emergence of specific traits of neuronal development, synaptic maturation, and circuit connectivity, thereby ultimately impacting cortical circuit function during human brain evolution.^{32,39–41,43,59,60} Interestingly, module 160 contains SLIT-ROBO Rho GTPase-activating protein 2⁶¹ (*Srgap2* in mammals, named *SRGAP2A* in humans; [Figure 3C](#)), a gene that has undergone two main partial duplications, leading to the emergence of the human-specific paralogs *SRGAP2B* and *SRGAP2C* approximately 3.5 and 2.4 million years ago (mya), respectively, corresponding approximately to the emergence of the Homo lineage.^{39,62}

Strikingly, the ancestral gene *SRGAP2A* (present in all mammals) and its human-specific paralogs *SRGAP2B/C* are the

only HSGDs expressed in human microglia ([Figure 4A](#)), and their temporal expression pattern in mouse and human microglia coincides with microglial maturation ([Figures S4E and S4F](#)), making them unique candidates for controlling microglial morphological and/or functional maturation in a human-specific manner. In human neurons, *SRGAP2B/C* encode truncated and intrinsically unstable proteins that remain able to interact with the ancestral full-length *SRGAP2A* protein and constitutively target ~50% of *SRGAP2A* protein for proteasome degradation, inhibiting all known functions of *SRGAP2A* in CPNs and phenocopying *SRGAP2A* haploinsufficiency ([Figure S4G](#)).^{39–42} We hypothesized that expression of *SRGAP2B/C* in human cortical microglia should phenocopy a partial *SRGAP2A* loss of function and might modulate the developmental timing of microglial morphological and functional maturation.

We first tested the phenotypic consequences of *SRGAP2* loss of function in mouse cortical microglia by generating a microglia-specific *Srgap2* deletion by crossing the microglia-specific inducible *Tmem119-Cre^{ERT2}*⁶³ with a conditional knockout mouse model (*Srgap2^{F/F}*) we previously generated and validated ([Figure 4B](#)).⁶⁴ Mice were treated with tamoxifen early during development (P8–9), achieving high recombination efficiency in cortical microglia ([Figures S5A–S5F](#)), to drive *Srgap2* conditional deletion ([Figure S5G](#)). Deletion of 1 copy (*MG-Srgap2^{F/+}*) or 2 copies (*MG-Srgap2^{F/F}*) of *Srgap2* specifically in microglia led to a dose-dependent increase in microglial tissue occupancy in the mouse cortex compared with littermates, tamoxifen-treated control mice (*Srgap2^{F/F}* mice not carrying the *Tmem119-Cre^{ERT2}* allele; [Figures 4C–4F](#)). However, the somatic density of adult cortical microglia was unchanged in mice from all genotypes ([Figures S5H–S5K](#)), suggesting that the increased coverage may be due to increased microglial morphological complexity. Indeed, *Srgap2*-deficient microglia showed a significant, dose-dependent increase in morphological complexity, displaying a hyper-ramified morphology at P35 ([Figures 4G–4I](#)). Notably, microglia from constitutive *Srgap2* heterozygous knockout mice (*Srgap2^{+/-}*) also displayed a hyper-ramified phenotype compared with control wild-type (WT) littermates at P35 ([Figures S5L–S5N](#)).

Because (1) *Srgap2* mRNA expression monotonically increases in mouse cortical microglia during the first 2–3 postnatal

Figure 4. *Srgap2* is a critical regulator of microglial morphological maturation in developing mouse cortex

(A) Dot plot heatmap depicting the mRNA expression of all assembled and annotated human-specific duplicated genes (indicated by red stars) and their ancestral gene copy (black star) in human microglia based on snRNA-seq data.⁵⁷

(B) Schematic representation of the genetic approach used to conditionally knockout *Srgap2* specifically in microglia in the mouse brain.

(C–E) Representative low-magnification images of cortical microglia stained with Iba1 immunofluorescence from WT (*Srgap2^{F/F}*; C), *MG-Srgap2^{F/+}* (*Tmem119^{CreERT2} Srgap2^{F/+}*; D), and *MG-Srgap2^{F/F}* (*Tmem119^{CreERT2} Srgap2^{F/F}*; E) P35 mice. Scale bar, 10 μ m.

(F) Quantification of the area covered by Iba1 immunofluorescent staining per field of view from these three genotypes. Data from six mice per genotype, with a line indicating the median and error bars the 95% confidence interval. Statistical differences were calculated using a one-way ANOVA (Tukey's multiple-comparison test); * $p \leq 0.05$.

(G) Representative images of individual Iba1+ microglia from these 3 genotypes. Scale bar, 10 μ m.

(H and I) Comparison of Sholl analysis (H) and morphological complexity (area under the curve [AUC]) (I) of Iba1+ cortical microglial cells from these three genotypes. Data from six mice per genotype, with 11–17 Iba1+ microglial cells per mouse, with a line indicating the median and error bars the 95% confidence interval. Statistical differences were calculated using a one-way ANOVA (Tukey's multiple-comparison test), using mice as biological replicates; ** $p \leq 0.01$; *** $p \leq 0.001$.

(J) Representative traces of microglia from these three genotypes at different ages.

(K) Morphological complexity of microglia from these three genotypes across time. Data from 3 to 8 mice per time point and genotype, with 8–22 cells per mouse. Curve data show the group mean of each time point, and error bars indicate the 95% confidence interval. Statistical differences were calculated using a two-way ANOVA (Sidak multiple-comparison test), using mice as biological replicates; # $p < 0.05$; * $p < 0.05$.

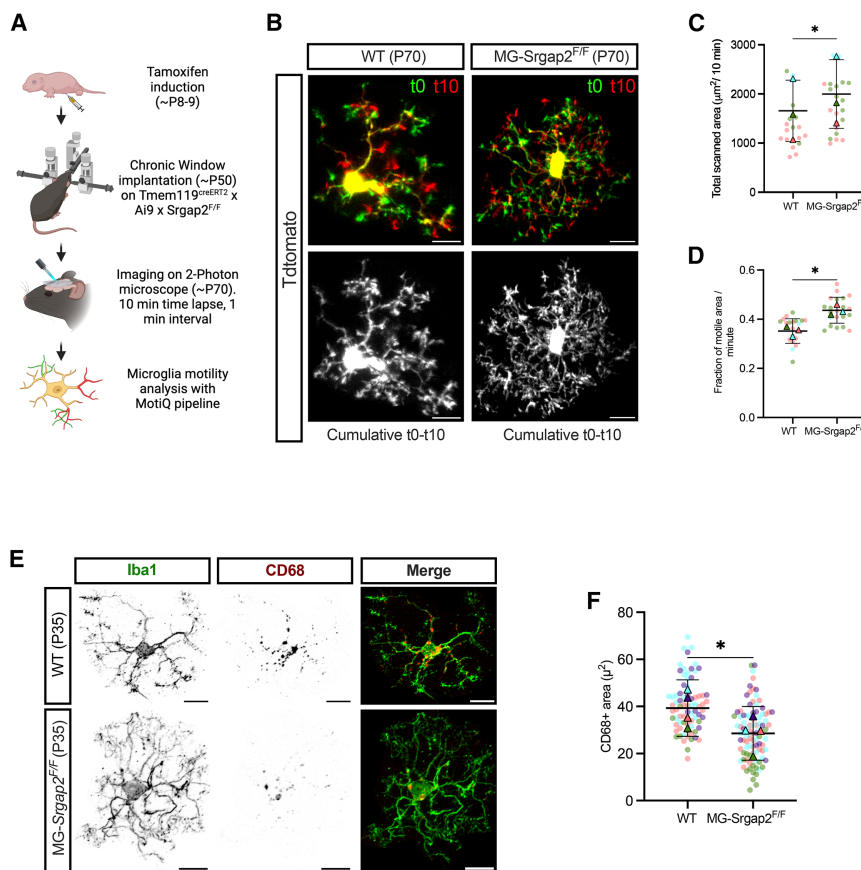


Figure 5. *Srgap2* regulates microglial surveillance and degradation potential

(A) Experimental approach to assess microglial process dynamics *in vivo* using time-lapse (1 images/min) 2P microscopy in the mouse primary somatosensory cortex at P50.

(B) Representative still frames (top, pseudo-colored t0 in green and t10 min in red) and cumulative time-projection of all frames (10 total; bottom in black and white) of tdTomato+ microglia from WT and *MG-Srgap2^{F/F}* mice, imaged over a 10-min period. Scale bar, 10 μ m.

(C and D) Microglia process motility quantified using MotiQ by measuring the total area scanned by processes over 10 min (C) and by fraction of non-overlapping pixels for fraction of motile processes per unit time (D) from WT and *MG-Srgap2^{F/F}* mice. Data from three mice per genotype, with $n = 19$ for WT mice and $n = 22$ for *MG-Srgap2^{F/F}* microglial cells, with a horizontal line indicating the median, and error bars the 95% confidence interval. Statistical differences were calculated using an unpaired *t* test, using mice as biological replicates; $*p < 0.05$.

(E) Representative images of Iba1+ (green) microglia from WT and *MG-Srgap2^{F/F}* mice at P35 co-stained with the phagolysosomal marker CD68 (red). Scale bar, 10 μ m.

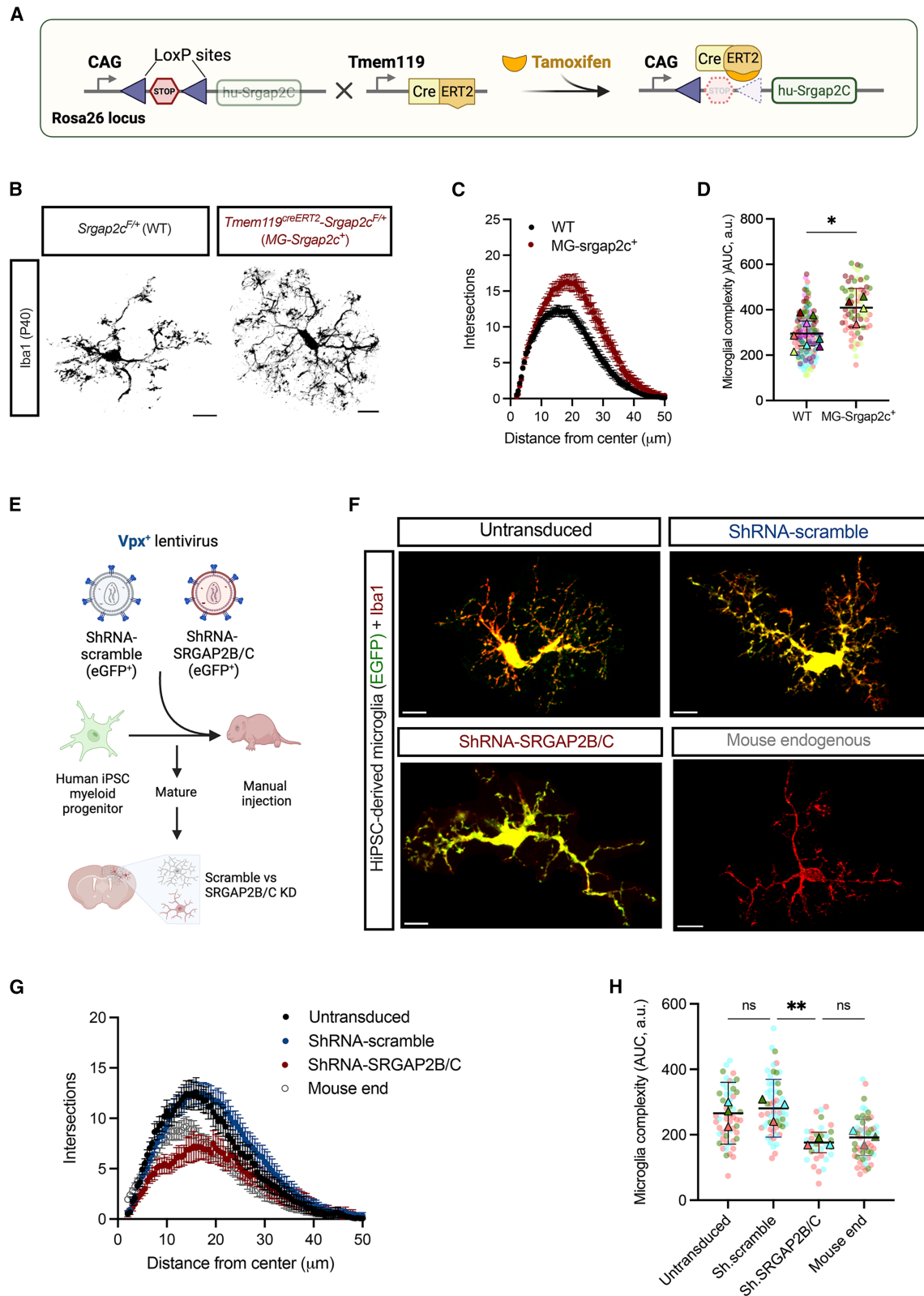
(F) Quantification of the total CD68+ area in WT and *MG-Srgap2^{F/F}* microglia. Data from four mice per genotype, with 10–29 cells per mouse, with a horizontal line indicating the median and error bars the 95% confidence interval. Statistical differences were calculated using an unpaired *t* test, using mice as biological replicates; $*p < 0.05$.

weeks of development and is maintained throughout adulthood (Figure S4E), and (2) microglia-specific deletion of *Srgap2* results in increased morphological complexity (Figures 4G–4I), we hypothesized that SRGAP2 function might regulate the developmental timing of microglial morphological maturation. To test this, we treated WT, *MG-Srgap2^{F/+}*, and *MG-Srgap2^{F/F}* mice with 4-hydroxytamoxifen shortly after birth (P2–3) and found that both *MG-Srgap2^{F/+}* and *MG-Srgap2^{F/F}* microglia reached peak morphological complexity at 2–4 weeks after birth but then displayed a delayed period of morphological simplification, only approaching WT morphological complexity levels after 1 year (Figures 4J and 4K). Altogether, our data suggest that SRGAP2 negatively regulates microglial morphological complexity and that its deletion results in a dose-dependent delay in morphological maturation, i.e., the neonatal retention of an “immature,” hyper-ramified morphology.

We then tested whether we could capture a transcriptional signature in *Srgap2*-deficient microglia consistent with that of a less mature, neonatal state. Bulk RNA-seq of fluorescence-activated cell sorting (FACS)-sorted WT and *MG-Srgap2^{F/F}* microglia from P30 mice, following tamoxifen induction at P8–9 (Figure S6A), validated the downregulation of *Srgap2* mRNA expression and revealed significantly higher expression of transcripts enriched for genes encoding cytoskeletal organization and actin dynamics, such as *Tmsb4x*, *Lsp1*, and *Stmn1*, compared with WT isochronic microglia

(Figure S6B). Indeed, Gene Ontology enrichment analysis of the top 100 genes showing increased expression in *MG-Srgap2^{F/F}* compared with WT microglia revealed a marked enrichment of biological processes involved in actin filament polymerization and regulation of cytoskeleton organization (Figure S6C). Interestingly, these genes are highly expressed at early postnatal stages, and their expression subsequently decreases with microglial maturation (Figure S6D), suggesting that high expression of these genes may correlate with a less mature microglial stage. In line with our previous findings, these data support our morphological observation that SRGAP2 expression is necessary for the morphological maturation of cortical microglia.

Finally, we investigated whether the neonatal maturation of *MG-Srgap2^{F/F}* microglia had an impact on their functional properties. Previous *in vivo* two-photon (2P) microscopy revealed that mouse cortical microglia display high levels of process dynamics (extension and retraction).^{12,65,66} We first assessed microglial process dynamics in the cortex of WT (*Tmem119-Cre^{ERT2};SRGAP2^{+/+}*) and *MG-Srgap2^{F/F}* (*Tmem119-Cre^{ERT2};SRGAP2^{F/F}*) mouse littermates at P70 using *in vivo* time-lapse 2P microscopy (Figure 5A; Videos S1 and S2), for which each strain was additionally crossed with a Cre-dependent tdTomato red fluorescent reporter (*Ai9* mouse line).⁶⁷ Semiautomatic analysis of microglial process dynamics using the MotiQ pipeline⁶⁸ revealed that *Srgap2*-deficient microglia exhibited increased process extension and retraction



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dynamics compared with WT microglia, even when normalized to the total area occupied by microglial processes (Figures 5B and 5C).

We also observed a decrease in the microglial area covered by the late phagolysosomal marker CD68 at P35, suggesting that although *MG-Srgap2^{F/F}* microglia have increased morphological complexity at this time point, they also show reduced phagolysosomal content (Figures 5D and 5E). To independently assess whether *MG-Srgap2^{F/F}* microglia display reduced degradation of synaptic elements *in vivo* compared with WT control mice at P35, we performed expansion microscopy (240 $\times$) using a previously published protocol⁶⁹ to improve spatial resolution and identify unambiguously presynaptic elements (positive for VGLUT2) colocalizing with CD68⁺ phagolysosomes inside Iba1⁺ microglia, as previously reported⁷⁰ (Figure S7). Our results demonstrate that *MG-Srgap2^{F/F}* microglia contain significantly fewer vGlut2⁺ phagolysosomes than WT microglia in layer 4 of the barrel-field representation in the primary somatosensory cortex (bS1), which could be due to either increased presynaptic engulfment and/or decreased degradation. In summary, our data suggest that the ancestral SRGAP2 protein is necessary for mouse microglial maturation, acting as a critical regulator of the developmental transition between functional and morphological maturation.

Expression of the human-specific gene *SRGAP2C* in mouse microglia is sufficient to induce increased morphological complexity

We next tested whether, as previously shown in CPNs,^{39,40,42} expression of human-specific *SRGAP2C* in mouse microglia phenocopies a *Srgap2* partial loss of function in these cells. We used a previously characterized transgenic mouse model in which a *loxP*-STOP-*loxP*:*SRGAP2C* cassette was knocked into the *Rosa26* locus (*Srgap2c^{LSL}*), enabling expression of *SRGAP2C* in a Cre-dependent manner, which, in turn, reduces *SRGAP2A* protein levels.^{39,42,43,64} We generated crosses between the *Tmem119-Cre^{ERT2}* mouse line and *Srgap2c^{LSL}* knockin mice and induced Cre-dependent *SRGAP2C* expression following tamoxifen induction at P8–9 (Figure 6A).⁴³ Analysis at P40 revealed that mouse Iba1⁺ microglia expressing human-specific *SRGAP2C* exhibit increased morphological complexity (Figures 6B–6D), reaching levels similar to those of *Srgap2*-deficient microglia at the same age (see

Figures 4G–4I). These results demonstrate that, in terms of morphological complexity, expression of human-specific *SRGAP2C* in mouse microglia is sufficient to phenocopy *Srgap2* loss of function *in vivo*, i.e., is sufficient to increase mouse cortical microglial complexity at P40, at a time point when WT cortical microglia have already reduced their morphological complexity (see Figures 1A and 1B).

Deletion of human-specific *SRGAP2B/C* paralogs in human microglia leads to a reduction in morphological complexity

We then assessed whether the human-specific genes *SRGAP2B/C* were required to induce the delayed hyper-ramification that characterizes developing human microglia. To do so, we performed the same xenotransplantation of hMGs in the neonatal mouse cortex following short-hairpin RNA (shRNA)-mediated downregulation of human-specific *SRGAP2B/C*. We used modified lentiviral particles packaging the simian immunodeficiency virus Vpx protein on their capsid to infect hiPSC-derived myeloid precursors. The Vpx protein has been shown to promote lentiviral infection of myeloid cells and to greatly increase transduction efficiency.^{71,72} These lentiviral particles contained either control (scrambled) shRNA or shRNA targeting the shared 3' UTR region of the human-specific paralogs *SRGAP2B* and *SRGAP2C*, thereby effectively downregulating the expression of both human-specific paralogs, as previously characterized.^{39,41} Following lentiviral infection of hiPSC-myeloid progenitors, we xenotransplanted these cells into the cortex of immune-compromised *Rag2^{-/-}* *il2rg^{-/-}* hCsf-1 knockout/knockin mouse neonates, where the transplanted cells differentiated into human microglial cells (Figure 6E). We found that the morphological complexity of control hMGs, transduced with scrambled shRNA lentivirus, was indistinguishable from that of untransduced hMGs at 3 MPT (Figures 6F–6H). Strikingly, cells transduced with shRNA *SRGAP2B/C* lentivirus displayed a significant decrease in morphological complexity compared with untransduced hMGs or hMGs expressing control scrambled shRNA at 3 MPT (Figures 6F–6H). The morphological complexity of hMGs expressing shRNA against human-specific *SRGAP2B/C* was not statistically different from that of the surrounding endogenous adult mouse microglia (Figures 6F–6H). This result demonstrates that the human-specific genes *SRGAP2B/C* are required for the morphological complexity observed in developing human microglia *in vivo*.

Figure 6. The human-specific *SRGAP2C* gene modulates microglial morphology

(A) Schematic representation of the genetic approach used to inducibly express the human-specific gene *Srgap2c* in mouse microglia. (B) Representative images of individual Iba1⁺ microglia from the cortex of WT and *MG-Srgap2c⁺* (*Tmem119^{CreERT2}* *Srgap2c^{F/F}*) mice at P40. Scale bar, 10 μ m. (C and D) Sholl analysis (C) for Iba1⁺ cortical microglia from both control (WT) and *MG-Srgap2c⁺* mice at P40 and morphological complexity (D) of microglia from these mice. Data from four to eight mice per genotype, with 14–26 cells per mouse, with a horizontal line indicating the median and error bars the 95% confidence interval. Statistical differences were calculated using an unpaired *t* test using mice as biological replicates; **p* < 0.05. (E) Experimental strategy to knock down the human-specific *Srgap2b/c* paralogs in hMGs. Human iPSC-derived myeloid progenitors were transduced with an EGFP-expressing Vpx⁺ lentivirus also encoding control (scrambled) shRNA or shRNA targeting both human-specific paralogs *SRGAP2B/C*, scrambled shRNA, or untransduced cells (constitutively expressing GFP). All three groups were xenotransplanted in P0 *Rag2^{-/-}* *il2rg^{-/-}* double-knockout/hCsf-1 knockin mouse recipients and analyzed at 3 MPT. (F) Representative images of untransduced hMGs, lentivirally transduced hMGs, or control endogenous mouse microglia from 3-month-old mice (3 MPT). Scale bar, 10 μ m. (G and H) Sholl analysis (G) and morphological complexity (H) of microglia from these mice. Data from 3 mice per condition, with 32–58 cells per condition, with a horizontal line indicating the median and error bars the 95% confidence interval. Statistical differences were calculated using a one-way ANOVA (Tukey's multiple-comparison test), using mice as biological replicates; ns *p* > 0.05, ***p* < 0.01.

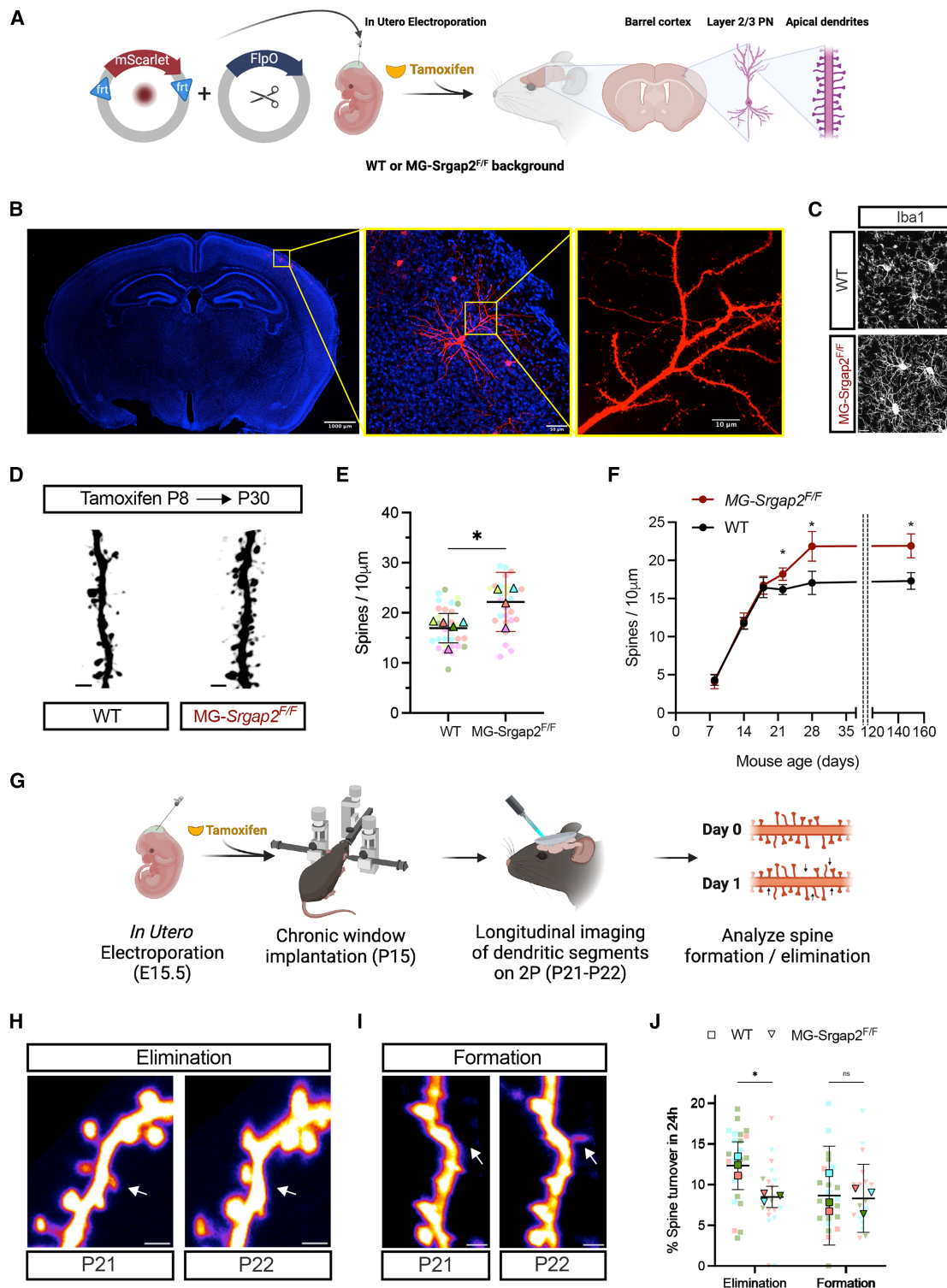


Figure 7. Microglia-specific deletion of *Srgap2* leads to increased dendritic spine density and reduced spine elimination in cortical L2/3 PNs from juvenile mice

(A) Schematic representation of the experimental approach used to obtain sparse fluorescent (mScarlet) labeling of layer 2/3 pyramidal neurons (L2/3 PNs) in a Flp-dependent manner in WT and MG-specific *SRGAP2* conditional knockout mice (*Tmem119^{CreERT2} Srgap2^{F/F}*).

(B) Representative confocal images of a brain section containing sparsely labeled, mScarlet-expressing L2/3 PNs, and a detail of their apical oblique dendrites featuring dendritic spines.

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Microglia-specific *Srgap2* deletion impacts synaptic development in CPNs in a cell-non-autonomous manner

Microglial cells are required for proper synaptic development in the developing brain and have been involved in promoting both synapse formation and elimination.^{1,2,7,8,73–75} Given that in the mouse cortex, synaptic formation and maturation peak during the second and third postnatal weeks, corresponding with the peak of microglial morphological complexity (see Figure 1), we tested whether the delayed microglial maturation observed in *MG-Srgap2^{F/F}* mice could affect the timing of synaptic development in CPNs in a cell-non-autonomous manner. To assess synaptic development and structural dynamics, we used *in utero* electroporation (IUE) to sparsely label layer 2/3 pyramidal neurons (L2/3 PNs) in the somatosensory cortex, using a FlpO-recombinase-dependent expression system (Figure 7A). Briefly, *MG-Srgap2^{F/F}* or control WT littermate mouse embryos were electroporated at E15 with plasmids encoding the red fluorescent protein mScarlet flanked by FRT regions, and low amounts of a plasmid encoding the recombinase FlpO, enabling sparse recombination and expression of mScarlet as a cell filler in L2/3 PNs (Figure 7B). This manipulation enables visualization of dendritic spines in L2/3 PNs in WT mice or mice where *Srgap2* is deleted in nearly 100% of microglia (*MG-Srgap2^{F/F}*). We verified that tamoxifen induction in these mice resulted in increased morphological complexity of Iba1+ microglia compared with control mice (Figure 7C).

We have previously shown in constitutive *Srgap2^{+/-}* mice,³⁹ or following neuron-specific downregulation of *SRGAP2* using shRNA or conditional deletion of *Srgap2*,^{40–42} that reducing *SRGAP2* levels in neurons results in a significant increase in dendritic spine density in apical oblique dendrites of L2/3 PNs, corresponding to an increase in cortico-cortical synaptic inputs received by these neurons.⁴³ Analysis of L2/3 PN apical oblique dendritic spines in mice carrying microglia-specific *Srgap2* deletion revealed a higher spine density at P30 compared with littermate controls (Figures 7D and 7E). These results demonstrate that induction of delayed morphological maturation of microglia in the mouse cortex contributes, in a cell-non-autonomous manner, to the increased synaptic density in CPNs *in vivo*.

We then assessed when this increased spine density emerged during development, as P30 is considered a mature stage for synaptic development under WT conditions. In WT mice, spine density in apical dendrites of L2/3 PNs quickly increased during the first 2 weeks after birth (concomitant with high morphological complexity in WT microglia) and remained stable afterward;

however, in mice carrying microglia-specific *Srgap2* deletion, the spine density of L2/3 PNs increased monotonically during the first 4 weeks after birth and reached a significantly higher density than that of WT mice, which was maintained in adult mice (Figure 7F). This result suggests that induction of *SRGAP2*-dependent neonatal microglial maturation results in a prolonged, cell-non-autonomous period of synaptic development in CPNs, ultimately leading to higher synaptic density.

To determine whether the increased dendritic spine density reached in mice carrying microglia-specific *Srgap2* deletion is the result of increased spine formation and/or reduced spine elimination during the peak of synaptogenesis in L2/3 PNs, we performed longitudinal *in vivo* 2P microscopy (Figure 7G). We implanted a cranial window above the primary somatosensory cortex (S1) of juvenile (P15) WT control mice or littermate mice carrying *MG-specific Srgap2* deletion induced with hydroxytamoxifen at P2–3 and tracked dendritic spine formation/elimination events in the apical dendrites of mScarlet-expressing L2/3 PN at P21 for 24 h (Figures 7H and 7I). We found that although both WT and *MG-Srgap2^{F/F}* neurons exhibited similar rates of spine formation, *MG-Srgap2^{F/F}* neurons displayed lower rates of spine elimination (Figure 7J). Altogether, our data suggest that the delayed increase in spine density characterizing mouse L2/3 PNs following microglia-specific *Srgap2* conditional deletion (Figure 7F) is correlated with decreased rates of synaptic elimination (Figures 7H–7J).

DISCUSSION

Altogether, our data indicate that *SRGAP2* is essential for the maturation of mouse and human microglia and that delayed microglial maturation may lead to extended periods of synaptic development. We uncovered that (1) human cortical microglia are characterized by a slow, neonatal timing of morphological, functional, and transcriptional maturation and that (2) the truncated proteins expressed by the human-specific genes *SRGAP2B/C*, by downregulating *SRGAP2A* protein,^{39,41,42} are necessary and sufficient to induce delayed morphological maturation of human microglia, which, in turn, influences the timing of synaptic maturation in CPNs.

SRGAP2B/C as human-specific genetic modifiers of microglial development

Recent scRNA-seq transcriptomic studies have highlighted the developmental trajectories of human microglia during fetal and

(C) Representative images of Iba1+ microglia from P30 WT and *MG-Srgap2^{F/F}* mice that had undergone *in utero* electroporation (IUE).

(D) Representative images of L2/3 PN dendritic segments from P30 WT and *MG-Srgap2^{F/F}* mice. Scale bar, 2 μ m.

(E) Quantification of dendritic spine density from apical oblique dendrites of L2/3 PNs from these two genotypes. Data from 2 dendritic segments per neuron, for a total of 14–16 neurons from 4 to 5 mice per genotype, with a horizontal line indicating the median and error bars the 95% confidence interval. Statistical differences were calculated using an unpaired *t* test using mice as biological replicates; **p* < 0.05.

(F) Dendritic spine density of L2/3 PNs from WT and *MG-Srgap2^{F/F}* mice throughout mouse cortical development. Data from 7 to 22 neurons from 3 to 7 mice per genotype and time point; curve data show the group mean of each time point, and error bars indicate the 95% confidence interval. Statistical differences were calculated using a two-way ANOVA (Sidak multiple-comparison test); **p* < 0.05.

(G) Strategy to longitudinal 2P microscopy imaging of spine dynamics in L2/3 PN dendritic segments *in vivo* in juvenile mice (P21–22).

(H and I) Representative examples of dendritic spine elimination (arrow in H) and formation (arrow in I) in L2/3 PNs during a 24-h time period. Scale bar, 2 μ m.

(J) Rates of dendritic spine formation and elimination in L2/3 PNs from WT and *MG-Srgap2^{F/F}* mice. Data from 3 mice per genotype, with 18–23 segments per genotype, with a horizontal line indicating the median and error bars the 95% confidence interval. Statistical differences were calculated using a two-way ANOVA (Sidak multiple-comparison test), using mice as biological replicates, ns *p* > 0.05, **p* < 0.05.

postnatal stages, although maturational trajectories over postnatal development, and at longer timescales, are scarcely available and still poorly characterized.^{33–36,76} Our results suggest that microglial maturation, in morphological, functional, and transcriptional terms, occurs over much slower, protracted periods of development, taking about a decade in human compared with a couple of months after birth in mouse microglia. We identify the human-specific genes *SRGAP2B/C* as critical modifiers of the timing of morphological microglial maturation in the developing human cortex.

Although we have identified the function of one candidate human-specific genetic modifier of microglia, i.e., the human-specific paralogs *SRGAP2B/C*, other potential human-specific modifiers could also have played a role during evolution in the acquisition of human-specific features of microglia. These include species-specific epigenetic⁷⁷ and metabolic⁷⁸ developmental programs setting the slow pace of human neuronal maturation, human-specific base pair substitutions in regulatory regions otherwise ultra-conserved throughout vertebrate evolution (termed Human Accelerated Regions) that might impact spatiotemporal patterns of gene regulatory network deployment,^{79–81} the occurrence of human-specific mRNA splicing events,⁸² longer protein half-lives,⁸³ or the presence of human-specific polymorphisms in genes involved in microglial function.^{38,84}

The increased number of synapses per neuron characterizing human cortical excitatory neurons^{85–87} has been proposed to represent a key human-specific modifier that might have enabled the emergence of our higher cognitive abilities during human evolution.^{4,32,88–90} Recently, using human CPN xenotransplantation and mouse genetic models, multiple studies have shown that human-specific genes (such as *SRGAP2B/C*) are necessary and sufficient to induce delayed, neonatal excitatory synapse maturation and ultimately contribute to the increased excitatory synapse number received by human CPNs.^{29,41} Our present results now suggest that the expression of the human-specific genes *SRGAP2B/C* in human cortical microglia also contributes to the neonatal timing of synaptic maturation characterizing human CPNs.

Interestingly, the slow neonatal developmental trajectory characterizing human microglial structural maturation is maintained *in vivo*, at least partially, in xenotransplanted hMGs, suggesting that certain features (such as the tempo of structural maturation) of microglial development are cell intrinsic. Conversely, our results also show that the developmental expression pattern of other markers, such as the microglia-specific phagolysosomal marker CD68 (also called *LAMP4*; pertaining to its ability to degrade intracellular cargo), is mainly driven by extracellular context (environmental and temporal) to which microglia are exposed during cortical development and that a temporal mismatch between the developmental “age” of microglia and their extracellular environment (as is the case for xenotransplanted hMGs in the mouse cortex) precludes the proper timing of expression of functional markers such as CD68.

Previous work from our lab and others demonstrated that neuronal *Srgap2* cell-autonomously regulates excitatory synapse density and the timing of synaptic maturation in mouse and human L2/3 PNs and that induction of human-specific

SRGAP2C expression in mouse L2/3 PNs leads to increased cortico-cortical connectivity, more reliable sensory-evoked responses, and improved behavioral performance in a sensory-discrimination task.^{39,40,43} Given the prominent role of microglia in fine-tuning synaptic connectivity during postnatal brain development, it is likely that the same mechanisms are at play during human postnatal brain development, and our results argue that, in the human cortex, the expression of the human-specific genes *SRGAP2B/C* in microglia contributes to the prolonged period of synaptic maturation and possibly to the increased synaptic density observed in adult human CPNs.

However, although the molecular mechanisms underlying *SRGAP2* function in excitatory and inhibitory synapse maturation have been well characterized in neurons and involve both interactions with postsynaptic scaffolding proteins such as Homer1 and inhibition of postsynaptic proteins SYNGAP1 and CTNND2, two negative regulators of synaptic maturation,^{39–41,91} the molecular and signaling mechanisms underlying *SRGAP2* function in microglial structural maturation remain unclear, as none of the *SRGAP2*-interacting proteins identified so far in neurons are expressed in microglia. *SRGAP2* was shown to regulate actin dynamics through actin severing (via bimodal regulation of formin-like proteins such as FMNL1) and through modulating Rac1 activity (via its Rac1-specific GTPase-activating protein [GAP] domain), at least in cell lines.^{64,92} Interestingly, FMNL2 and FMNL3 are highly expressed in developing and adult microglia.^{57,93} Future studies will need to investigate the signaling effectors linking *SRGAP2* to actin remodeling and to the process dynamics of microglia *in vivo*.

Species-specific temporal coordination of microglial and synaptic development

Our estimate of the timing of human microglial maturation largely coincides with the neonatal timing of synapse maturation during human cortical development.^{4,6,16,57} Overall, our results highlight the fact that the timing of microglial and synaptic maturation must be synchronized in a species-specific manner during brain development. Indeed, our data suggest that the timing of morphological maturation of microglia during cortical development is developmentally synchronized with the delayed timing of synapse maturation in both mouse and human—albeit at strikingly different absolute and relative timescales. As shown in Figure 1E, even when normalized to a developmental milestone such as sexual maturity (~P50 [~7% of lifespan] in mice and ~12 years [~17% of lifespan] in humans^{47,48}), the peak of microglial morphological complexity (~P21 in mice versus ~7 years in humans) and the peak of dendritic spine density (P30 in mice versus ~10–15 years in humans in primary sensory areas and the prefrontal cortex, respectively^{30,49}) are extended not only in absolute time but also relative to lifespan or sexual maturity. This remarkable difference in the timing of maturation between mouse and human microglia seems to be partly regulated by the ability of the human-specific proteins *SRGAP2B/C* to inhibit *SRGAP2A* function and could therefore also be dependent on the regulatory mechanisms controlling the timing of *SRGAP2A* and *SRGAP2B/C* mRNA expression: in Figures 3 and S3, we leveraged existing single-cell transcriptomics data to show that *SRGAP2* belongs to a module of genes whose expression

monotonically increases during postnatal development to peak at ~P20 in mouse cortical microglia and at ~6 years after birth in human cortical microglia. These transcriptomic data, together with our gain- and loss-of-function assessments of SRGAP2 and SRGAP2B/C function in mouse and human microglia, suggest that (1) SRGAP2 is necessary and sufficient to promote the rate of microglial structural maturation and that (2) SRGAP2B/C, by inhibiting SRGAP2A function, delay the timing of structural maturation in human microglia. Finally, we show that microglia-specific deletion of SRGAP2 in mouse microglia delays their structural maturation (Figure 4) and delays dendritic spine development (Figures 7A–7F), presumably by delaying spine elimination (Figures 7H–7J).

Our results also highlight a set of genes (module 160) characterized by a temporal pattern of expression (monotonically increasing), coinciding with the predicted maturation of human microglia. Interestingly, these genes are overrepresented in transcriptomic signatures from primary human microglia obtained from patients with Alzheimer's disease.⁹⁴ Given the specificity of this transcriptional signature for human microglia,³⁸ the impact of these genes on microglial functional and structural development has been previously overlooked. Finally, SRGAP2 has recently been shown to be enriched in microglial subsets that express high levels of the disease-associated microglial (DAM) transcriptional program.⁹⁴ Future work will shed light on the role of the genes contained in module 160, including SRGAP2, in shaping the human microglial response to neurodegenerative diseases. Indeed, in the context of disease, polymorphisms observed in the human APOE and TREM2 gene loci have been shown to endow hiPSC xenotransplanted microglia with distinct transcriptional responses to amyloid beta plaques,³⁸ suggesting that this is another potential mechanism by which microglia can exhibit human-specific structural and/or functional features. Future studies will need to assess the impact of these other factors on the acquisition of human-specific features in microglia.

Limitations of the study

Compared with other hMG models (such as *in vitro* or organoid-based co-cultures), the hMG xenotransplantation model is considered to mirror postnatal development of human microglia most closely, both morphologically and transcriptionally.^{33,52,53,95} However, hMGs still reside and develop within a mouse brain environment, and as such, the role of extracellular cues in microglial development may also depend on species-specific interactions. For example, our results reveal that the temporal profile CD68 expression is different in hMGs xenotransplanted into the mouse cortex compared with human cortical microglia *in vivo* and, therefore, we hypothesize that CD68 and the lysosomal degradation potential of microglia may be driven by extrinsic, environmental cues rather than by cell-intrinsic cues. In this sense, a new xenotransplantable tri-culture organoid system was recently developed, where most of the extracellular cues (such as Csf1) can be provided by isochronic human astrocytes and neurons present in the organoid.⁹⁶ Interestingly, mouse microglial development has been shown to be partially dependent on cues from the microbiota and its interaction with other immune cells, mainly CD4⁺ T cells.^{97,98} Whether such interactions play a role in human mi-

croglial maturation, and whether they are not fully captured in the hMG xenotransplantation model, remains to be addressed. The relatively short lifespan of the inbred mouse lines commonly used, such as C57BL/6 (~2 years), currently limits our ability to fully assess hMG maturation longitudinally and its relationship with human primary microglia, as exemplified by our finding that even 10 months after xenotransplantation, human microglia have not started to undergo morphological simplification.

Furthermore, microglial function and morphology can be influenced by the close crosstalk between neighboring neurons and astrocytes. Although the genetic manipulations used in the present study to elucidate the role of Srgap2 in microglia are microglia specific, it is formally possible that Srgap2 deletion in microglia alters neuronal activity and the timing of circuit maturation, which, in turn, could influence microglial maturation and thereby contribute to some of the observed morphological phenotypes.

Our observation that Srgap2-deficient mouse microglia contain fewer engulfed synaptic proteins such as VGLUT2 (Figure S6), and that neurons in MG-Srgap2 juvenile mice have lower rates of synapse elimination (Figure 7J), correlates microglial engulfment with synapse elimination. Further work will need to directly assess how Srgap2-deficient microglia engulf synapses *in vivo* and shape neuronal connectivity.

Here, we have described the morphological maturation trajectories of human and mouse cortical microglia throughout cortical development, using features such as microglial density, morphology, gene expression, and CD68 content to comparatively assess these in both absolute timing and also relative to developmental milestones such as sexual maturation (Figure 1E). Although the endolysosomal marker CD68 is widely used across the macrophage literature to report on the phagocytic capacity of macrophages and microglia,^{44,45} it is not a direct measure of microglial phagocytic activity because it also plays a role in other degradation mechanisms, such as autophagy upon autophagosome fusion with lysosomes. Still, we believe that reporting CD68 content across development adds informative value to the maturational stage of microglia as a descriptor for their degradation potential across time. More investigation will be required to better understand the molecular mechanisms influencing the developmental maturation of microglial phagocytic capacity versus its morphological maturation, which is controlled in part by SRGAP2.

Our results obtained using microglia-specific deletion of SRGAP2 in mice *in vivo* (shown in Figures 4J and 4K) demonstrate delayed morphological maturation of microglia, which, in turn, induces delayed dendritic spine development (Figures 7A–7F). Therefore, our interpretation is that SRGAP2A regulates the timing of structural maturation in mouse microglia and that it is likely that SRGAP2B/C expression in human microglia, by inhibiting SRGAP2A expression, plays a role in the timing of human microglial morphological maturation. However, our quantification of SRGAP2B/C knockdown in human microglial xenotransplantation relies on a single time point (3 MPT), at which it induces a reduction in human microglial complexity compared with control human microglia, which is compatible with a change in the timing of their maturation, but we cannot exclude the possibility that SRGAP2A and its human-specific paralogs SRGAP2B/C regulate overall microglial complexity.

Lastly, we highlight a transient hyper-ramified state of microglial morphology that correlates with the timing of synaptic maturation. Although we have identified this transitional state in two systems (human and mouse), it is possible that it is restricted to the specific brain regions tested and dependent on extrinsic factors, such as levels of neuronal activity, wake state, anesthesia, or microbiota composition, as previously reported.^{10,13,97} Although it is widely accepted that fully mature, homeostatic microglia exhibit limited transcriptional heterogeneity in non-diseased physiological conditions,^{36,76,99} it will be important to test whether the timing of microglial maturation differs across brain regions outside the neocortex in response to the local environment.¹⁰⁰ For example, it has been shown that cerebellar microglia exhibit region-specific functional and developmental differences compared with cortical microglia.^{101,102} Future research will elucidate whether the molecular mechanisms described in our study regulate the timing of microglial maturation in other brain regions.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to, and will be fulfilled by, the lead contact, Franck Polleux (fp2304@columbia.edu).

Materials availability

All new unique reagents generated for this study and listed in the [key resources table](#) are available either through repositories such as Addgene or upon request addressed directly to the [lead contact](#) following completion of a materials transfer agreement.

Data and code availability

Raw sequences for the bulk RNA-seq performed on FACS-purified microglial cells in [Figures S6A–S6C](#) are deposited in GEO under accession no. GSE335485. Analysis and plotting code used for the single-cell RNA-seq analysis ([Figures 3, 4A, and S3](#)) are available on Zenodo (DOI: <https://doi.org/10.5281/zenodo.20618142>). All other data and source material should be requested from the [lead contact](#), Franck Polleux (fp2304@columbia.edu).

ACKNOWLEDGMENTS

We thank Sergio Bernal Garcia for valuable input and technical assistance during the optimization of image acquisition and processing of two-photon microscopy data. We thank Cynthia Jia for support during image data analysis. We thank Dr. Nathaniel Landau at NYU for generously providing the Vpx expression plasmids and Dr. Dorothy Schafer for insightful discussion and experimental guidance. We thank Sergio Bernal Garcia, Aleksandra Recupero, and other Polleux lab members for extensive discussions. We thank Dr. Pierre Vanderhaeghen for help editing the manuscript. Some of the schematic illustrations were generated with BioRender. We thank Qiaolian Liu for excellent technical assistance with mouse colony management. Imaging was performed at the Zuckerman Institute's Cellular Imaging Platform with assistance from Dr. Darcy Peterka. This work was supported by a K99/R00 award (K99NS138587; J.K.), an NIH-NIMH grant (R01MH132689; A.B.), a Revson Senior Fellowship in Biomedical Sciences (REVSON 23-17; C.D.-S.), a career award from the NIH-NINDS (R35 NS127232; F.P.), and an award from the NO-MIS Foundation (F.P.).

AUTHOR CONTRIBUTIONS

Conceptualization, C.D.-S. and F.P.; methodology, C.D.-S., J.K., M.K., J.Y., P.R.N., and F.P.; primary human data acquisition and analysis, J.K. and C.D.-S.; human iPSC work and xenotransplantation, M.K.; scRNA-seq data analysis, J.Y. and P.R.N.; investigation, C.D.-S., J.K., J.Y., and P.R.N.; re-

sources, C.D.-S., F.P., A.B., and R.J.; project administration, C.D.-S. and F.P.; supervision, F.P., A.B., and M.P.; funding acquisition, C.D.-S., M.K., R.J., M.P., and F.P.; writing – original draft, C.D.-S., J.Y., P.R.N., and F.P.; writing – review and editing, C.D.-S. and F.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.neuron.2026.07.007>.

Received: August 29, 2024

Revised: April 18, 2026

Accepted: July 7, 2026

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Iba1 (clone E4O4W)	Cell Signaling	Cat#17198S; RRID: AB_2820254
CD68 (Clone FA-11)	BioRad	Cat#MCA1957; RRID: AB_322219
CD45 (clone 104) (Flow Cytometry)	Tonbo	Cat#25-0454; RRID: AB_2621630
CD11b (clone M1/70) (Flow Cyt.)	Tonbo	Cat#20-0112; RRID: AB_2621556
CX3CR1 (clone SA011F11) (Flow Cyt.)	Biolegend	Cat#149015; RRID: AB_2565699
Human CD68 (clone KP1)	eBioScience	Cat#14-0688-82; RRID: AB_11151139
Vglut2 (polyclonal)	Sigma Aldrich	Cat#AB2251-I; RRID: AB_1587626
Alexa488 Goat α Rabbit	Thermo Fisher Scientific	Cat#A-11008; RRID: AB_143165
Alexa555 Goat α Rat	Thermo Fisher Scientific	Cat#A-21434; RRID: AB_141733
Alexa555 Goat α Rabbit	Thermo Fisher Scientific	Cat#A-21428; RRID: AB_2535849
CF568 Goat α Rat	Biotium	Cat#20096; RRID: AB_10559813
CF568 Goat α Rabbit	Biotium	Cat#20801; RRID: AB_3678764
Biological samples		
Healthy developing and adult human cingulate cortex tissue	Mercedes Paredes lab at UCSF	Table S1
Chemicals, peptides, and recombinant proteins		
Phosphate Buffer Saline (PBS)	Cytiva	Cat#SH30256.FS
Goat Serum	Invitrogen	Cat#16210-064
Fetal Bovine Serum	Thermo Fisher Scientific	Cat#16141002
Triton X-100	Sigma-Aldrich	Cat#T9284
4',6 Diamidino 2 Phenylindole, Dihydrochloride (DAPI)	Thermo Fisher Scientific	Cat#D1306
Formaldehyde Aqueous Solution, 32%, Liquid (PFA)	Electron Microscopy Solutions	Cat#15714-S
Prolong Glass Antifade	Thermo Fisher Scientific	Cat#P36980
Tamoxifen	Sigma-Aldrich	Cat#T5648
(Z)-4-hydroxy Tamoxifen	TargetMol	Cat#T4420
TRIzol	Thermo Fisher Scientific	Cat#15-596-018
Percoll	Sigma-Aldrich	Cat#P4937
NEBNext® Ultra™ II RNA Library Prep kit	New England Biolabs	Cat#E7770S
NEBNext Poly(A) mRNA Magnetic Isolation Module	New England Biolabs	Cat#E7490
NEBNext Multiplex Oligos for Illumina	New England Biolabs	Cat#E7335S
ROCK inhibitor	EMD Millipore	Cat#688000
BMP-4	Thermo Fisher Scientific	Cat#RHBMP-4
SCF	Life Technologies	Cat#PHC2115
VEGF-121	Life Technologies	Cat#PHC9394
M-CSF	Life Technologies	Cat#PHC2015
IL-3	Life Technologies	Cat#PHC0034
Protamine sulfate	Thermo Scientific	Cat#J62926.06
Zeocin	Thermo Scientific	Cat#R25001
Dulbecco's Modified Eagle Medium (DMEM)	Cytiva	Cat#05890
Lenti-X GoStix Plus tests	Takara Bio	Cat#631280

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Polyethylenimine, 25000 Molecular Weight, Linear (PEI)	Polysciences	Cat#23966-100
Low toxicity silicone adhesive	World Precision Instruments	Cat#KWIK-SIL
Parkell C&B Metabond Quick Adhesive Cement System (Dental cement)	Parkell	Cat#04-PKL-S380
Artificial Cerebrospinal Fluid (aCSF)	Tocris	Cat#3525
3 mm round coverglasss, #0 thickness	Warner Instruments	Cat#64-0726
Loctite Super Glue Ultra Liquid control	Loctite	Cat#1647358

Deposited data

Bulk RNA sequencing (Figure S6A-C)	Polleux lab	GEO: GSE335485
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Experimental models: Cell lines

HEK-293T cell line	ATCC	Cat#CRL-3216
WIBR-hiPS-SNCA ^{A53T-Corr}	Jaenisch Lab ¹⁰³	N/A

Experimental models: Organisms/strains

Mouse: 129S2/SvPasCrl mice (129 mice)	Charles River	287
Mouse: B6.Cg-Gt(ROSA) 26Sor ^{tm9(CAG-tdTomato)Hze/J} (Ai9)	The Jackson Laboratory	007909
Mouse: C57BL/6- Tmem119 ^{erm1(Cre/ERT2)Gfng/J} (Tmem119 ^{CreERT2})	The Jackson Laboratory	031820
Mouse: Constitutive Srgap2 knockout mouse	Polleux Lab ³⁹	N/A
Mouse: Conditional Srgap2 knockout mouse (Srgap2 ^{F/F})	Polleux Lab ⁶⁴	N/A
Mouse: Rosa26 ^{SRGAP2C(F/+)} knockin mouse (Srgap2c ^{LSL})	Polleux Lab ⁴³	N/A
Mouse: C;129S4.Rag2 ^{tm1.1Flv} Csfl ^{tm1(CSF1)Flv} Il2rg ^{tm1.1Flv/J} (Rag2 ^{-/-} il2rg ^{-/-} hCsf-1 knockin)	The Jackson Laboratory	017708

Recombinant DNA

pAAV-CAGGS-EGFP	Jaenish lab ¹⁰⁴	Addgene #22212
pCAG-FlpO	Scanziani lab ¹⁰⁵	Addgene #60662
pAAV-CAG-fDIO-mNeonGreen	Gradinaru lab ¹⁰⁶	Addgene #99133
pAAV-EF1a-DIO-mGreenLantern	Petsko lab ¹⁰⁷	Addgene #164468
pmScarlet_C1	Gadella lab ¹⁰⁸	Addgene #85042
pAAV-CAG-fDIO-mGreenLantern-FRT (mGreenLantern-FRT)	Polleux lab (this study)	N/A
pCAFNF-fDIO-mScarlet-FRT (mScarlet-FRT)	Polleux lab ¹⁰⁹	N/A
shRNA Scramble	Polleux lab ³⁹	N/A
shRNA targeting human <i>SRGAP2B/C</i>	Polleux Lab ^{39,41}	N/A
pMDL-X	Landau Lab ⁷¹	N/A
pCMV:VSV-G	Landau Lab ⁷¹	N/A
pCMV:HIVRev	Landau Lab ⁷¹	N/A
pCMV:Vpx	Landau Lab ⁷¹	N/A

Software and algorithms

Analysis and plotting code for single cell RNA sequencing and k-means clustering	Badhuri lab ⁵⁶	DOI:10.5281/zenodo.20618142
Fiji and ImageJ plugins	Cardona lab ¹¹⁰	https://imagej.net/software/fiji/
Prism version 11.0.2	GraphPad	https://www.graphpad.com/features

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
NIS Element image acquisition and analysis software	Nikon	https://www.microscope.healthcare.nikon.com/products/software/nis-elements
pyDESeq2- Differential gene expression analysis bulk RNA sequencing	Andreux lab ^{111,112}	https://pydeseq2.readthedocs.io/en/stable/
Image analysis for expansion microscopy samples	Imaris- Oxford Instruments	https://imaris.oxinst.com/imaris-viewer
Software driving Bergamo II 2-photon microscope	ThorImage ® LS	https://www.thorlabs.com/thorimage-r-ls?tabName=Overview

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human tissue collection

Eight de-identified human brain specimens were collected during autopsy, with a post-mortem interval of fewer than 48 hours (Table S1). Tissue was collected with the previous patient's consent in strict observance of the legal and institutional ethical regulations of the University of California, San Francisco Committee on Human Research. Protocols were approved by the Human Gamete, Embryo, and Stem Cell Research Committee and the Committee on Human Research (institutional review board) at the University of California, San Francisco.

Human induced pluripotent stem cell (hiPSC) culture, myeloid precursor differentiation, lentiviral infection and xenotransplantation

Human iPSCs lines used in this study were previously generated and characterized in the Jaenisch Lab.¹⁰³ The cell lines used for the morphological characterization of wild-type human microglia, WIBR-hiPS-SNCA^{A53T-Corr}, stably expressed Green Fluorescent Protein (GFP) from the AAVS1-locus after targeting with a Zinc-Finger GFP-expressing AAV, as previously described.^{113,114} The cell line used for *Srgap2b/c* knockdown experiments, WIBR-hiPS-SNCA^{A53T-Corr}, was unlabeled. This human iPSC lines were maintained in feeder-free conditions and regularly tested for mycoplasma contamination. HiPSCs were differentiated into myeloid precursors (MPs) using a previously published protocol.^{114,115} Briefly, hiPSCs were cultured for four days in embryoid body medium (containing ROCK inhibitor, BMP-4, SCF, and VEGF-121) and then cultured in hematopoietic medium (containing M-CSF and IL-3) for a maximum of 1.5 months, during which cells were harvested. For *Srgap2b/c* knockdown experiments, cultured MPs were infected a mix of lentivirus and protamine sulfate (5 µg/mL, Thermo Fisher Scientific) to enhance lentiviral integration into the cells and infected cells were subsequently selected with zeocin (Thermo Fisher scientific) at 1 mg/mL 72 hours after infection. For xenotransplantation, cultured MPs were resuspended in phosphate-buffered saline without calcium and magnesium prior to injection at a concentration of 10⁵ cells/µL. Postnatal day 0 (P0) to P3 mouse pups of either sex were manually injected with 4.10⁵ MPs in the lateral ventricles (1 anterior and 1 posterior injection site per brain hemisphere) using glass micropipettes.

Mice

All animals were handled according to protocols approved by the institutional animal care and use committee (IACUC) at Columbia University, New York. All experiments involving xenotransplantation were performed according to the standards set forth by the Department of Comparative Medicine and Massachusetts Institute of Technology. Mice of both sexes were used at approximately equal ratio and there was no influence of sex on any of the findings reported in this study. All mice used in these experiments were maintained on a regular 12 h light/dark cycle at 20 to 22 °C, humidity between 30 to 70%, and given feed and water *ad libitum*. Tmem119^{creERT2} mice⁶³ and Ai9 mice⁶⁷ were obtained from Jax. Recipient mice for xenotransplantation (*Rag2*^{-/-}*IL2rg*^{-/-} mice carrying the transgene for the human *Csf1* allele, C;129S4-*Rag2*^{tm1.1Flv} *Csf1*^{tm1(CSF1)Flv} *Il2rg*^{tm1.1Flv/J}) were obtained from Jackson Labs. Previously generated and validated mouse lines constitutive *Srgap2* knockout,³⁹ conditional *Srgap2*^{fl/fl} knockout,⁶⁴ and *Srgap2c*^{LSL} knockin mice⁴³ were generated in house and previously validated. All mouse lines used to compare differences across genotypes were littermates (except those generated to analyze microglial surveillance) and received the same tamoxifen dosage, and genotype was blinded to the experimenter. Individual strains were kept in a C57BL/6J background crossed with the outbred strain 129S2/SvPasCrl mice, for the purpose of improving pup viability and maternal care following *in utero* electroporation. For experiments performed on adult mice (>P28) mice received two doses (75 µg/g body weight, BW) of tamoxifen (Sigma-Aldrich) at P7-8. For experiments performed on younger mice (<P28), mice received two doses (50 µg/g BW) of 4-hydroxytamoxifen (TargetMol) at P2-3. hMGs and *Srgap2*^{-/-} mice did not receive any tamoxifen.

METHOD DETAILS

In utero Electroporation

In Utero Electroporation (IUE) was performed on isoflurane-anesthetized timed-pregnant females at E15.5 as previously described.¹¹⁶ Briefly, after exposing the embryos from the abdominal cavity, an endotoxin-free DNA mix containing dose-limiting amounts (5 ng/μl for [Figures 7B–7E](#), and 20 ng/μl for [Figures 7F–7I](#)) of a FlpO plasmid, and 1 μg/μl of a flp-dependent fluorescent protein-encoding plasmid (mScarlet for [Figures 7B–7E](#), and mGreenLantern for [Figures 7F–7I](#)) were injected into the ventricles of mouse embryos using a heat-pulled glass pipette. mScarlet-FRT and mGreenLantern-FRT plasmids were generated by subcloning the mScarlet and mGreenLantern coding sequence (Addgene #85042 and #164468, respectively) inside the FRT-flanking region of a CAG-promoter plasmid (Addgene #99133). Electroporation into cortical neurons was performed applying 4 pulses of 42 V for 50 ms with 500 ms intervals, using a 3 mm diameter platinum tweezer electrode (Nepa Gene) and a square wave electroporator (ECM 830, BTX). Embryos were placed back into the abdominal cavity and the incision was sutured, allowing the mouse to recover on a heated pad and give birth naturally.

Cortical Window Implantation

Adult (~P50 for [Figures 5A–5C](#)) or juvenile (P15, for [Figures 7F–7I](#)) mice were anesthetized with isoflurane and given analgesic (rimadyl, 10 μg/g BW) and topical anesthetic (lidocaine 10 μg/g BW), prior to exposing the dorsal skull with a scissors. After performing a craniotomy, a glass window (3 mm diameter, Warner Instruments) was placed over the primary somatosensory cortex and secured with cyanoacrylate (Loctite super glue). Ice-cold sterile artificial cerebrospinal fluid (aCSF, Tocris) was applied when necessary to prevent excessive heating and dryness throughout the craniotomy. A custom 3D-printed headplate was secured onto the skull using dental adhesive cement (Parkell). Coverslip was protected with silicone (World Precision Instruments) until imaging and reapplied between imaging sessions. After surgery, mice were injected with 20 μl/g BW of sterile PBS and allowed to recover under a heated pad. Mice received two additional daily analgesic injections after surgery and were imaged after 2 weeks for adult mice, and 6 days for juvenile mice.

Lentivirus production

Vpx-containing Lentiviruses were produced as described.⁷¹ Briefly, HEK293 cells were cultured in DMEM High Glucose (Cytiva) according to standard protocol and transfected using polyethylenimine (PEI) with the following plasmids: pMDL-X, VSV-G, HIVRev, Vpx, and shRNA-encoding plasmid at a mass ratio of 10:7:5:2:28. pMDL-X, pCMV:VSV-G, pCMV:HIVRev, pCMV:Vpx -encoding plasmids were a gift of Dr. Nathaniel Landau (NYU). Plasmids encoding shRNA against SRGAP2B/C and scramble shRNA were generated and previously validated.^{39,41} 72 hours after transfection, the viral supernatant was collected, centrifuged at 2,000rpm for 5 min to remove cell debris, filtered through a 0.45 μm PES membrane, and further concentrated by centrifuging at 16,000 rpm for 2h at 4°C on a tabletop centrifuge. Pellets were stored at -80°C. Viral titers were estimated using Lenti-X GoStix tests (Takara Bio).

Mouse brain slice preparation, immunostaining, confocal imaging and analysis

At the indicated age, mice were anesthetized with isoflurane and perfused intracardially with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences). Brain was left to fix on PFA for 2h at room temperature (RT) and then wash on PBS at 4°C overnight (O/N). Brains were sectioned coronally at 100μm thickness on a vibratome 9Leica VT1200S). When immunostained, slices were incubated in blocking buffer (5% Goat Serum, 0.5% Triton X in PBS) for 30 min at RT, and stained with their respective primary antibodies (Iba1 clone E4O4W, Cell Signaling; and CD68 Clone FA-11, BioRad) in staining buffer (2% Goat Serum, 0.5% Triton X in PBS) at RT O/N. The following day, slices were washed twice with PBS and incubated with fluorescent secondary antibody in staining buffer. Slices were finally washed and incubated in DAPI (1:5000 in PBS) and mounted on slides with Prolong Glass Antifade (Thermo Fisher Scientific). All antibodies used in this study are listed on the reagent's table. Slices for microglia morphological analysis were imaged using a Nikon A1 laser-scanning confocal microscope controlled by NIS-elements (Nikon) at 1024x1024 using a 20x Plan Apo 0.8 NA objective (Nikon) to quantify microglia numbers and a 60x Plan Apo 1.4 NA oil immersion objective (Nikon) to quantify their morphology. For morphology measurements, a 12 μm z-section was acquired with a 0.3 μm z-step and converted to Maximum Intensity Projections (MaxIP) of each z-stack for analysis. For experiments involving dendritic spine quantification, images were acquired on a CSU-W1 Yokogawa Spinning Disk Field Scanning Confocal System (Nikon) using a 100x Plan Apo 1.35 NA silicone-immersion objective (Nikon). Images were acquired with a 0.15 μm z-step and variable z-section thickness dependent on dendritic segment orientation. Microglial morphological complexity from MaxIP images was quantified on Fiji ImageJ using 2D sholl analysis. Briefly, individual microglia were manually cropped, binarized while excluding particles <20pixels, and a pointer was added to the cell center for sholl analysis. Dendritic spine density from L2/3 pyramidal neurons was manually quantified across the z-section (3D). Large brain slice-wide images were obtained with a 4X Plan Apo 0.2 NA (Nikon).

RNA sequencing sample preparation, library generation, and data analysis

Fresh cortices from ice-cold PBS-perfused anesthetized mice were quickly homogenized made into a single-cell suspension by passing it through a 100 μm filter. Immune cells were isolated by centrifuging a brain homogenate on a 40% Percoll gradient at

2000 rpm for 5 min at 4°C. Myelin and fat debris were removed, cells washed with PBS, and incubated (2% FBS in PBS) with the following antibodies for 30 min at 4°C: CD11b-APC (1:200, Tonbo), CD45-APCCy7 (1:200 Tonbo), Cx3Cr1-PECy7 (1:200 Biolegend). After washing twice, microglia (~100,000 cells / cortex) were sorted directly into TRIzol (Thermo Fisher Scientific) as CD11b⁺, CD45^{low}, CX3CR1⁺ cells on a Sony MA900 (SONY) sorter with a 100 µm microfluidic chip. TRIzol containing the cell lysates was mixed with chloroform at a 5/1 ratio, shaken and spun at 12,000 g for 15 min at 4°C. Clear supernatant was transferred to a fresh tube and washed with 70% ethanol. RNA cleanup was performed on a RNeasy Mini (Qiagen) according to manufacturer instructions. RNA quantity and quality was assessed with a 2100 Bioanalyzer (Agilent). cDNA Libraries were prepared from samples with >8.5 RIN using the NEBNext Ultra II RNA Library Prep kit, the NEBNext Poly(A) mRNA Magnetic Isolation beads, and the NEBNext Multiplex Oligos for Illumina (all New England Biolabs), all according to manufacturer's instructions. Samples were run on a NextSeq 2000 analyzer (Illumina) according to manufacturer's instructions. Reads were aligned to the GRCh38 mouse genome using BaseSpace DRAGEN RNA app (Illumina). Differential gene expression analysis was performed with pyDESeq2.^{111,112} Genes with less than 10 read counts in total were filtered out. ERT2 expression from the *Tmem119^{creERT2}* locus was mapped to the *Esr1* gene and was thus filtered out ($\log_2\text{FC}=\text{xx}$, $\text{padj}=\text{ppp}$) for visualization. Genes with $|\log_2\text{FC}(\log_2 \text{ fold change})| > 0.5$ and adjusted p-value ($\log\text{FDR}$) < 0.01 between MG-Srgap2^{F/F} and WT samples were assigned as up- or down-regulated.

Expansion Microscopy and Synaptic material engulfment analysis

Mice were anesthetized with isoflurane and perfused with PFA as described in main [STAR Methods](#). Brains were similarly sectioned, fixed, and immunostained similarly as described. Slices were stained with αIba1 , αCD68 and αVglut2 antibodies to identify microglial cells, phagolysosomes, and pre-synaptic puncta in the S1 barrel cortex, respectively. A pre-expansion image of the region of interest was taken to calculate the expansion coefficient. Slice fixation, gelation, digestion and expansion were performed as previously described (PMID: 38370645). Briefly, slices were first incubated with 0.1 mg/ml AcX solution in PBS overnight to prepare proteins for anchoring to the swellable gel matrix. After washing with PBS, a polyacrylamide-based gel mix (sodium acrylate, acrylamide, bis-acrylamide monomers with APS and TEMED polymerization initiators) was added onto the slices and gelation was carried out at 37°C for 2h in the dark in a humidity chamber. Next, slices were digested with Proteinase K 0.2mg/ml in Tris-EDTA buffer overnight at RT. Finally, gels were expanded by washing them 3 times in ddH₂O for 20min at RT. Expansion coefficient for expanded sections was ~4x. Microglial cells from the S1 barrel cortex were imaged at 60X and their engulfed Vglut2 content was assessed using Imaris software (Oxford instruments) by creating a nested CD68⁺ mask within each Iba1⁺ cell.

Human tissue Immunostaining, confocal imaging, and analysis

Frozen slices were thawed overnight at 4°C and allowed to equilibrate at room temperature for 3 hours. For antigen retrieval, slides were boiled at 95–100°C in 10 mM sodium citrate buffer (pH 6.0) for 5–10 mins and then cooled at room temperature. Samples were permeabilized with 0.05 % Triton X-100 in PBS for 10 mins, then incubated in 1% H2O2 in PBS for 1 hour and blocked with TNB blocking buffer (0.1 M Tris-HCl; pH 7.5; 0.15 M NaCl; 0.5% blocking reagent from PerkinElmer, cat# FP1012) for 1 hour. For the adult human tissues, samples were permeabilized with 0.2 % Triton X-100 in PBS for 10 mins and blocked with 2 % TNB blocking buffer. Slides were incubated with primary antibodies in the TNB buffer overnight at 4°C (Reagent's table), followed by incubation with biotinylated secondary antibodies, diluted 1:250 in TNB solution for 2.5 hours at room temperature. Next, slides were incubated with horseradish peroxidase (HRP)-conjugated streptavidin, diluted 1:200 in TNB for 30 mins, before incubation with tyramide-conjugated fluorophores (Akoya) diluted 1:100 in amplification buffer (PerkinElmer) for 5 mins. Images were acquired using a Leica Stellaris 8 Tau STED confocal Microscope with a 40X objective (HC PL APO 40X/1.30 Oil CS2). For morphology measurements, a 20 µm z-section was acquired with a 0.3 µm z-step, and converted to MaxIP for analysis. Microglial morphological complexity from MaxIP images was quantified on Fiji ImageJ using 2D sholl analysis. Briefly, individual microglia were manually cropped, binarized while excluding particles <4pixels, and a pointer was added to the cell center for Sholl analysis.

In vivo 2-photon microscopy: image acquisition, processing, and analysis

Animals were imaged under isoflurane anesthesia on a custom-built Bergamo II two-photon microscope running ThorImage LS version 4.1 and Thorsync (Thorlabs), using a 25x SCALEVIEW-A2 1.0 NA objective (Olympus) at 920 nm wavelength (for imaging mGreenLantern, [Figures 7F–7I](#)) or 980 nm wavelength (for imaging Tdtomato, [Figures 5A–5C](#)) using a Ti-Sapphire laser (Coherent). Imaging was performed ~30–100 µm below the pial surface at 1 µm z-step. For microglia surveillance ([Figures 5A–5C](#)), images (20 cumulative frames) were acquired at 3X zoom with 0.155 µm/pixel XY resolution, imaging a 20 µm z-section each minute for 10 minutes. For dendritic spine longitudinal imaging ([Figures 7F–7I](#)), regions of interest were manually re-located 24h after initial imaging based on overlaying cranial blood vessel architecture, and images (50 cumulative frames) were acquired at 4.1X zoom with 0.117 µm/pixel XY resolution and variable z-section thickness dependent on dendritic segment orientation. Motion correction and Image registration were performed on Fiji ImageJ. Still images ([Figures 7F–7I](#)) were aligned using rigid body registration with the StackReg Plugin. Time-lapse images ([Figures 5A–5C](#)) were converted to Hyperstacks and aligned using rigid body registration with the HyperStackReg Plugin. Registered images were bleach-corrected using histogram-matching and converted to Maximum Intensity Projections (MaxIP) of each z-stack. Using the MaxIP time-lapse images, Microglia surveillance was then quantified using the MotiQ 2D Analyzer Plugin.⁶⁸ Dendritic spines were quantified manually across the z-section (3D), and individual spines were matched across days. Spine dynamics were quantified as percentage of spines eliminated or formed on day 2 compared to day 1.

WGCNA and K-means clustering for single cell RNA sequencing **Module generation and analysis using WGCNA**

Transcriptional signatures of human and mouse microglia were obtained using human datasets from Velmeshev et al.⁵⁷ (available at <https://pre-postnatal-cortex.cells.ucsc.edu>) and mouse datasets from Hamed et al.⁵⁸ (GEO series GSE200202) and generating subsets containing exclusively microglia. The human microglia subsets were generated from Velmeshev et al.⁵⁷ by binning the multiple development stages in the original dataset into ‘stage’ groups to reflect developmental milestones and to include comparable number of microglia per group. The mouse microglia subsets were generated from Hamed et al.⁵⁸ maintaining the annotated developmental stages but including exclusively cells annotated as “Juvenile” or “Adult” microglia. Modules were generated from datasets using WGCNA (ver. 1.72.5 on R 4.3.1) as previously described.¹¹⁷ The activity of these gene modules was evaluated using the module activity metric described in Nano et al.⁵⁶, in which the activity of each module is evaluated for each cell by calculating the average normalized counts per million (CPM) detected for each module gene.

Grouping WGCNA module by K-means clustering

The WGCNA module activity value for each module was used to perform k-means clustering (packages `factoextra_1.0.7`, `cluster_2.1.6` and `dplyr_1.1.4` on R4.3.2) to assign the modules to different groups. The number of clusters were determined by calculating within-cluster squared errors (WSS) and the elbow methods, to choose a number of clusters whereby adding another cluster does not further improve the total WSS.

QUANTIFICATION AND STATISTICAL ANALYSIS

All quantitative data with a single timepoint is represented as superplots, where biological replicates (individual mice) are represented by differently colored filled triangles, overlaid onto the technical replicates (individual cells or dendritic segments) which are represented by semi-opaque circles of the same color. A horizontal line indicating the median, and error bars indicate the 95% confidence interval. All temporal (except from transcriptomic data) and Sholl curve data show the group mean of each timepoint and genotype, and error bars indicate the 95% confidence interval. Populations were tested for normality (Kolmogorov-Smirnov test) prior to performing statistical analyses. For populations with normal distributions, statistical analyses were performed using standard t-test when only two groups were compared, one-way ANOVA when three or more conditions were compared across the same magnitude, or two-way ANOVA when two conditions were compared across two magnitudes. For populations with non-normal distributions, statistical analyses were performed using Kolmogorov-Smirnov cumulative distribution test when only two groups were compared. Statistical significance was represented according to GraphPad format: ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Data was plotted and analyzed using Prism software except for K-means clustering and transcriptomic data from [Figure S2](#).

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Supplemental information

**Human-specific SRGAP2 paralogs
synchronize neotenic microglial maturation
and synaptic development**

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Figures S1-S6
Table S1

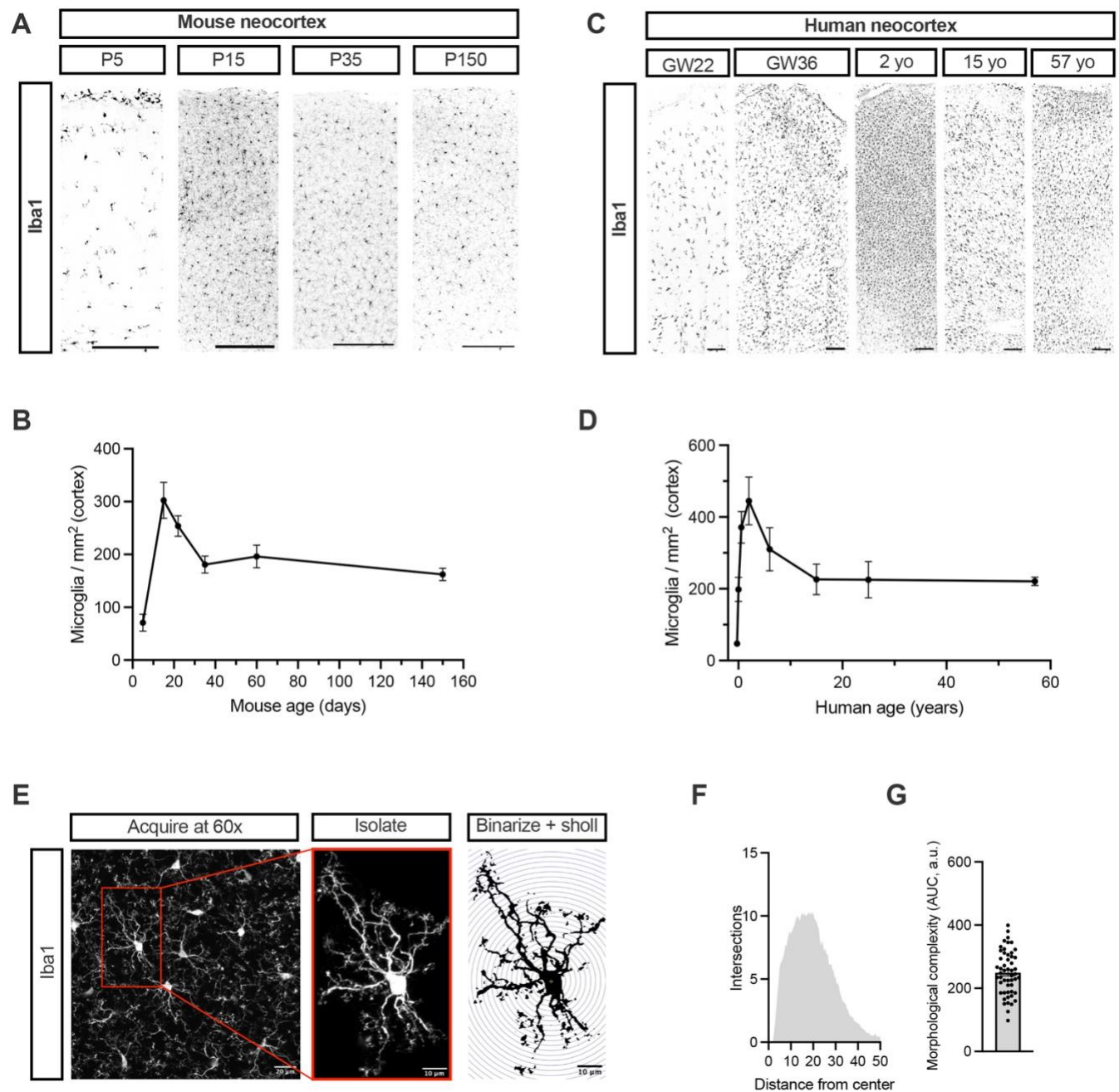


Figure S1. Microglial density in mouse and human cortex across development, and approach to analyze microglial complexity (related to Figure 1). **(A)** and **(C)** Representative images of mouse **(A)** and human **(C)** cortical microglial density throughout brain development. Scale bar, 250 μm . **(B)** and **(D)** Quantification of cortical microglia in mouse **(B)** and human **(D)** brains throughout development. Data from 3 to 7 mice per timepoint, 2 to 4 fields per mouse, and 1 human tissue sample per timepoint, 3-5 fields per timepoint. **(E-G)** Analytical approach to assess microglial morphology. Microglia (as revealed

by Iba1 staining) from layer 2/3 of the barrel cortex were imaged using confocal microscopy (E), isolated, binarized, and subjected to Sholl analysis, counting the intersections of concentric circles with microglia branches (F). The Area under the curve (AUC) of the Sholl traces was computed as a measure of microglial morphological complexity (G).

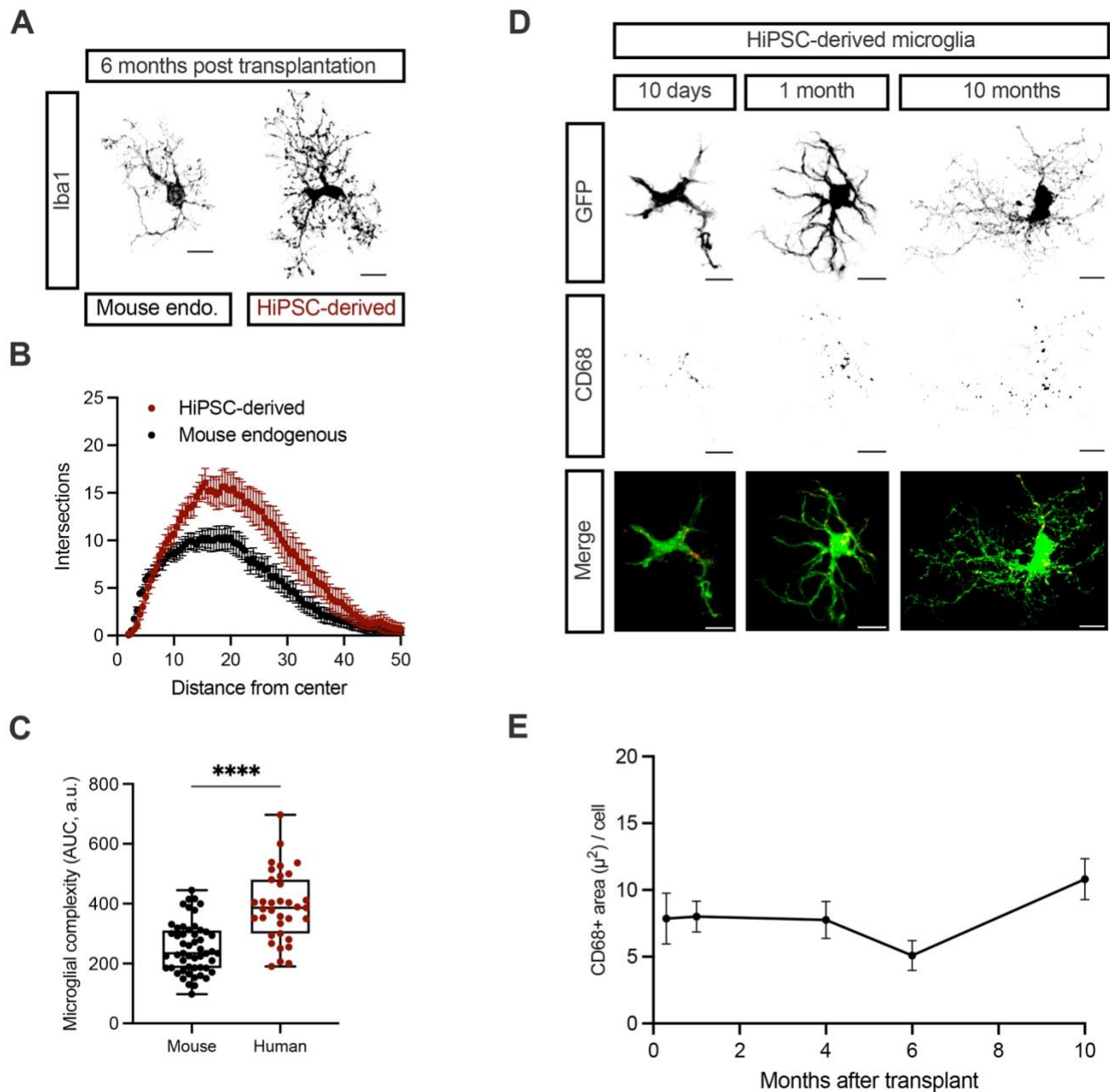


Figure S2. HiPSC-derived Microglia (hMG) are more ramified than their mouse endogenous counterparts (related to Figure 2). **(A)** Representative images of hMG and mouse endogenous microglia from a 6 month-old mouse. Scale bar, 10 μm . **(B and C)** Comparison of sholl analysis (B) and morphological complexity (computed as the AUC of sholl traces) (C) of hMG and mouse endogenous microglia. Data from three mice, 8-22 cells per mouse. Statistical differences were calculated using an unpaired t test. **(D)** Representative images of hMG microglia stained with Iba1 and the phago-lysosomal marker CD68 at different timepoints post transplantation. **(E)** Phagocytic potential (expressed as area of all CD68+ puncta per microglia throughout hMG development. Data from three mice per timepoint, 8-18 cells per mouse. Statistical differences were calculated using a one-way ANOVA (Tukey multiple comparisons test).

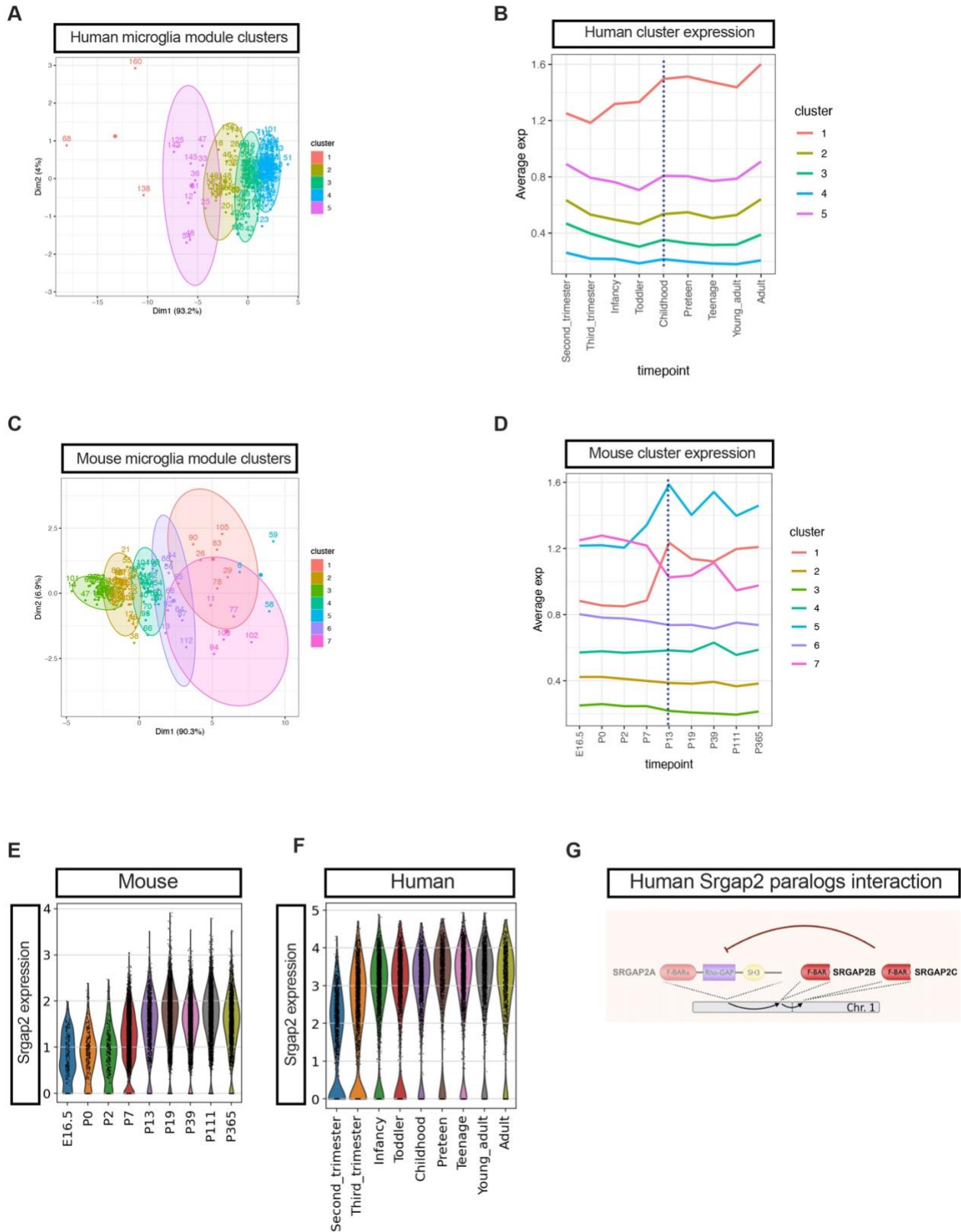


Figure S3. Unsupervised analysis of human and mouse microglial transcriptomic dynamics during development and maturation (related to Figure 3). **(A and C)** k-Means clustering of Weighted Gene Correlation Network Analysis (WGCNA) modules of co-expressed genes during human (A) or

mouse (C) microglia development. (**B and D**) Average activity of module clusters throughout human (B) or mouse (D) microglia development. (**E-F**) Violin plot depicting expression of the *Srgap2* gene in mouse (E) and human (F) microglia throughout development. (**G**) Scheme of *Srgap2* partial gene duplications and protein interactions, depicting partial inhibition of *Srgap2* functions by its human-specific paralogs.

A

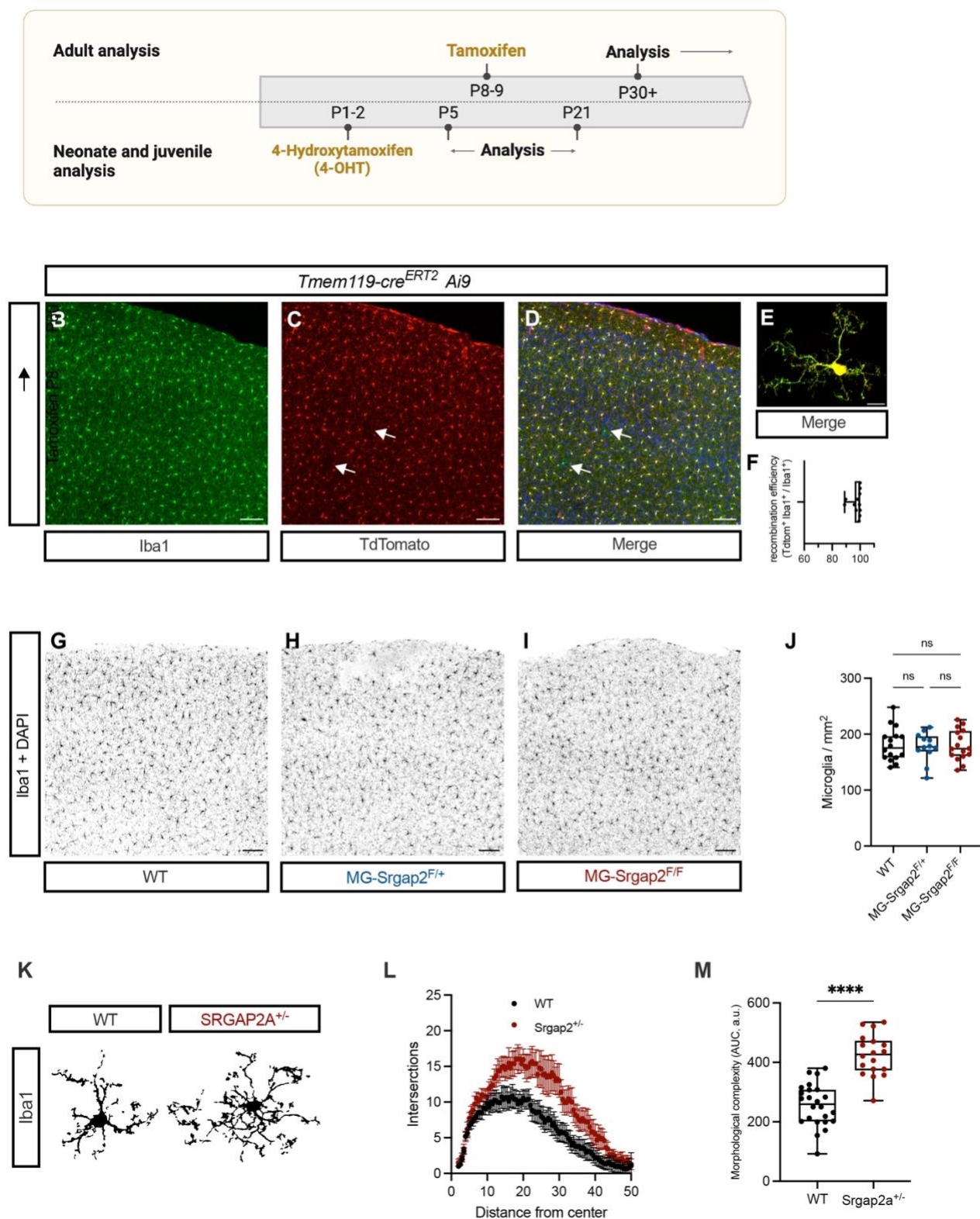
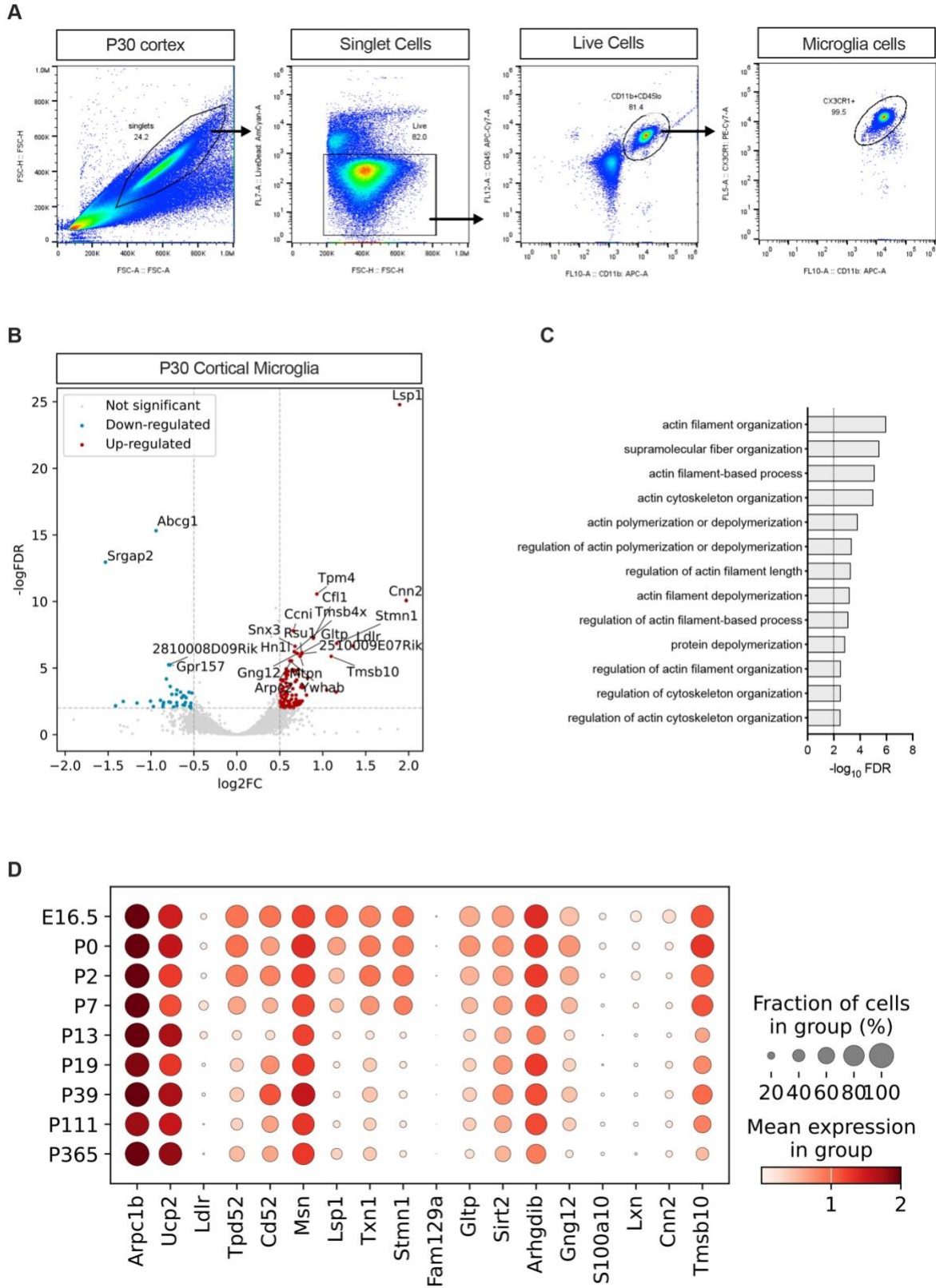


Figure S4. Experimental approach for tamoxifen induction of microglial-specific expression in *Tmem119^{CreERT2}* mouse driver and morphological complexity in constitutive knockout *Srgap2^{+/-}*

mice (related to Figure 4). **(A)** Tamoxifen dosing and timing approach employed to drive Cre-mediated *Srgap2* gene excision and *Srgap2C* inducible expression in mouse microglia, for analysis at mature stages or during development. **(B-D)** Representative images of cortical microglia from P60 *Tmem119^{CreErt2}* crossed with *Ai9^{F/+}* mice, showing high recombination efficiency upon tamoxifen administration at P8-9. White arrows, rare events of Iba1-positive but tdTomato-negative microglia where Cre-dependent recombination did not occur. Scale bar, 10 μ m. **(E)** High magnification image of Tdtomato-expressing microglia stained with Iba1 (green), showing overlap between both intracellular proteins. **(F)** Quantification of recombination efficiency in microglia from these mice. Data from four mice, three fields per mouse. **(G-I)** Representative images of cortical microglial in adult WT, *MG-Srgap2^{F/+}* and *MG-Srgap2^{F/F}* mice. Scale bar, 100 μ m. **(J)** Quantification of cortical microglia density in these mice. Data from 4 to 6 mice per genotype, 2 to 3 fields per mouse. Statistical differences were calculated using a one-way ANOVA (Tukey multiple comparisons test). **(K)** Representative binary traces of microglia from constitutive *Srgap2^{+/-}* mice and littermate control WT mice. **(L-M)** Comparison of Sholl analysis (L) and morphological complexity (M) of microglia from these mice. Data from 2 mice per genotype, 8-14 cells per mouse. Statistical differences were calculated using an unsupervised t test.



sequencing. (B) Volcano plot representing differentially expressed genes between WT and *MG-Srgap2^{F/F}* microglia obtained by bulk RNA sequencing of FACS-sorted cortical microglia. Data from 3-4 biological replicates (individual mice). Note that *Srgap2* mRNA (blue point on left) is significantly downregulated in microglia of *MG-Srgap2^{F/F}* compared to WT cortex at P30, validating the conditional targeting strategy. (C) Gene Ontology enrichment analysis displaying overrepresented pathways in *MG-Srgap2^{F/F}* microglia at P30, when compared to WT microglia. (D) Developmental expression profile of top overexpressed genes in *MG-Srgap2^{F/F}* microglia.

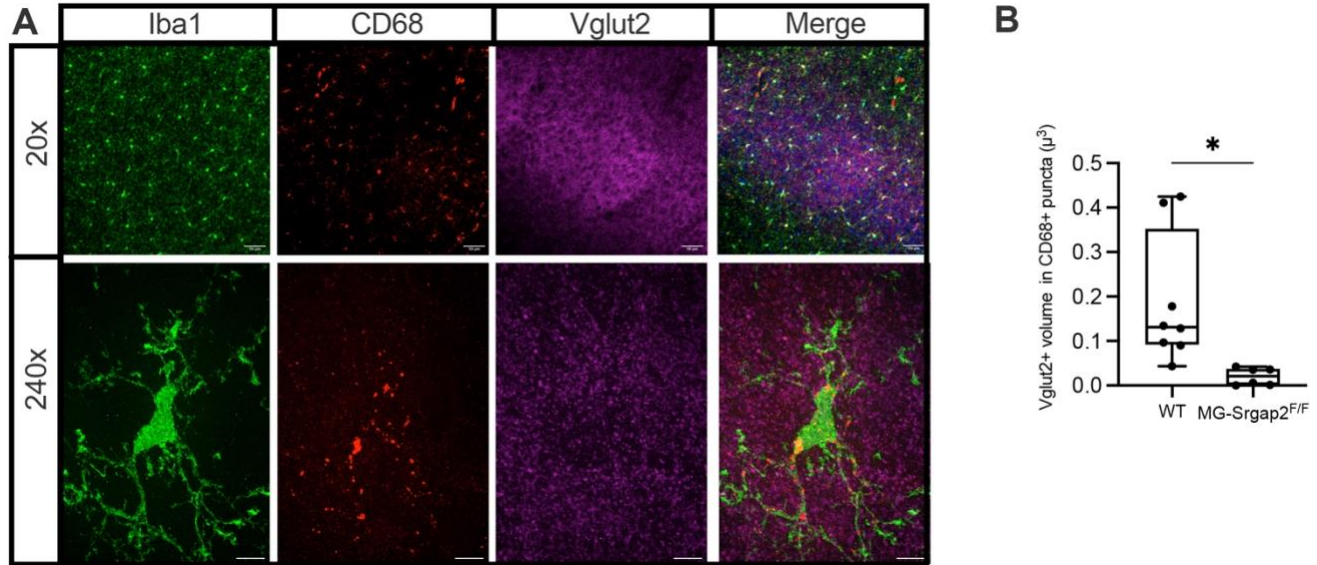


Figure S6. *Srgap2*-deficient microglia engulf fewer presynaptic materials at juvenile stages (related to Figure 5). (A) Representative images of S1 barrel cortex (layer 4) area stained with the microglial marker Iba1 and cortical microglia from juvenile P22 mice, stained with the microglial marker Iba1 (green), the phagolysosomal marker CD68 (red), and the presynaptic marker Vglut2 (magenta), obtained before (20X) and after- (240X) expansion microscopy. (B) Quantification of the volume of presynaptic Vglut2⁺ puncta overlapping with microglial CD68⁺ phagolysosomes of WT or *MG-Srgap2^{F/F}* microglia. Data from 3 mice per genotype, 2-3 cells per mouse. Statistical significance was tested using an unsupervised t test. * p<0.05.

Table S1. Clinical details of human cortex samples used in present study. Related to data presented in Figure 1 and Figure S1A-D.

Eight de-identified human specimens were collected during autopsy, with a post-mortem interval of fewer than 48 hours. See Methods section for details.

Case	Age	Clinical diagnosis	Sex	Cortical region
1	22GW	Elective pregnancy termination	U	Cingulate cortex
2	36GW	Cardiac anomaly	F	Cingulate cortex
3	7mo	Wiskott-Aldrich	M	Cingulate cortex
4	2yo	Leukemia	M	Cingulate cortex
5	6yo	Lung hypoplasia	F	Superior frontal gyrus
6	15yo	Leukemia	M	Cingulate cortex
7	25yo	Chronic myeloid leukemia (CML)	M	Visual cortex
8	57yo	Metastatic papillary thyroid carcinoma	F	Cingulate cortex