TAM receptors regulate multiple features of microglial physiology

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Microglia are damage sensors for the central nervous system (CNS), and the phagocytes responsible for routine non-inflammatory clearance of dead brain cells1. Here we show that the TAM receptor tyrosine kinases Mer and Axl2 regulate these microglial functions. We find that adult mice deficient in microglial Mer and Axl exhibit a marked accumulation of apoptotic cells specifically in neurogenic regions of the CNS, and that microglial phagocytosis of the apoptotic cells generated during adult neurogenesis is normally driven by both TAM receptor ligands Gas6 and protein S3. Using live two-photon imaging, we demonstrate that the microglial response to brain damage is also TAM-regulated, as TAM-deficient microglia display reduced process motility and delayed convergence to sites of injury. Finally, we show that microglial expression of Axl is prominently upregulated in the inflammatory environment that develops in a mouse model of Parkinson’s disease6. Together, these results establish TAM receptors as both controllers of microglial physiology and potential targets for therapeutic intervention in CNS disease.

Microglia, the tissue macrophages of the brain and spinal cord, have fundamental roles in CNS homeostasis. They are mobilized in response to nearly any CNS perturbation, and can act to both resolve and exacerbate CNS disease1,7. Their importance notwithstanding, the signalling networks that regulate microglial function are only beginning to be deciphered. We asked whether the TAM receptor tyrosine kinases might comprise one such system. These receptors, Tyro3, Axl, and Mer, regulate the innate immune response in dendritics and macrophages8,9, mediate the engulfment of apoptotic cells by phagocytes10–12, promote the infection of cells by enveloped viruses13, and contribute to the growth and metastasis of human cancers14. In the CNS, Tyro3 is abundant in neurons15,16, whereas Mer and Axl are more prevalent in the periphery and the phagocytes responsible for routine non-inflammatory clearance of apoptotic cells2,8,9, mediate the engulfment of apoptotic cells by microglia and astrocytes2,8,9,12. Consistent with the minimal expression of Axl, no accumulation of apoptotic cells was detected in the Axl−/− SVZ (Extended Data Fig. 3a). In contrast, the MerT−/− SVZ contained many cCasp3+ apoptotic cells, although this number was around fourfold lower than that seen in Axl−/− MerT−/− double mutants (Extended Data Fig. 3a). We counted 733 ± 359 (± s.e.m.) cCasp3+ cells per mm² in sections of the MerT−/− SVZ, and 2942 ± 262 cCasp3+ cells per mm² in the Axl−/− MerT−/− SVZ. This synergistic effect of an Axl mutation on the background of an existing MerT−/− mutation has been noted previously10,11,12.

We demonstrated that accumulation of apoptotic cells in the MerT−/− SVZ and RMS is due to the loss of Mer specifically from microglia, by analysing a new mouse line carrying conditional floxed alleles of the MerT gene (Extended Data Fig. 4; see Methods) crossed to a tamoxifen-inducible oestrogen receptor (ER) Cre driver controlled by the Cx3cr1 promoter24. In the absence of tamoxifen (upon vehicle injection alone), Mer was present in Cx3cr1CreER+/Mertkfl/fl Iba1+ microglia (Extended Data Fig. 5a), and there were no cCasp3+ apoptotic cells in the SVZ or RMS (Fig. 1d). However, 1 week after tamoxifen injection, microglial Mer expression was lost (Extended Data Fig. 5a), and accumulation of apoptotic cells, comparable to that seen in MerT−/− mice, was detected in the Cx3cr1CreER+/Mertkfl/fl SVZ and RMS (Fig. 1d). Microglia remained

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Figure 1 | TAM signalling mediates microglial phagocytosis of apoptotic cells in brain neurogenic regions. a, SVZ sections adjacent to the lateral ventricle (LV) of wild-type (WT) or Axl−/− Mertk−/− Cx3cr1GFP+ brains visualized for GFP (green), cCasp3 (magenta), and NeuN (cyan). b, RMS of wild-type and Axl−/−Mertk−/− brains immunostained for cCasp3 (green) and NeuN (red). c, Immunostaining of wild-type and Axl−/−Mertk−/− SVZ with anti-Iba1 (green) and anti-NeuN (red), or anti-Iba1 (green) and anti-CD169 (red). Arrowheads mark Iba1+ microglia with an amoeboid morphology (lower left) and Iba1+ CD169+ double-positive microglia (lower right); open arrowhead is an Iba1+ CD169− cell outside the SVZ. d, No cCasp3+ apoptotic cells accumulate in the SVZ or RMS of Cx3cr1CreER+/Mertkfl/fl mice 1 week after vehicle injection (−tamoxifen), but many are evident in the SVZ and RMS at 1, 3 and 7 weeks after injection with vehicle and tamoxifen to induce Cre expression in Cx3cr1+ microglia (+tamoxifen). All sections in a–d are co-stained with nuclear Hoechst 33258 (blue). Representative images from analyses performed in 3 (a, b, d) and 2 (c) mice. Scale bars, 50 μm.

Mer-negative (Extended Data Fig. 5a) and accumulation of apoptotic cells was maintained at 3 and 7 weeks after tamoxifen injection (Fig. 1d), by which time most Cx3cr1CreER+/ gene-deleted cells outside the CNS have been replaced by monocytes and/or haematopoietic progenitors24. Mer expression in brain microvascular endothelial cells25, an important Mer reservoir in the CNS25, persisted following tamoxifen treatment (Extended Data Fig. 5b).

We assessed the consequences of defective clearance of apoptotic cells on neurogenesis by pulse labelling dividing cells in the SVZ of adult mice with bromodeoxyuridine (BrdU), and then counting BrdU+ cells that had migrated to the granule cell and glomerular layers of the olfactory bulb 35 days after the pulse (Fig. 2a, b). We found that accumulation of apoptotic cells in the Axl−/−Mertk−/− SVZ did not reduce the number of BrdU+ cells in the olfactory bulb, none of which were apoptotic (Extended Data Fig. 2c) or microglia (Fig. 2c). Indeed, we observed a notable ~70% increase in the number of BrdU+ cells in the Axl−/−Mertk−/− olfactory bulb relative to the wild type (Fig. 2b). This translated to an increased cellular density in the combined granule cell and glomerular layers of the Axl−/−Mertk−/− olfactory bulb, from 87.7 ± 2.2 nuclei per 104 μm2 (± s.e.m.) in wild type to 99.8 ± 2.4 for the double mutants (n = 6 for both genotypes; P = 0.004). These results are consistent with the possibility that a fraction of phosphatidylserine (PtdSer)-expressing, but nonetheless viable, SVZ-derived cells are normally ‘eaten alive’ by microglia, in a process termed ‘phagoptosis’26, that this process occurs continuously in a non-pathogenic environment.
and that it is TAM-dependent. Many of the BrdU+ cells that had migrated to the Axl+/−/Mertk−/− olfactory bulb were NeuN+ (Fig. 2d), and some expressed markers appropriate to their location (Extended Data Fig. 6a, b).

Genetic analyses in vivo indicated that both protein S (Pros1) and Gas6 function as Mer agonists for engulfment of apoptotic cells by microglia. The Gas6−/− SVZ displayed a wild-type phenotype (Extended Data Fig. 3b), as did the SVZ of Gas6−/−Pros1fl/fl mice, in which one Pros1 allele is floxed with loxP sites and the other is inactivated72. (The complete Pros1−/− knockout is embryonic lethal72.) In contrast, Gas6−/− Mertk−/− mice displayed a marked accumulation of cCasp3+ cells in the SVZ and RMS, comparable to that seen in Axl−/− Mertk−/− mice (Extended Data Fig. 3c). We counted 3,638 ± 282 (± s.e.m.) cCasp3+ cells per mm2 in the Gas6−/− Mertk−/− mice SVZ. This is consistent with the fact that Pros1, the only TAM ligand remaining in the Gas6−/− Mertk−/− mice, does not activate Axl5, the only microglial TAM receptor remaining in these mice. Thus, as for Mer-dependent phagocytosis in the retina56, only half of the wild-type level of only a single TAM ligand was sufficient to drive wild-type levels of microglial phagocytosis.

We also quantified phagocytosis of apoptotic cells by microglia cultured from Cx3cr1GFP+ mice12 (Extended Data Fig. 7a). When incubated with apoptotic cells12 in medium containing 10% serum, where Pros1 is present at ~30 nM, wild-type microglia were exceptionally active phagocytes (Extended Data Fig. 7b). Phagocytosis was reduced in Axl−/− Mertk−/− cells (Extended Data Fig. 7b). In serum-free medium, phagocytosis of apoptotic cells was further reduced, which was mostly TAM-dependent (Extended Data Fig. 7c, d). (This TAM dependence is consistent with the fact that microglia express endogenous Gas6 and Pros1 mRNA12.) When we supplemented serum-free medium with Pros1 or Gas6, we found that both ligands stimulated TAM-dependent phagocytosis (Extended Data Fig. 7c, d). Cultured astrocytes engulfed less apoptotic cells than microglia, and this phagocytosis could be stimulated only modestly by Gas6 (Extended Data Fig. 7e). Stimulation of microglial phagocytosis by both Gas6 and Pros1 demonstrates that it is mediated principally by Mer, as Axl is activated only by Gas612.

Although microglia in an uninjured brain are essentially fixed in position, their processes are in constant motion, and survey the entirety of the CNS parenchyma every few hours28. As process extension is also required for phagocytosis12, we asked if TAM signalling regulates microglial extension velocity. We used in vivo two-photon microscopy to measure the movement of microglial processes outside of the neurogenic regions, in the visual cortex of wild-type Cx3cr1GFP+ and Axl−/− Mertk−/−/Cx3cr1GFP+ mice (Supplementary Videos 2 and 3). Selected video stills, with individual wild-type and Axl−/− Mertk−/− processes tracked during imaging, are shown in Fig. 3a. These measurements demonstrated that Axl−/− Mertk−/− microglia, in an uninjured brain, display a ~19% reduction in process extension velocity relative to wild type (Fig. 3b). We also assessed whether TAM signalling was required for microglial responses to injury. We disrupted the blood-brain barrier at the level of individual capillaries with a laser lesion28, and then measured the velocity of microglial process extension towards the lesion site using live two-photon imaging (Supplementary Videos 4 and 5). Selected video stills, with individual extensions towards the lesion tracked in wild-type and Axl−/− Mertk−/−/Cx3cr1GFP+ brains, are shown in Fig. 3c. We found that extension towards the laser lesion was ~39% slower in the Axl−/− Mertk−/− microglia (Fig. 3d). These results demonstrate that routine microglial process activity and the response to injury are both regulated by TAM. They are consistent with the finding that Mertk−/− macrophages exhibit compromised cellular migration and a disrupted cytoskeleton in vitro59.

For many macrophages, Axl and Mer segregate to inflammatory and tolerogenic environments, respectively12. Inflammatory stimuli such as polyinosinic–polycytidylic acid (poly(I:C)) and interferon γ (IFN-γ) upregulate Axl expression, whereas immunosuppressive drugs such as dexamethasone upregulate Mer12. We saw similar responses in cultured microglia (Extended Data Fig. 8a, b). As Axl is an inflammatory marker, we asked whether its microglial expression might be elevated in neurodegenerative disease1, and examined a transgenic mouse model of Parkinson’s disease. In this model, an alanine 53 to threonine (A53T) mutated form of human α-synuclein (SNCAAS3T), which leads to a hereditary form of Parkinson’s disease, is expressed in neurons, most prominently in the spinal cord, under the control of the mouse Thy1 promoter4. This transgenic expression results in late-onset neurodegeneration, and death at ~8–10 months6. Measurement of a panel of inflammatory marker mRNAs demonstrated that the spinal cords, and to a lesser extent the brains, of aged Thy1-SNCAAS3T transgenic mice displayed elevation of these markers, whereas inflammation was undetectable in the spleen (Fig. 4a, Extended data Fig. 8c).

The aged transgenic spinal cord, where SNCAAS3T expression is high (ref. 29 and Fig. 4b), showed markedly elevated expression of Iba1 (Fig. 4b). We also detected upregulation of both Axl and soluble Axl (sAxl) ectodomain, an inflammatory marker12, in the transgenic cord (Fig. 4c and Extended Data Fig. 8d). In contrast, Axl upregulation was undetectable in Thy1-SNCAAS3T spleen and minimal in brain (Extended Data Fig. 8d). No change in Mer expression was detected in the spleen.
of the transgenic mice (Extended Data Fig. 8d), with only a very modest increase in the spinal cord (Fig. 4c and Extended Data Fig. 8e). Axl induction in the Thy1-SNCA\textsuperscript{A53T} spinal cord was exclusively associated with Iba1\textsuperscript{+} microglia (Fig. 4d). Expression of SNCA\textsuperscript{A53T} in spinal motor neurons (ref. 29 and Fig. 4b) leads to progressive ataxia, paralysis, and death, with an onset at ~120 days in the transgenic population (Fig. 4e). A 50% reduction in Mer and Axl resulted in no change in this time course, but the loss of both receptors modestly extended survival (Fig. 4e). A 50% reduction in Mer and Axl resulted in no change in this time course, but the loss of both receptors modestly extended survival (Fig. 4e). We do not know the reason for this modest life extension; however, we speculate that wild-type microglia may execute TAM-dependent ‘phagoptotic’ engulfment\textsuperscript{26} of distressed, PtdSer-displaying motor neurons, thereby speeding up the death of the mice.

Together, the above results identify Mer and Axl as regulators of multiple features of microglial physiology. The elevation in microglial Axl that we document in the Thy1-SNCA\textsuperscript{A53T} spinal cord is in keeping with the demonstration that Axl is an inflammatory response receptor in macrophages\textsuperscript{12}, and that elevated levels of sAxl are observed during multiple human disease and trauma states (ref. 8, and references therein). In this regard, we note that a recent longitudinal study in humans has identified elevated Axl in CSF as among the most reliable indicators of the early appearance of Aβ pathology and the subsequent development of Alzheimer’s disease\textsuperscript{50}.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Figure 4**| Microglial Axl is upregulated in a mouse model of Parkinson’s disease. **a**, Comparison of the mean expression (± s.e.m.) of the indicated inflammatory mediator/marker mRNAs in the spinal cords of 3 wild-type and 3 Thy1-SNCA\textsuperscript{A53T} (SNCA\textsuperscript{A53T}) mice at 8–10 months of age. **b**, Sections from the spinal cords of aged (8–9 month) wild-type and SNCA\textsuperscript{A53T} mice, immunostained for phosphorylated neurofilament (SMI31) and α-synuclein, or NeuN and Iba1. The α-synuclein antibody recognizes both the endogenous mouse protein and the transgenic human protein. Scale bars, 100 μm. **c**, Western blots of spinal cord extracts from 2 wild-type mice (lanes 1, 2) and 4 SNCA\textsuperscript{A53T} mice (lanes 3–6) for the indicated proteins at 9–10 months of age, with Gapdh as a loading control. **d**, Sections from wild-type and SNCA\textsuperscript{A53T} spinal cords immunostained with anti-Axl and anti-Iba1 antibodies. Scale bar, 10 μm. **e**, Kaplan–Meier survival curves for mice of the indicated genotypes. n = 62 SNCA\textsuperscript{A53T} mice; 20 Axl\textsuperscript{+/+}/Mertk\textsuperscript{+/+}/SNCA\textsuperscript{A53T}; and 13 Axl\textsuperscript{−/−}/Mertk\textsuperscript{−/−}/SNCA\textsuperscript{A53T} mice. log-rank (Mantel–Cox) test, P = 0.72 between SNCA\textsuperscript{A53T} and Axl\textsuperscript{−/−}/Mertk\textsuperscript{−/−}/SNCA\textsuperscript{A53T}; and P = 0.04 between SNCA\textsuperscript{A53T} and Axl\textsuperscript{−/−}/Mertk\textsuperscript{−/−}/SNCA\textsuperscript{A53T}. Representative images from n = 2 wild-type and 3 SNCA\textsuperscript{A53T} mice (b and d).
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Author Contributions L.F. and P.G.T. designed experiments, performed apoptotic cell, BrdU, immunohistochemical, and genetic analyses, and contributed equally to the paper; Y.T., L.F. and H.L.-B. performed and analysed in vivo two photon imaging; E.D.L. prepared TAM ligands; P.G.B. performed brain histology; P.C. analysed cytokine profiles; A.Z. analysed Axl expression in Parkinson’s disease transgenics; C.V.R. provided floxed Merk alleles; A.N. designed and implemented two-photon imaging; and G.L. designed experiments and wrote the paper. All authors edited the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to G.L. (lemke@salk.edu) or C.V.R. (carla.rothlin@yale.edu; for requests for floxed Merk alleles).
METHODS

Mice. The Axl+/−, 31, Mer−/−, Axl−/−Mer−/−, Gas6−/−, Pros−/−NesCreG, Pros−/−NesCreβ, Cx3cr1GFP−/−, Cx3cr1CreER24, S100βCre−/− and SNCAAS37 (ref. 6) strains have been described previously. The Mer−/− mouse line diagrammed in Extended Data Fig. 4 was generated by inGenious Targeting Laboratory (ITL, Ronkonkoma NY), using ITL C57BL/6 embryonic stem (ES) cells. This line targets exon 18, a 137 nucleotide (nt) sequence that encodes residues W779–824 of the Mer kinase domain. Cre-mediated deletion of this exon introduces a frame shift and a stop codon one amino acid downstream of exon 17. This truncated, kinase-dead protein and/or its mRNA are apparently unstable, as antibodies directed against the Mer extracellular domain do not detect a truncated protein upon Cre-mediated excision (see text). Deletion of exon 18 therefore effectively generates a protein null. The complete Mer−/− mouse knockout14 deletes exon 17, a 160 nt sequence that encodes M725–778 within the Mer kinase domain. (Exon 17 was numbered as exon 18 in the original description of the Mer−/− allele1.) This single exon deletion also introduces a frame shift (five amino acids downstream of exon 16) producing an unstable protein, and all results in a Mer protein null12. The Neo cassette was removed via Flp-mediated recombination by crossing high-percentage chimaeric mice to C57Bl6/F1Lp mice. Neo deletion was confirmed by PCR. These Mer−/− mice, together with PCR-based protocols for their genotyping, are available upon request from the Rothlin laboratory (contact C.V.R.). Recombination (inactivation) of the Mer−/− allele in Cx3cr1GFP+/−Mer−/− mice was achieved using tamoxifen injection. Cx3cr1CreER24/Mer−/− mice (16 weeks) received a dose (150 mg kg−1 body weight) of tamoxifen (Sigma) as a solution in corn oil (Sigma) by intraperitoneal (i.p.) injection. Control mice received an i.p. injection of vehicle (corn oil) alone. Mice were analysed for the expression and apoptosis (cCasp3) of Mer at 1 week, 3 weeks or 7 weeks after injection. Mice analysed at 1 week received a single dose of tamoxifen or oil; mice analysed at 3 and 7 weeks received two successive injections 48 h apart. All animals, with the exception of the Mer−/−/− alleles, have been backcrossed for > 9 generations to a C57BL/6 background. All animal procedures were conducted according to protocols approved by the Salk Institute Animal Care and Use Committee (Protocol No. 11-00051). Mice (both males and females) were randomly allocated to experimental groups (three to six mice per group) and investigators were blinded to group allocation during the experiment. Investigators were not blinded to sample identity. Group size was based on previous literature. No statistical methods were used to predetermine sample size.

Reagents and antibodies. Dexamethasone, 5-Bromo-2-deoxyuridine and DMSO were from Sigma-Aldrich. Poly(I:C) was from Invivogen. Lipopolysaccharide (LPS) and Dexamethasone, 5-Bromo-2-deoxyuridine and DMSO were not blinded to sample identity. Group size was based on previous literature. Investigators were blinded to group allocation during the experiment. Investigators were randomly allocated to experimental groups (three to six mice per group) and were conducted according to protocols approved by the Salk Institute Animal Care and Use Committee (Protocol No. 11-00051). Mice (both males and females) were randomly allocated to experimental groups (three to six mice per group) and investigators were blinded to group allocation during the experiment. Investigators were not blinded to sample identity. Group size was based on previous literature. No statistical methods were used to predetermine sample size.

Immunocytochemistry. Cells were fixed for 10 min in 4% PFA in PBS. Immunocytochemistry was fluorophore-conjugated anti-goat (A-11055 from Jackson ImmunoResearch Laboratories and anti-Rabbit (NA934V) from GE Healthcare. Secondary antibodies for immunocytochemistry were horseradish-peroxidase-conjugated anti-goat (Jackson ImmunoResearch Laboratories) and anti-rabbit (Abd, 1:2500 dilution in blocking buffer). Blots were then washed in PBStwice and incubated for 2 h at 22–24 °C with the dark with Hoechst stain and fluorophore-coupled donkey secondary antibody (identified above) diluted in blocking buffer. Coverslips were washed and mounted on slides with Fluoromount-G (SouthernBiotech) and stored at 4 °C. Images were acquired with a Zeiss axio Imager Z1 using 20× objective and analysed with Imago. For quantitation, BrdU−/− cells in granule cell layer and gloomerular layer of the olfactory bulb were counted in two consecutive sections per animal and averaged per animal.

Immunoblot. Cultured cells were washed with ice-cold DPBS and lysed on ice in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT and 2% Triton-X100, 0.25 mM sodium azide, and protease and phosphatase inhibitors (Roche). Tissues were snap frozen in liquid nitrogen before lysis. For immunoblot analysis, equal amounts of protein in LDS sample buffer (Invitrogen) were separated by electrophoresis through 4–12% Bis-Tris polyacrylamide gels (Novex, Life Technologies) and transferred to PVDF membranes (Millipore). For Axl immunoprecipitation, tissue lysates were precleared overnight at 4 °C with Protein G-Sepharose (Amersham). This was then removed and lysates were incubated for 2 h with 0.2 μg anti-Axl (M20) for 0.5 mg protein in cell lysate. Fresh Protein G–Sepharose was added for 2 h and immunoprecipitates were washed twice with 1 ml of lysis buffer containing 0.5 mM NaCl and once with 1 ml of 50 mM Tris-HCl (pH 7.5). Immunoprecipitates were eluted in LDS buffer, separated by electrophoresis through polyacrylamide gels and transferred to PVDF membranes. Non-specific binding was blocked with TBST (50 mM Tris-HCl (pH 7.5), 0.15 mM NaCl and 0.1% Tween-20) containing 5% BSA, and membranes were incubated overnight at 4 °C with primary antibodies. Blots were then washed in TBST and incubated for 1 h at 22–24 °C with secondary horseradish peroxidase–conjugated antibodies in 5% skim milk in TBST. After repeating the washes, signal was detected with enhanced chemiluminescence reagent.

Reverse transcription (RT)–qPCR. Total cellular RNA was isolated with an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen).
DNA was removed by on-column digestion with DNeasy (Qiagen). An RT Transcriber First Strand cDNA Synthesis Kit (Roche) with anchored oligonucleotide (dT) primers (Roche) was used for reverse transcription. Quantitative PCR was run in a 384-well plate format on a Viia7 Real-Time PCR System (Applied Biosystems) with 2 × SYBR Green PCR Master Mix (Applied Biosystems). Primers are listed in Supplementary Table 1. Expression was analysed by the threshold cycle (ΔΔCt).

Microglia and astrocyte culture. Postnatal day 30 (P30) to P50 mice (C57Bl/6J, Axl−/−, Merk−/−, C3H/HeJ G9G8) brains were dissociated using Neural Dissociation kit. PNS7 Neurons and the gentleMACS dissociator according to the manufacturer’s instructions (Miltenyi). Single cell suspensions were resuspended in 30% Percoll in HBSS solution and centrifuged 15 min at 700 g to remove myelin. Cells were grown for 7 days in DMEM-F12 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin before being processed for immunostaining or phagocytosis assay. Cytosine β-d-arabinofuranoside (Ara-C, 5 μM) was added after 5 days in vitro to limit fibroblast proliferation. When astrocytes were also isolated, microglia were first purified using C11b MicroBeads (Miltenyi) and grown for 7 days in DMEM-F12 with 10% FBS and 1% penicillin/streptomycin while astrocytes were grown for 10 days in MACS Neuro Medium with 2% MACS NeuroBrew-21 and 1% penicillin/streptomycin.

Phagocytosis assay. For the generation of apoptotic cells, thymocytes were isolated from 3- to 6-week-old mice, red blood cells were lysed with ACK buffer and remaining cells were incubated for 6 h in RPMI medium containing 5% FBS and 2 μM dexamethasone to induce apoptosis. This routinely resulted in 70% apoptotic and ≤ 5% necrotic cells. Apoptotic cells were then stained for 30 min with 100 ng ml−1 pHrodo-s.e. (Invitrogen) as described previously35,36. Labelled cells were washed twice in PBS containing 1% BSA (to block remaining pHrodo-SE) and 1 mM EDTA (to remove any bound Gas6 and protein S) and once with DMEM. Apoptotic cells were then incubated for 10 min with recombinant mouse Gas6 or purified human protein S, added to microglia or astrocyte cultures at a ratio of 10:1 (apoptotic cell:phagocytes), and incubated for 1 h at 37°C. Microglia or astrocytes were then briefly washed in DPBS, incubated for 10 min at 37°C in tropsin (0.25%), and then placed on ice and detached by vigorous pipetting. Astrocytes were labelled using anti-ACSA2-APC antibody37. Phagocytosis was assessed by flow cytometry with post-acquisition data analysis with FlowJo software (TreeStar). pHrodo fluorescence was measured with excitation at 561 nm and emission filters for phycerythrin (574–590 nm) on a LSR II (BD Biosciences) at the Flow Cytometry Core of the Salk Institute, as described previously35,37. Microglia were gated as GFP+ cells and astrocytes were gated as APC+ cells.

Two-photon imaging. Adult male mice (3–6 months old) were anaesthetized with isoflurane (1.5–2.5% in 100% oxygen at 0.8–1.0 l min−1). Body temperature was kept at 36–37°C, and hydration status was maintained using subcutaneous physiological saline injections (0.1 ml per 25 g body weight every 1–2 h). For head plate implantation, hair, skin and periosteum overlying the neocortex were removed. After cleaning exposed skull areas, a custom metal head plate was affixed to the skull using OptiBond (31514; Kerr) and dental acrylic (H100335, Coltene Whaledent), keeping the intended imaging area over somatosensory or visual cortex uncovered. A polished and reinforced thinned skull window (~2–3-mm diameter; ~20–50 μm remaining bone thickness) was then prepared, as described previously38,39. A movable objective microscope (Sutter Instrument) equipped with a pulsed femtosecond Ti:Sapphire laser (Chameleon Vision II or Ultra II, Coherent), two fluorescence detection channels (565DXCR dichroic, ET525/70M-2P and ET605/70M-2P emission filters, Chroma; H7422–40 GaAsP photomultiplier tubes, Hamamatsu), and a water immersion objective (LUMPlanFL N 40XW 0.8NA; Olympus) was used for two-photon imaging. Imaging was performed as described previously28,39 using 920–940 nm centre excitation wavelengths. Average laser powers used for transcranial optical recordings depended on imaging depth (typically ~10–30 mW at ~150–200 μm depth from the pia). Images were typically acquired using a 6 Hz frame rate, 256 × 256 pixel resolution and a 5-frame average. Image stacks were acquired every 1.5–2 min for up to 5h and typically contained 20–30 images per stack with 1 μm axial image spacing. Fields-of-view had a typical side length of 65–100 μm. Imaging settings were kept constant during time-lapse recordings. For quantitative image data analysis, ImageJ or Fiji software was used. First, maximum intensity images were produced from individual image stacks. Then, lateral image shifts in time-lapse recordings were corrected using a custom-written Imagemj alignment plugin based on the position shift of the peak in cross-correlation images, typically using the first projection image as the reference image. Structural dynamics of individual microglial cell processes was quantified manually using the MTTrackJ plugin in Fiji. Image analysis was done blind with respect to experimental condition. Videos were also created with Fiji.

Laser lesion. To target blood vessels for focal laser lesion, blood plasma was stained by tail vein injection of biocytin-TMR (2–5% in saline, T-12921, Life Technologies). Lesions were performed following a baseline recording period of 30–45 min, during which z-stacks were acquired as described above. To induce lesions, the Ti:Sapphire laser was transiently tuned to 800 nm and a confined area (8–15 μm diameter, ~1 μm axial extent) of a horizontally oriented cortical capillary at 150–220 μm depth was exposed to 70–130 mW for 10–30 s. Laser lesions caused extravasation of dye, indicating disruption of the blood-brain barrier. Following fusal lesion, image stack acquisition was resumed using the same laser and recording parameters as during the baseline recording period. Although Supplementary Videos 4 and 5 run for only ~12 min (the time required for microglial processes to reach the lesion site), time-lapse recording of the same cortical volume continued for 2–4 h after the lesion.
Extended Data Figure 1  | Mer is expressed by microglia. 

**a.** Brain (hippocampus) sections from Cx3cr1^{GFP/+} mice that were wild-type (top row) or Axl^{-/-} Merk^{-/-} (bottom row) were visualized by confocal microscopy for GFP (1st column), anti-Mer (red, 2nd column), or anti-GFAP (cyan, 3rd column) immunoreactivity; 4th column, merged images. Scale bars, 10 μm. Axl immunostaining signal is too low to be visualized in unactivated microglia (not shown; but see Fig. 4d). 

**b.** Mer expression does not co-localize with S100b^{+} cells. Immunostaining of Cx3cr1^{GFP/+} brain sections with anti-Mer (red, 2nd panel) and anti-S100b (cyan, 3rd panel); 4th panel, merged images. 

**c.** Mer co-localizes with Iba1, but not GFAP or GFP in S100b^{GFP/+} mice. Brain sections were visualized by confocal microscopy for anti-Iba1 (top) or anti-Gfap (bottom) (both cyan, 1st column), anti-Mer (red, 2nd column), or GFP (green, 3rd column) immunoreactivity; 4th column, merged images. Scale bars (b, c), 20 μm. Representative images from analyses performed in n = 2 mice (a–c).
Extended Data Figure 2 | Accumulation of apoptotic cells is confined to neurogenic and derivative migratory regions of the Axl<sup>−/−</sup>Mertk<sup>−/−</sup>CNS. a, A low power tiled image of a section through the Axl<sup>−/−</sup>Mertk<sup>−/−</sup> subventricular zone and surrounding brain tissue, stained for cCasp3, illustrates that apoptotic cells are confined within the SVZ. b, A low power tiled image of a section through the Axl<sup>−/−</sup>Mertk<sup>−/−</sup> rostral migratory stream (RMS) and surrounding brain tissue illustrates that cCasp<sup>3</sup> apoptotic cells are confined within the RMS. c, A low power tiled image of the granule cell and mitral cell layers (gcl and mcl, respectively) of the Axl<sup>−/−</sup>Mertk<sup>−/−</sup> olfactory bulb, stained for cCasp3, illustrates that there are no apoptotic cells detected in the double mutant bulb. Scale bars (a–c), 200 μm. Representative images from analyses performed in \( n = 3 \) mice (a–c).
Extended Data Figure 3 | Mer is the principal microglial TAM receptor required for phagocytosis of apoptotic cells in the SVZ. a, Sections of the SVZ from Axl−/− (top row) and Mertk−/− (bottom row) mice immunostained for cCasp3 and NeuN (green and red, respectively; left panels), or Iba1 and NeuN (green and red, respectively; right panels) reveal the accumulation of cCasp3+ apoptotic cells only in the Mertk−/− SVZ. b, Sections of the SVZ (top) and RMS (bottom) of Gas6−/− mice, illustrating no accumulation of apoptotic cells (similar to both wild type and Axl−/−). c, Sections of the SVZ (top) and RMS (bottom) of Mertk−/− Gas6−/− mice, illustrating a massive accumulation of apoptotic cells, similar to that seen in Axl−/− Mertk−/− mice. Scale bars, 50 μm. See main text for quantification. Representative images from analyses performed in n = 2 mice for Gas6−/− and Mertk−/− Gas6−/−, and n = 3 mice for Axl−/− and Mertk−/−.
Extended Data Figure 4 | Conditional Mertk knockouts. The knockout strategy targets exon 18 of the wild-type mouse Mertk gene, which encodes residues W779–L824 of the tyrosine kinase domain (1st line). Deletion of this exon leads to a functional and null protein (see Methods, and Extended Data Fig. 5). The targeting vector (2nd line) had a PGK-Neo cassette for selection in embryonic stem (ES) cells, and contained loxP and FRT sites, recognized by Cre and Flp recombinases, respectively, at the indicated positions. Five ES cell lines with homologous recombination at the Mertk locus were identified by Southern blots of MfeI-digested DNA, using the indicated Pb 1/2 (external) and Pb 3/4 (internal) probes (3rd line). Introduction of Flp recombinase, achieved by crossing high percentage chimaeras (obtained from blastocyst injection of these ES cells) to C57Bl/6 FLP mice, removed the Neo cassette, leaving exon 18 flanked by loxP sites (4th line). Cre-mediated recombination at these loxP sites deletes exon 18. Mertk\(^{fl/fl}\) mice, together with PCR-based protocols for their genotyping, are available upon request from the Rothlin laboratory (contact C.V.R.). See Methods for further information.
Extended Data Figure 5 | Persistence of microglial-specific Mer ablation following tamoxifen injection of Cx3cr1CreERT2; Mertkfl/fl mice.

a, Mice were injected intraperitoneally with oil vehicle alone (−tamoxifen, top row) or with tamoxifen (+tamoxifen, bottom row) (see Methods), and brain sections were immunostained for Mer protein expression (red panels in 2nd, 4th columns) in Iba1+ microglia (green panels in 1st, 3rd columns) at 1 week (left four panels) and 7 weeks (right four panels) after injection. Sections counter-stained with Hoechst 33258 to visualize nuclei (blue).

b, Brain sections containing a brain capillary 7 weeks after injection of vehicle (top) or tamoxifen (bottom), showing that although Mer expression in microglia is eliminated upon tamoxifen-mediated Cx3cr1-restricted induction of Cre activity, Mer expression in CD31+ microvascular endothelial cells (arrows) is maintained. Representative images of n = 2 mice per time point.
Extended Data Figure 6 | Identity of immigrant BrdU\(^+\) cells in the olfactory bulb. a, A group of the BrdU\(^+\) cells in the glomerular layer (gl), visualized 35 days after injection of BrdU (red) and presumed immigrant descendants of SVZ cells in S phase at the time of injection, are also positive for tyrosine hydroxylase (TH, green) in both wild-type (left panel) and Axl\(^{-/-}\)Mertk\(^{-/-}\) (right panel) mice. Arrowheads are examples of TH\(^+\) BrdU\(^+\) cells. b, Similar comparative granule cell layer (gcl) sections stained with anti-BrdU (red) and calretinin (CR, green). Arrowheads are examples of CR\(^+\) BrdU\(^+\) cells. Sections were co-stained with Hoechst 33258 to visualize nuclei. Scale bars, 50 μm. Representative images of \(n = 2\) per genotype.
Extended Data Figure 7 | Both Gas6 and Pros1 drive microglial phagocytosis of apoptotic cells in vitro. **a**, Cultured microglia express Mer but little or no Axl under basal conditions. Microglia were cultured from wild-type Cx3cr1<sup>GFP+</sup> mice, visualized for GFP (1st column), and immunostained for Iba1 (3rd column), Mer (2nd column, top), and Axl (2nd column, bottom). Scale bar, 10 μm. **b–d**, In vitro pHrodo-based assay of phagocytosis of apoptotic cells by microglia (see Methods). **b**, In serum–containing medium (10% FBS), wild-type microglia are effective phagocytes; mean phagocytic activity is substantially reduced in Axl<sup>−/−</sup> Mertk<sup>−/−</sup> (A<sup>−/−</sup> M<sup>−/−</sup>) microglia. **c**, **d**, Both purified Gas6 (c) and purified Pros1 (d) stimulate AC phagocytosis by cultured microglia in serum-free medium, and this stimulation is entirely TAM-dependent. **e**, The phagocytic activity of cultured astroglia prepared from Cx3cr1<sup>GFP+</sup> mice that were either wild-type or Axl<sup>−/−</sup> Mertk<sup>−/−</sup> was measured in the same pHrodo-based assay in serum-free medium ± Gas6. For this FACS-based assay, astrocytes were gated using an astrocyte-specific surface antigen-2 (ACSA-2) antibody (see Methods). Bar graphs represent mean phagocytic activity (± s.e.m.); n = 2 replicates from 2 mice per genotype (b–d), and 2 replicates from 4 mice per genotype (e).
Extended Data Figure 8 | Regulation of microglial Axl by neuroinflammation. a, b, Axl (a) and Mer (b) regulation in purified (GFP+) cultured microglia by the tolerogenic stimulus dexamethasone (Dex) and the two proinflammatory stimuli IFNγ and poly(I:C), as assessed by immunostaining. Axl expression (a) is very low in the absence of an added stimulus, is not elevated by Dex, but is strongly upregulated by both IFNγ and poly(I:C). In contrast, Mer expression (b) is readily detected in the absence of an added stimulus, is further elevated by Dex, but is modestly suppressed by both IFNγ and poly(I:C). Scale bar, 10 μm. c, In contrast to the spinal cord (see Fig. 4a), there is no upregulation of the indicated inflammatory mediator/marker mRNAs (mean expression ± s.e.m.) in the spleens, and only modest upregulation in the brains, of SNCA\textsuperscript{A53T} mice at 8–10 months of age. n = 3 mice for each genotype. d, Western blot analysis of spleen (left blots) and brain and spinal cord (right blots) extracts from two different wild-type mice and four or three different SNCA\textsuperscript{A53T} mice at 9–10 months, for the indicated proteins, with Gapdh as a loading control. Note that soluble Axl ectodomain (sAxl) is upregulated in the SNCA\textsuperscript{A53T} spinal cord concomitantly with Axl. e, Although Axl is strongly upregulated in Iba1+ microglia in the SNCA\textsuperscript{A53T} spinal cord (see Fig. 4d), no upregulation of Mer is observed in these same cells. Scale bar, 10 μm. n = 2 wild-type and 3 SNCA\textsuperscript{A53T} mice.