Neuroscience

A novel monomeric amyloid β-activated signaling pathway regulates brain development via inhibition of microglia

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Abstract

Amyloid β (Aβ) forms aggregates in the Alzheimer's disease brain and is well known for its pathological roles. Recent studies show that it also regulates neuronal physiology in the healthy brain. Whether Aβ also regulates glial physiology in the normal brain, however, has remained unclear. In this article, we describe the discovery of a novel signaling pathway activated by the monomeric form of Aβ that plays essential roles in the regulation of microglial activity and the assembly of neocortex during development. We find that activation of this pathway depends on the function of amyloid precursor (APP) and heterotrimeric G proteins in microglia and inhibits microglial immune activation at transcriptional and post-transcriptional levels. Genetic disruption of this pathway during neocortical development results in microglial dysregulation and excessive matrix proteinase activation, leading to basement membrane degradation, neuronal ectopia, and laminar disruption. These results uncover a previously unknown function of Aβ as a negative regulator of brain microglia and substantially elucidate the molecular mechanisms underlying this regulation. Considering the prominence of Aβ and neuroinflammation in the pathology of Alzheimer's disease, they also highlight a potentially overlooked role of Aβ monomer depletion in the development of the disease.

eLife assessment

The study describes a link between beta-amyloid monomers, regulation of microglial activity and assembly of neocortex during development. It brings **valuable** findings that have theoretical and practical implications in the field of neuronal migration, neuronal ectopia and type II lissencephaly. Unfortunately, the evidence is **incomplete** and the manuscript would benefit from additional experiments to clarify the relationship between Ric8a and APP and bolster the findings.

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Introduction

Aβ, a core component of amyloid plaques in the Alzheimer's disease brain, is well known to form oligomers under disease conditions. Studies have shown that the oligomers formed by Aβ are highly toxic, with wide-ranging effects including inhibition of neurotransmitter release, depletion of synaptic vesicle pools, disruption of postsynaptic organization and function, and impairment of multiple forms of synaptic plasticity (Gulisano et al., 2018; He et al., 2019; Kim et al., 2013; Lauren et al., 2009; Lazarevic et al., 2017; Parodi et al., 2010; Puzzo et al., 2008; Shankar et al., 2008; Walsh et al., 2002; Yang et al., 2015; Zott et al., 2019). These effects likely significantly underpin the pathogenic role of Aβ in Alzheimer's disease and contribute to neuron loss and cognitive decline in patients. Besides its pathological roles, recent studies show that Aβ is also produced in the healthy brain by neurons in a neural activity-dependent manner and regulates the normal physiology of neurons (Cirrito et al., 2005; Fogel et al., 2014; Galanis et al., 2021; Garcia-Osta and Alberini, 2009; Gulisano et al., 2018; Gulisano et al., 2019; Morley et al., 2010; Palmeri et al., 2017; Puzzo et al., 2008; Zhou et al., 2022). For example, consistent with studies showing that Aβ monomers and low molecular weight oligomers positively regulate synaptic function and plasticity, administration of these molecules in vivo has been found to improve learning and memory in animals (Fogel et al., 2014; Garcia-Osta and Alberini, 2009; Gulisano et al., 2018; Gulisano et al., 2019; Morley et al., 2010; Palmeri et al., 2017; Puzzo et al., 2008). Recent studies have further shown that Aβ monomers directly promote synapse formation and function and homeostatic plasticity, processes crucial to normal cognitive function (Galanis et al., 2021; Kamenetz et al., 2003; Zhou et al., 2022). Together, these findings have provided crucial insights into the physiological roles that Aβ plays in regulating normal neuronal function in the brain. However, it remains unclear if Aβ also regulates the physiology of glia, nonneuronal cells that also play important roles in normal brain function.

Microglia and astrocytes, two of the major glial cell types in the brain, are known to play critical roles in the normal development, function, and plasticity of the brain circuitry (Barres, 2008; Schafer and Stevens, 2015). They coordinately regulate, among others, the spatiotemporally specific expression of immune cytokines in the brain that regulate numerous processes in brain circuit development, function, and plasticity (Zipp et al., 2023). For example, in the thalamus, a key relay station in the visual pathway, populations of astrocytes have been found to activate the expression of interleukin-33 in a neural activity-dependent manner, induce activity-dependent elimination of supernumerary synapses, and promote the maturation of the visual circuitry in early postnatal life (Vainchtein et al., 2018). In the adult hippocampus, in contrast, astrocytes have been found to activate the expression of interleukin-33 under neuronal activity blockade and induce homeostatic synaptic plasticity that maintains circuit activity balance (Wang et al., 2021). In the striatum and the neocortex, not only have astrocytes but also have microglia been observed to activate the expression of TNFα upon changes in neural circuit activity and induce homeostatic synaptic plasticity that dampens circuit perturbation (Heir et al., 2024; Lewitus et al., 2016; Stellwagen and Malenka, 2006). In the clinic, the induction of microglial release of cytokines such as TNFα also underpins the application of repetitive transcranial magnetic stimulation, a noninvasive brain stimulation technique frequently used to induce cortical plasticity and treat pharmaco-resistant depression (Eichler et al., 2023). Furthermore, in neurodegenerative diseases such as Alzheimer's disease, glial activation and brain cytokine elevation are key pathologic factors in disease development (Colonna and Butovsky, 2017; Patani et al., 2023). Elevated TNFα expression by microglia also underlies interneuron deficits and autism-like phenotype linked to maternal immune activation (Yu et al., 2022). Thus, the precise regulation of glial cytokine expression in the brain plays a key role in the normal development and function of the brain and its dysregulation is linked to common neurodevelopmental and neurodegenerative diseases. However, how glial cytokine expression is mechanistically regulated by cell-cell communication in the brain have remained largely unknown.

In this article, we report the discovery of a novel microglial signaling pathway activated by Aβ, the neuron-produced peptide at the center of Alzheimer's disease, that plays a crucial role in precisely regulating the levels of microglial cytokine expression and activity and ensuring the proper assembly of neuronal laminae during cerebral cortex development. We first came across this pathway in our study of the function of *ric8*a, a gene encoding a molecular chaperone for heterotrimeric G proteins (Gabay et al., 2011; Ma et al., 2012; Ma et al., 2017; Tall et al., 2003). We found that deletion of *ric8a* during cortical development resulted in cortical basement membrane degradation, neuronal ectopia, and laminar disruption. However, unlike in classic models of cobblestone lissencephaly, these phenotypes resulted not from *ric8a* deficiency in brain neural cell types, but from deficiency in microglia. The phenotypes also resemble those in mutants of amyloid precursor protein (APP) family and pathway genes. Indeed, we found that *app* deficiency in microglia also underpins ectopia formation in *app* family gene mutants. Furthermore, we found that APP and Ric8a form a functional pathway in microglia that is specifically activated by the monomeric form of Aβ and this pathway normally inhibits the transcriptional and post-transcriptional expression of immune cytokines by microglia.

Results

Cortical ectopia in *ric8a-emx1-cre* mutants results from non-neural deficiency

To study of the function of *ric8a* in neocortical development, we deleted a conditional *ric8a* allele (Ma et al., 2012; Ma et al., 2017) using *emx1-cre,* a *cre* line designed to target dorsal forebrain neural progenitors in mice (Gorski et al., 2002). We found it result in ectopia formation exclusively in the lateral cortex of the perinatal mutant brain (**Fig. 1a-d**). Birth-dating showed that the ectopia consisted of both early and late-born neurons (**Supplemental Fig. 1**). Consistent with this observation, neurons in the ectopia also stained positive for both Ctip2 and Cux1, genes specific to lower and upper-layer neurons, respectively. Interestingly, in cortical areas without ectopia, radial migration of early- and late-born neurons appeared largely normal as shown by birth-dating as well as Cux1 and Ctip2 staining (**Supplemental Fig. 2**). This suggests that cell-autonomous defects in neurons are unlikely the cause of the ectopia. At E16.5, clear breaches in the pial basement membrane of the developing cortex were already apparent (**Supplemental Fig. 3**). However, unlike classic models of cobblestone lissencephaly, where radial glial fibers typically retract, radial glial fibers in *ric8a* mutants instead extended beyond the breaches. This argues against radial glial cell adhesion defects since they would be predicted to retract. Furthermore, in areas without ectopia, we also observed normal localization of Cajal-Retzius cells, expression of Reelin, and splitting of the preplate, arguing against primary defects in Cajal-Retzius cells. In cobblestone lissencephaly, studies show that ectopia result from primary defects in radial glial maintenance of the pial basement membrane (Beggs et al., 2003; Graus-Porta et al., 2001; Moore et al., 2002; Satz et al., 2010). In *ric8a* mutants, we observed large numbers of basement membrane breaches at E14.5, almost all associated with ectopia (**Supplemental Fig. 4**). In contrast, at E13.5, although we also observed significant numbers of breaches, none was associated with ectopia. This indicates that basement membrane breaches similarly precede ectopia in *ric8a* mutants. However, at E12.5, despite a complete lack of basement membrane breaches, we observed increased numbers of laminin-positive debris across the lateral cortex, both beneath basement membrane segments with intact laminin staining and beneath segments with disrupted laminin staining, the latter presumably sites of future breach (**Supplemental Fig. 5**). As a major basement membrane component, the increased amounts of laminin debris suggest increased degradative activity within the developing cortex. Thus, these results indicate that excessive basement membrane degradation, but not defective maintenance, is likely a primary cause of cortical ectopia in *ric8a* mutants.

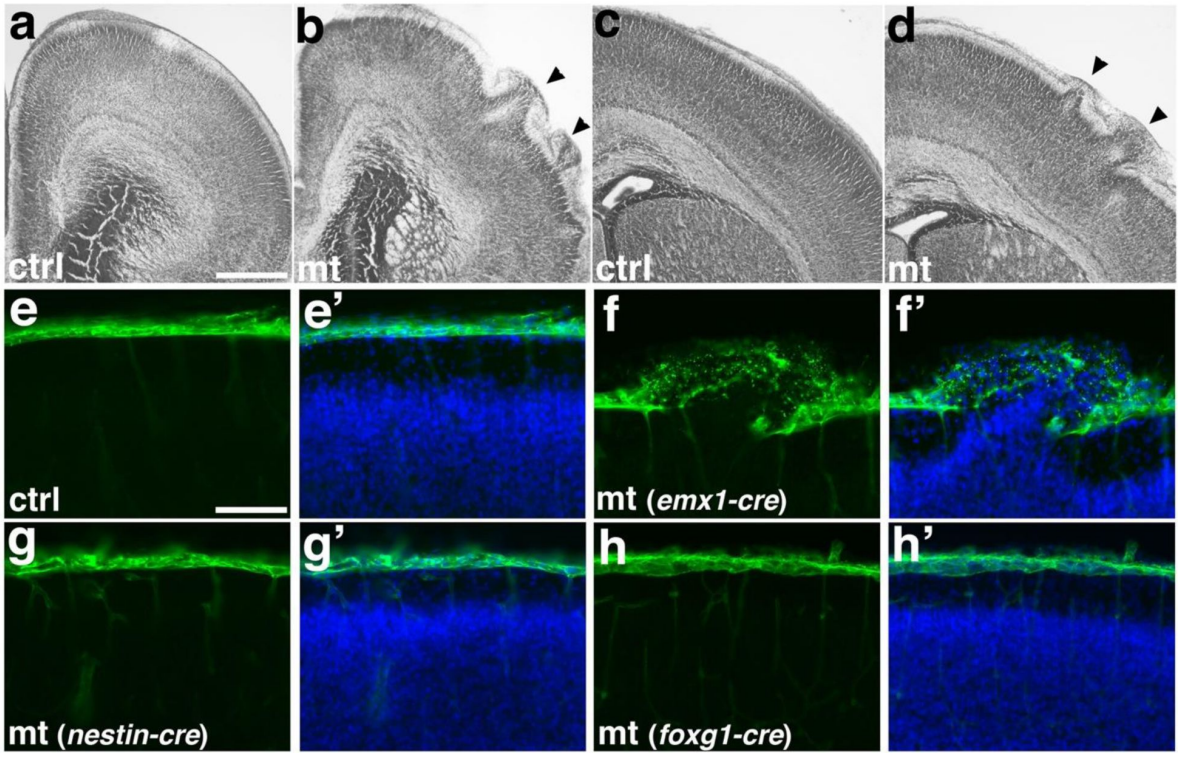

Fig. 1.

Deletion of *ric8a* using *emx1-cre* results in cortical ectopia due to non-neural deficits.

(**a-d**) Nissl staining of control (ctrl, **a**&**c**) and mutant (mt, **b**&**d**) anterior motor (**a-b**) and posterior somatosensory (**c-d**) cortex at P0.
(e-e') Laminin (LN, in green) and nuclear (DAPI, in blue) staining of control cortices at P0. A continuous basement membrane is observed at the pia, beneath which cells are well organized in the cortical wall.
(**f-f'**) Staining of *ric8a-emx1-cre* mutant cortices at P0. Basement membrane breach and neuronal ectopia are observed following *ric8a* deletion by *emx1-cre*, a *cre* line expressed in cortical radial glial progenitors beginning at E10.5.
(g-g') Staining of *ric8a-nestin-cre* mutant cortices at P0. No obvious basement membrane breach or neuronal ectopia is observed following *ric8a* deletion by *nestin-cre*, a *cre* line expressed in cortical progenitors beginning around E12.5.
(**h-h'**) Staining of *ric8a-foxg1-cre* mutant cortices at P0. No obvious basement membrane breach or neuronal ectopia is observed following *ric8a* deletion by *foxg1-cre*, a *cre* line expressed in forebrain neural progenitors from E9.0.
Scale bars, 640μm for (**a-b**), 400μm for (**c-d**), and 100μm for (**e-h'**).

To determine the cell type(s) genetically responsible for cortical basement membrane degradation and ectopia in *ric8a* mutants, we employed a panel of *cre* lines (**Fig. 1e-h'**). To target Cajal-Retzius cells, we employed *wnt3a-cre* (Yoshida et al., 2006) but found *ric8a* deletion using *wnt3a-cre* did not result in ectopia. To target postmitotic excitatory and inhibitory neurons, we employed *nex-cre* (Goebbels et al., 2006) and *dlx5/6-cre* (Stenman et al., 2003) respectively but similarly found neither result in ectopia. These results point to *ric8a* requirement in cell types other than post-mitotic neurons. To test the involvement of neural progenitors, we employed *nestin-cre* (Graus-Porta et al., 2001). Previous studies show that deletion of *β1 integrin* and related genes by *emx1-cre* and *nestin-cre* results in similar ectopia phenotypes (Belvindrah et al., 2006; Graus-Porta et al., 2001; Huang et al., 2006; Niewmierzycka et al., 2005). To our surprise, deletion of *ric8a* by *nestin-cre* did not result in ectopia (Ma et al., 2017) (**Fig. 1g-g'**). Since *nestin-cre*-mediated deletion in neural progenitors is inherited by post-mitotic neurons and astrocytes, this indicates that the combined deletion of *ric8a* from all these cell types does not lead to ectopia. The onset of *nestin-cre* expression is, however, developmentally slightly later than that of *emx1-cre* (Gorski et al., 2002). To assess the potential contribution of this temporal difference, we employed *foxg1-cre*, a *cre* line expressed in forebrain neural progenitors starting from E10.5 (Hebert and McConnell, 2000). We found that *ric8a* deletion using *foxg1-cre* still failed to produce ectopia (**Fig. 1h-h'**). Thus, these results strongly argue against the interpretation that *ric8a* deficiency in neural cell lineages is responsible for basement membrane degradation and ectopia in *ric8a* mutants.

During embryogenesis, the neural tube undergoes epithelial-mesenchymal transition giving rise to neural crest cells (Leathers and Rogers, 2022). This process involves region-specific basement membrane breakdown that resembles the *ric8a* mutant phenotype. To determine if ectopic epithelial-mesenchymal transition plays a role, we examined potential changes in neuro-epithelial cell fates in the mutant cortex. We found that cortical neural progenitors expressed Pax6, Nestin, and Vimentin normally (**Supplemental Fig. 6**). Cell proliferation in the ventricular zone was also normal. Furthermore, although *ric8a* regulates asymmetric cell division in invertebrates (Afshar et al., 2004; Couwenbergs et al., 2004; David et al., 2005; Hampoelz et al., 2005; Wang et al., 2005), we observed no significant defects in mitotic spindle orientation at the ventricular surface. Additionally, no ectopic expression of neural crest markers or Wnt pathway activation was observed (**Supplemental Fig. 7**). Altogether, these results further indicate that non-neural-cell deficiency is responsible for ectopia formation in *ric8a* mutants.

Microglial *ric8a* deficiency is responsible for ectopia formation

To assess the role of non-neural cell types, we turned our attention to microglia since RNA-seq studies show that brain microglia express *emx1* at a significant level (Zhang et al., 2014). To determine if *emx1-cre* is expressed and active in microglia, we isolated microglia from *ric8a-emx1-cre* mutants. We found that *emx1-cre* mediated *ric8a* deletion indeed resulted in altered cytokine expression in microglia (**Supplemental Fig. 8**). This indicate that *emx1-cre* is expressed and active in microglia and deletes *ric8a*. To determine the specific significance of *ric8a* deletion from microglia alone, we next employed a microglia-specific *cx3cr1-cre* (Yona et al., 2013). Like *emx1-cre* mutants, *ric8a-cx3cr1-cre* mutant microglia also showed elevated cytokine secretion and transcription in comparison to control microglia upon stimulation by lipopolysaccharide (LPS) (**Fig. 2a-b**). Similar results were also obtained with stimulation by polyinosinic-polycytidylic acid (poly I:C), an intracellular immune activator. Thus, these results indicate that *ric8a* deficiency in microglia results in broad increases in microglial sensitivity to immune stimulation.

To determine if microglial *ric8a* deficiency alone is sufficient to cause cortical ectopia in vivo, we examined *ric8a-cx3cr1-cre* mutants but found that it did not affect either basement membrane integrity or cortical layering (**Fig. 2c**). We reasoned that this may be related to the fact that *ric8a* mutant microglia only show heightened activity upon stimulation but not under basal unstimulated conditions (**Fig. 2a-b**) but elevated microglial activity may be needed for basement membrane degradation and ectopia formation. To test this possibility, we employed in

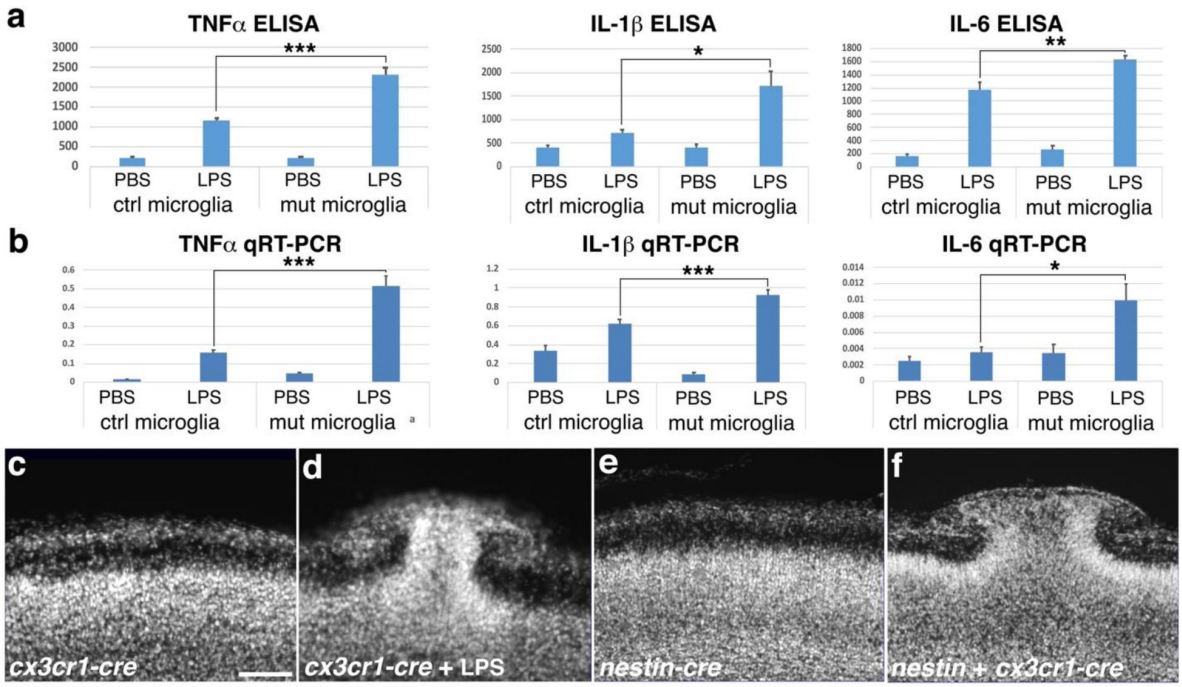

Fig. 2.

ric8a deficiency in microglia is responsible for cortical ectopia.

(a) TNFα, IL-1β, and IL-6 secretion (pg/ml) in control and *ric8a/cx3cr1-cre* mutant microglia following LPS stimulation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n = 6-8 each group.
(b) TNFα, IL-1 β, and IL-6 mRNA expression in control and *ric8a/cx3cr1-cre* mutant microglia following LPS stimulation. *, $P < 0.05$; ***, $P < 0.001$; n = 5-6 each group.
(**c**-**d**) Nuclear (DAPI, in grey) staining of *ric8a-cx3cr1-cre* mutant cortices at P0 in the absence (**c**) or presence (**d**) of LPS treatment during embryogenesis.(**e-f**) Nuclear (DAPI, in grey) staining of *ric8a-nestin-cre* single *cre* (**e**) and *ric8a-nestin-cre+cx3cr1-cre* double *cre* (**f**) mutant cortices at P0.
Scale bar in (**c**), 100 μm for (**c**-**f**).

utero LPS administration to activate microglia during cortical development. We found that over 50% of *ric8a-cx3cr1-cre* mutant neonates showed ectopia when administered LPS at E11.5-12.5 (10 of 19 mutant neonates examined) (**Fig. 2d**). In contrast, no cortical ectopia were observed in any of the 32 littermate controls that were similarly administered LPS. This indicates that only the combination of microglial *ric8a* deficiency and immune activation leads to ectopia formation. In *emx1-cre* mutants, ectopia develop without LPS administration (**Fig. 1**). We suspect that this may be due to concurrent *ric8a* deficiency in neural cell types, which may result in deficits that mimic immune stimulation. To test this, we next additionally deleted *ric8a* from neural cells in the *ric8a-cx3cr1-cre* microglial mutant background by introducing *nestin-cre*. We have shown that *ric8a* deletion by *nestin-cre* alone does not result in ectopia (**Fig. 1g-g'**). However, we found that, like deletion by *emx1-cre*, *ric8a* deletion by the dual *cre* combination of *cx3cr1-cre* and *nestin-cre* also resulted in ectopia in all double *cre* mutants (6 of 6 examined) (**Fig. 2f**). Thus, these results indicate that elevated immune activation of *ric8a* deficient microglia during cortical development is responsible for ectopia formation.

Microglial *app* deficiency also results in ectopia formation

The exclusive localization of ectopia to the lateral cortex in *ric8a* mutants is reminiscent of the phenotypes observed in APP family and pathway mutants including *app/aplp1/2* triple (Herms et al., 2004) and *apbb1/2* double knockouts (Guenette et al., 2006). Independent studies also point to a role of non-neural cells in ectopia formation in these mutants. For example, unlike the triple knockout, specific *app* knockdown in cortical neurons during development results in under- but not over-migration of neurons (Young-Pearse et al., 2007). To test the role of microglia in ecotopia formation in APP pathway mutants, we first analyzed *app* mutant microglia. To this end, we employed *cx3cr1-cre* to delete a conditional allele of *app* from microglia and found that microglia cultured from *app-cx3cr1-cre* mutants showed reduced TNFα and IL-6 secretion as well as muted IL-6 transcription upon stimulation (**Fig. 3a**, **Supplemental Fig. 9**). This indicates that *app* plays a previously unrecognized, cell-autonomous role in microglia in regulating microglial activity. Microglia exhibit attenuated immune response following chronic stimulation, especially when carrying strong loss-of-function mutations in anti-inflammatory pathways (Chamberlain et al., 2015; Sayed et al., 2018). We suspect that the attenuated response by *app* mutant microglia may result from similar effects following in vitro culture. To test effects of *app* mutation under conditions that more closely resemble in vivo conditions, we next isolated fresh, unelicited peritoneal macrophages and acutely analyzed their response to immune stimulation. We found that *app* mutant macrophages showed significantly elevated secretion of all cytokines tested (**Fig. 3b**). At the transcriptional level, mRNA induction was also increased for all cytokines (**Fig. 3c**). Thus, like that of *ric8a*, the normal function of *app* also appears to be to suppress the inflammatory activation of microglia.

To determine if microglial *app* deficiency is also responsible for ectopia formation in *app* triple knockout mutants, we next asked if activating microglia in microglia-specific *app* mutants similarly results in pial ectopia during cortical development. To this end, we administered LPS in utero at E11.5-12.5 to *app-cx3cr1-cre* mutant animals as we did to *ric8a-cx3cr1-cre* mutants above. We found that, while none of the 81 littermate controls administered LPS showed ectopia, a significant number of mutant neonates showed ectopia (6 of 31 neonates examined, ~19%) (**Fig. 3e**). Thus, *app* deficient microglia, when activated, also results in cortical ectopia during development. The reduced severity of the ectopia observed, as compared to that in *ric8a/cx3cr1-cre* mutants, likely in part results from the reduced LPS dosage (by ~3 folds) we had to use in these animas due to the enhanced immune sensitivity of their strain genetic background. Other *app* gene family members are also expressed in microglia (Zhang et al., 2014) and may in addition compensate for the loss of APP. Thus, these results indicate that *app* normally plays a cell-autonomous role in microglia that negatively regulate microglial activation and its loss of function underlies ectopia formation. The similarities between *app* and *ric8a* mutant phenotypes suggest that they form a previously unknown anti-inflammatory pathway in microglia.

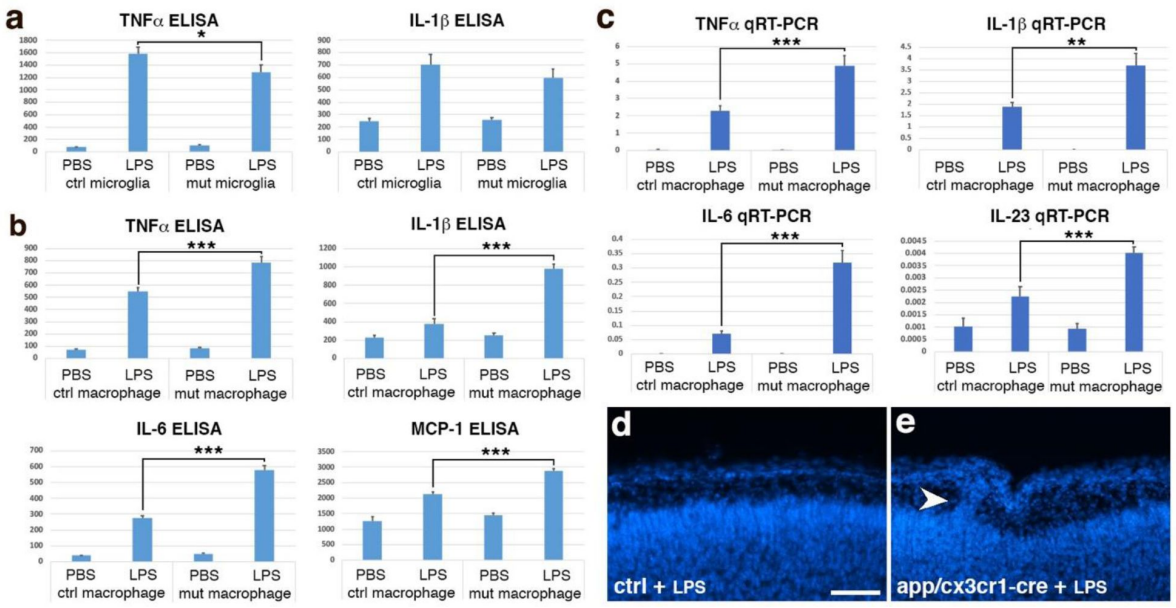

Fig. 3.

app deficiency results in hypersensitive microglia and cortical ectopia.

(a) TNFα and IL-1β secretion (pg/ml) in cultured control and *app/cx3cr1-cre* mutant microglia following LPS stimulation. *, $P < 0.05$; n = 7-9 each group.
(b) TNFα, IL-1β, IL-6, and MCP1 secretion (pg/ml) in fresh unelicited control and *app/cx3cr1-cre* mutant peritoneal macrophages following LPS stimulation. ***, $P < 0.001$; n = 7-10 each group.
(c) TNFα, IL-1 β, IL-6, and IL-23 mRNA expression in fresh unelicited control and *app/cx3cr1-cre* mutant peritoneal macrophages following LPS stimulation. **, $P < 0.01$; ***, $P < 0.001$; n = 6 each group
(**d-e**) Nuclear (DAPI, in blue) staining of control (**D**) and LPS-treated *app/cx3cr1-cre* mutant (**E**) cortices at P0. Note cortical ectopia in the mutant cortex (arrowhead).
Scale bar in (**d**), 200μm for (**d-e**).

Monomeric Aβ suppresses microglial inflammatory activation via APP and Ric8a

The possibility that *app* and *ric8a* may form a novel anti-inflammatory pathway in microglia raises questions on the identity of the ligands for the pathway. Several molecules have been reported to bind to APP and/or activate APP-dependent pathways (Fogel et al., 2014; Milosch et al., 2014; Rice et al., 2012), among which Aβ is note-worthy for its nanomolar direct binding affinity (Fogel et al., 2014; Shaked et al., 2006). Aβ oligomers and fibrils have been shown by numerous studies to be pro-inflammatory, while non-fibrillar Aβ lack such activity (Halle et al., 2008; Huang, 2023, 2024; Lorton et al., 1996; Muehlhauser et al., 2001; Tan et al., 1999). In contrast, when employed under conditions that favor the monomer conformation, Aβ inhibits T cell activation (Grant et al., 2012). This suggests that, unlike Aβ oligomers, Aβ monomers may be anti-inflammatory. To test this possibility, we dissolved Aβ40 peptides in DMSO, which has been shown to preserve the monomeric conformation (LeVine, 2004; Stine et al., 2011). We found that Aβ monomers as prepared potently suppressed the secretion of large numbers of cytokines (**Fig. 4a**, **Supplemental Fig. 10**) and showed similar effects on microglia no matter if they were activated by LPS or poly I:C (**Fig. 4b**). We also found that the Aβ monomers similarly strongly inhibited the induction of cytokines at the transcriptional level (**Fig. 4c**, **Supplemental Fig. 10**). In addition, we observed these effects with Aβ40 peptides from different commercial sources. Thus, these results indicate that monomeric Aβ possesses a previously unreported anti-inflammatory activity against microglia that strongly inhibits microglial inflammatory activation.

To determine whether monomeric Aβ signals through APP, we employed *app-cx3cr1-cre* mutant microglia. We found that, unlike that of control microglia, Aβ monomers failed to suppress the secretion of all tested cytokines by *app* mutant microglia (**Fig. 4d**, **Supplemental Fig. 10**). Interestingly, this blockade appeared to be specific to *app* since Aβ monomers still significantly suppressed cytokine secretion by *aplp2* mutant microglia. At the transcriptional level, Aβ monomers also failed to suppress cytokine induction in *app* mutant microglia (**Fig. 4e**, **Supplemental Fig. 10**). Together, these results indicate that APP is functionally required in microglia for Aβ monomer inhibition of cytokine expression at both transcriptional and post-transcriptional levels. Cultured microglia from *app-cx3cr1*-cre mutants showed attenuated immune activation (**Fig. 3**). To assess whether this may affect the efficacy of Aβ monomer inhibition, we next tested the response of fresh, unelicited macrophages. We found that, like that of control microglia, cytokine secretion by control macrophages was also strongly suppressed by Aβ monomers (**Fig. 4f**, **Supplemental Fig. 10**). However, even though *app* mutant macrophages showed elevated response to immune stimulation in comparison to control macrophages, they still failed to respond to Aβ monomers and displayed levels of cytokine secretion that were indistinguishable from those of DMSO-treated cells (**Fig. 4f**, **Supplemental Fig. 10**). Thus, these results further indicate that APP function is required in microglia for mediating the anti-inflammatory effects of Aβ monomers.

The similarity of *ric8a* ectopia to *app* ectopia phenotype (**Figs. 2** & **3**) also suggests that Ric8a functions in the same pathway as APP in mediating Aβ monomer anti-inflammatory signaling in microglia. This is consistent with previous studies showing that heterotrimeric G proteins are coupled to APP and mediate APP intracellular signaling in vitro and vivo (Fogel et al., 2014; Milosch et al., 2014; Nishimoto et al., 1993; Ramaker et al., 2013) and that Ric8a is a molecular chaperone essential for the post-translational stability of heterotrimeric G proteins (Gabay et al., 2011; Tall et al., 2003). To directly test if Ric8a is part of this pathway, we next employed *ric8a-cx3cr1-cre* mutant microglia. We found that, indeed, like that of *app* mutant microglia, Aβ monomers also failed to suppress the secretion of TNFα and IL-6 by *ric8a* mutant microglia (**Fig. 4g**). This indicates that heterotrimeric G proteins function is likely required in the same pathway of APP in microglia for the suppression of TNFα and IL-6 secretion. However,

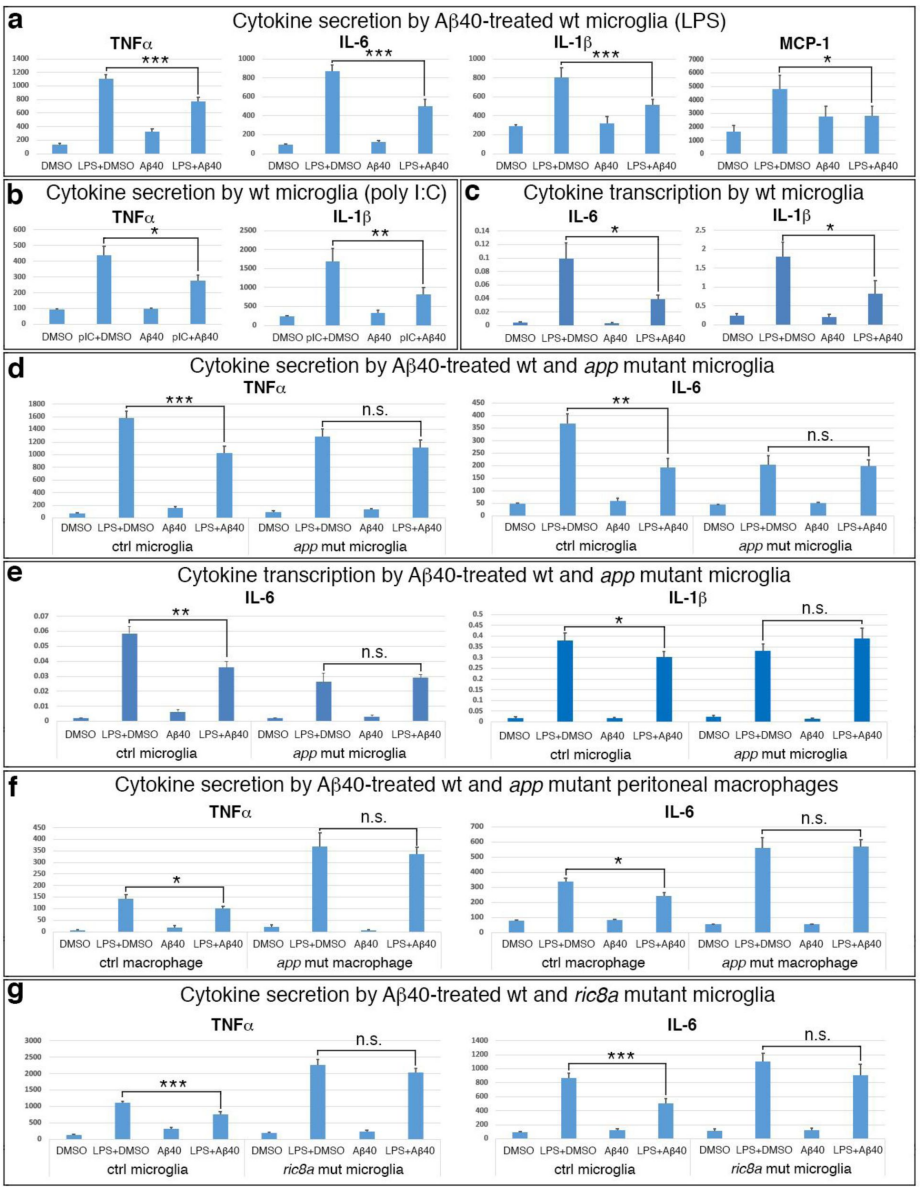

Fig. 4.

Monomeric Aβ40 suppresses microglia via an APP and Ric8a.

(a) TNFα, IL-6, IL-1β, and MCP1 secretion (pg/ml) by wildtype microglia following LPS stimulation in the absence or presence of Aβ40 (200 or 500nM). *, $P < 0.05$; ***, $P < 0.001$; n = 8-14 each group.
(b) TNFα and IL-1β secretion (pg/ml) by wildtype microglia following poly I:C stimulation in the absence or presence of Aβ40 (500nM). *, $P < 0.05$; **, $P < 0.01$; n = 6-7 each group.
(c) IL-6 and IL-1β mRNA induction in wildtype microglia following LPS stimulation in the absence or presence of Aβ40 (500nM). *, $P < 0.05$; n = 6 each group.
(d) TNFα and IL-6 secretion (pg/ml) by control and *app/cx3cr1-cre* mutant microglia following LPS stimulation in the absence or presence of Aβ40 (200nM). **, $P < 0.01$; ***, $P < 0.001$; n = 8 each group.
(e) IL-6 and IL-1β mRNA induction in control and *app/cx3cr1-cre* mutant microglia following LPS stimulation in the absence or presence of Aβ40 (200nM). *, $P < 0.05$; **, $P < 0.01$; n = 6 each group.
(f) TNFα and IL-6 secretion (pg/ml) by control and *app/cx3cr1-cre* mutant peritoneal macrophages following LPS stimulation in the absence or presence of Aβ40 (500nM). *, $P < 0.05$; n = 6-7 each group
(g) TNFα and IL-6 secretion (pg/ml) by control and *ric8a/cx3cr1-cre* mutant microglia following LPS stimulation in the absence or presence of Aβ40 (200nM). ***, $P < 0.001$; n = 12-14 each group.

unlike APP, we found that Ric8a appears to be dispensable for Aβ monomer regulation of other cytokines. For example, unlike that of TNFα and IL-6, Aβ monomers still suppressed IL-1β secretion by *ric8a* mutant microglia (**Supplemental Fig. 10**). It also appears to be dispensable for the regulation of cytokine transcription since Aβ monomers similarly suppressed IL-6 transcriptional induction in both control and *ric8a* mutant microglia. These results suggest that heterotrimeric G proteins function may only mediate some of the anti-inflammatory signaling of monomeric Aβ. Thus, APP and Ric8a-regulated heterotrimeric G proteins form part of a novel anti-inflammatory pathway activated by monomeric Aβ in microglia.

Elevated matrix metalloproteinases (MMPs) cause basement membrane degradation

We have shown that heightened microglial activation due to mutation in the Aβ monomer-activated APP/Ric8a pathway results in basement membrane degradation and ectopia during cortical development. To further test this interpretation, we sought to test the prediction that inhibition of microglial activation in these mutants suppressed the formation ectopia. To this end, we employed dorsomorphin and S3I-201, inhibitors targeting Akt, Stat3, and other mediators in pro-inflammatory signaling (Lee et al., 2016; Qin et al., 2012). Consistent with their anti-inflammatory activity, we found that dorsomorphin and S3I-201 both suppressed astrogliosis associated with neuroinflammation in the cortex of *ric8a-emx1-cre* mutants (**Supplemental Fig. 11**). Furthermore, they also suppressed the formation of ectopia in *ric8a-emx1-cre* mutants, reducing both the number and the size of the ectopia observed (**Fig. 5a-f**). Most strikingly, the combined administration of dorsomorphin and S3I-201 nearly eliminated all ectopia in *ric8a-emx1-cre* mutants (**Fig. 5d**, **5e**). Thus, these results indicate that excessive inflammatory activation of microglia is responsible for ectopia formation in *ric8a* mutants.

Under neuroinflammatory conditions, brain cytokines frequently induce MMPs, which lead to breakdown of the extracellular matrix and contribute to disease pathology (Pagenstecher et al., 1998; Wang et al., 2000). Since *ric8a* mutant microglia are hyperactive in inflammatory cytokine production, we wonder if induction of MMPs may underlie the laminin degradation and cortical basement membrane break observed in *ric8a* mutants. To test this, we examined the activities of MMP2 and MMP9 in the developing cortex using gelatin gel zymography. We found that the activity of MMP9 in the mutant cortex was significantly increased (**Fig. 5i**, **Supplemental Fig. 12**). In contrast, that activity of MMP2 remained unaffected. Similarly, at the protein level, we found that the immunoreactivity for MMP9 was increased in *ric8a-emx1-cre* mutants (**Fig. 5g-h**). To test if the increased MMP activity is responsible for the ectopia observed, we next employed BB94, a broad-spectrum inhibitor of MMPs. We found that BB94 administration significantly suppress both the number and the size of the ectopia in *ric8a* mutants (**Fig. 5j-m**). To narrow down the identity of MMPs responsible, we further employed an inhibitor specific for MMP9 and 13 and found that it similarly suppressed both the number and the size of the ectopia (**Fig. 5l-m**). Furthermore, consistent with its near complete suppression of cortical ectopia (**Fig. 5a-f**), we found that the co-administration of dorsomorphin and S3I-201 also reduced MMP9 activity in the mutant cortex to the control level (**Supplemental Fig. 12**). Thus, these results indicate this Aβ monomer-activated anti-inflammatory pathway normally promotes cortical development through suppressing microglial activation and MMP induction.

Discussion

The spatiotemporal expression of immune cytokines by glial cells in the brain plays critical roles in the normal development, function, and plasticity of the brain circuitry (Barres, 2008; Schafer and Stevens, 2015; Zipp et al., 2023). In this article, we have identified a novel microglial anti-inflammatory pathway activated by monomeric Aβ that inhibits microglial cytokine expression and plays essential roles in the normal development of the cerebral cortex. We have found that

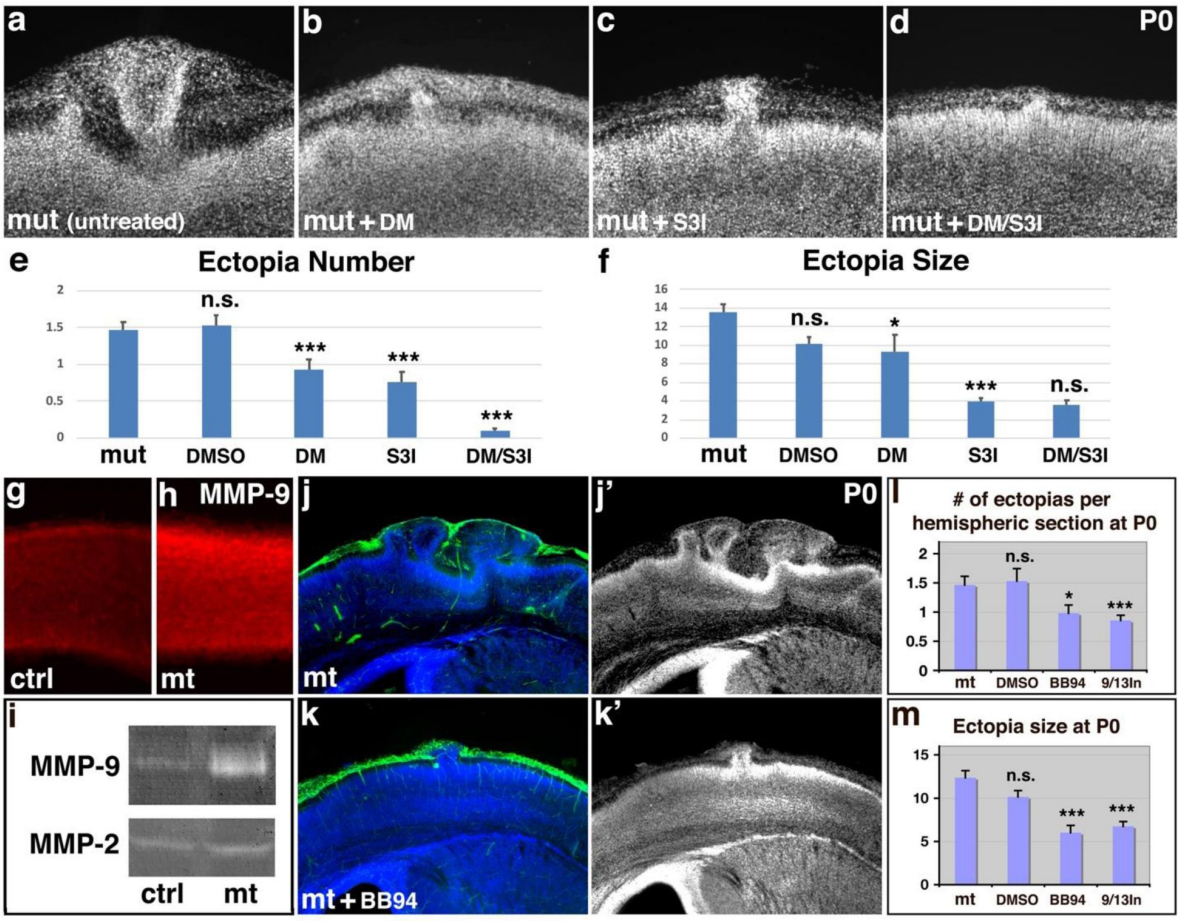

Fig. 5.

Inhibition of both microglial inflammatory activation and cortical MMP9 activity suppresses basement membrane breach and neuronal ectopia.

(**a-d**) Nuclear (DAPI, in grey) staining of untreated (**a**), anti-inflammatory drug dorsomorphin (DM) (**b**), Stat3 inhibitor S3I-201 (S3I) (**c**), and DM/S3I (**d**) dual treated mutant cortices at P0.
(**e-f**) Quantitative analysis of ectopia number (**e**) and size (**f**) in the neonatal mutant cortex after DMSO, DM, S3I, and DM/S3I dual treatment at E12.5. *, $P < 0.05$; ***, $P < 0.001$; all compared to untreated mutants. The reduction in ectopia size after dual treatment is not statistically significant, likely due to the small number of ectopias that remained.
(**g-h**) MMP9 (in red) staining of control (**g**) and mutant cortices (**h**) at E13.5. Quantification shows statistically significant increases in mutants (control, 24.8 ± 0.2 AU (Arbitrary Units); mutant, 35.7 ± 1.7 AU; $P = 0.002$; n = 6).
(i) Gel zymography of control and mutant cortical lysates at E13.5. Increased levels of MMP9 but not of MMP2 were observed in mutants (control, 1.00 ± 0.06 AU; mutant, 3.72 ±1.86 AU; $P = 0.028$; n = 4)
(**j-k'**) Laminin (in green) and nuclear (DAPI, in blue) staining of mutant cortices untreated (**H**) or treated (I) with BB94.
(**l-m**) Quantitative analysis of ectopia number and size following MMP inhibitor BB94 or MMP9/13 inhibitor treatment. *, $P < 0.05$; ***, $P < 0.001$; all compared to untreated mutants.

this pathway is mediated by APP and heterotrimeric G proteins in microglia and its activation leads to the inhibition of microglial cytokine induction at transcriptional and post-transcriptional levels (**Figs. 1**-**4**). We further show that a key function of this pathway is to suppress the activity of MMP9 during corticogenesis and disruption of this regulation results in cortical basement membrane degradation and neuronal ectopia development (**Figs. 1**-**3**, **5**). Furthermore, we find that this pathway is activated specifically by the monomeric form of Aβ (**Fig. 4**), identifying, for the first time, an isoform specific activity of Aβ against microglia. These results provide novel insights into the neuron-glia communication mechanisms that coordinate the regulation of immune cytokines, key regulators of Hebbian and non-Hebbian synaptic plasticity, by glial cells in the brain. The discovery of the novel activity of monomeric Aβ as a negative microglial regulator may also facilitate the further elucidation of Alzheimer's disease pathogenesis.

Microglial activity regulation during cortical development

Among the glial cell populations in the brain, astrocytes and oligodendrocyte are both born within the nervous system at the end of cortical neurogenesis. As such, they play limited roles in the early steps of cortical development. In contrast, microglia are not only of a distinct non-neural lineage that originates from outside the nervous system but also begin to populate the brain at the onset of corticogenesis (Ginhoux et al., 2010; Hattori et al., 2023). As such, they play unique roles throughout cortical development. Indeed, microglial activity has been found to regulate the size of the cortical neural precursor pool (Cunningham et al., 2013). Microglia-secreted cytokines have also been found to promote both neurogenesis and oligodendrogenesis (Shigemoto-Mogami et al., 2014). As such, the precise regulation of microglial activity is critical to the normal development of the neocortex from an early stage. In this study, we have shown that immune over-activation of microglia due to disruption of a monomeric Aβ-activated pathway results in excessive cortical matrix proteinase activation, leading basement membrane degradation and neuronal ectopia. Previous studies have shown that reductions in the expression of microglial immune and chemotaxis genes instead lead to the failure of microglia to populate the brain (Iyer et al., 2022). These results together thus highlight the importance of precisely regulating the level of microglial activity during brain development. The dramatic destructive effects of microglial hyperactivity we have observed during corticogenesis also foreshadow the critical roles it plays in brain dysfunction and disease at later stages of life.

In this study, we have also shown that the anti-inflammatory regulation of microglia in corticogenesis depends on a pathway composed of APP and Ric8a-regulated heterotrimeric G proteins. This has revealed new insight into the intercellular signaling mechanisms regulating microglial activity in the brain. Heterotrimeric G proteins are well-known mediators of G protein-coupled receptor (GPCR) signaling. In this study, we have found that they also function in the same pathway as APP. To our knowledge, ours is the first study to report an in vivo anti-inflammatory function of this pathway in microglia and has significantly advanced knowledge in microglial biology. This is also consistent with previous studies showing that heterotrimeric G proteins interact with the APP cytoplasmic domain and mediate APP signaling from invertebrates to mammals in several other cell types (Fogel et al., 2014; Milosch et al., 2014; Nishimoto et al., 1993; Ramaker et al., 2013). In this study, we have in addition shown that this pathway is specifically activated by the monomeric form of Aβ, a peptide produced by neurons in the brain (Cirrito et al., 2005), providing further insight into the biological function of this pathway. In the early cortex, neurogenesis is just beginning, and most neurons born are in an immature state. It is unclear if this pathway is activated by Aβ at this stage. However, studies have shown that other APP ligands such as pancortin, a member of the olfactomedin family proteins known to inhibit innate immunity (Liu et al., 2010), are expressed in the cortex at this stage (Rice et al., 2012). It will be interesting to determine if these innate immune regulators play a role in regulating this pathway.

Neuronal activity, glial cytokine expression, and brain circuit plasticity

Activity-dependent competitive and homeostatic plasticity is a foundational rule that regulates the development, maturation, and function of neural circuits across brain regions. Studies have shown that glial cells, through regulating the spatiotemporal expression of immune cytokines, play a pivotal role in this process. In the developing thalamus, by activating interleukin-33 expression in an activity-dependent manner, astrocytes have been found to promote the segregation of eye-specific axonal projection and the maturation of the visual circuitry (He et al., 2022; Vainchtein et al., 2018). In the visual cortex, astrocytic expression of TNFα similarly mediates activity-dependent homeostatic upscaling of cortical synapses following peripheral monocular deprivation (Barnes et al., 2017; Heir et al., 2024; Kaneko et al., 2008). In this study, we have shown that Aβ monomers inhibit expression of cytokines by brain microglia via a novel APP/heterotrimeric G protein-mediated pathway. Aβ is primarily produced by neurons in the brain in a neural activity-dependent manner and form oligomers when large quantities are produced (Cirrito et al., 2005). Aβ oligomers, in contrary to monomers, are proinflammatory and increase glial cytokine expression (Halle et al., 2008; Huang, 2023; Lorton et al., 1996; Muehlhauser et al., 2001; Tan et al., 1999). These findings thus suggest that different levels of neural circuit activity in the brain may differentially regulate glial cytokine expression through inducing different levels of Aβ. High levels of neural activity may lead to high levels of Aβ and the formation of Aβ oligomers that activate glial cytokine production, while low levels of neural activity may produce low levels of Aβ, maintain Aβ as monomers, and inhibit glial cytokine production. Thus, Aβ in the brain may not only be a reporter of the levels of neural circuit activity but may also serve as an agent that directly mediate activity level-dependent plasticity. Following sensory deprivation, for example, Aβ levels may be lowered due to loss of sensory stimulation. This may lead to the relief of monomeric Aβ inhibition of cytokines such as TNFα and as a result trigger homeostatic upscaling of cortical synapses in the visual cortex (Barnes et al., 2017; Heir et al., 2024; Kaneko et al., 2008). In contrary, when neural activity levels are high, large quantities of Aβ may be produced, leading to formation of Aβ oligomers that may in turn induce expression of cytokines such as IL-33 that promote synaptic pruning. A large body of evidence strongly indicates that Aβ and related pathways indeed mediate homeostatic and competitive plasticity in the visual and other systems of the brain (Galanis et al., 2021; Huang, 2023, 2024; Kamenetz et al., 2003; Kim et al., 2013). Our discovery of the Aβ monomer-activated pathway has therefore provided novel insights into a universal mechanism that senses neural circuit activity pattern and translates it into homeostatic and competitive synaptic changes in the brain, a mechanism with fundamental roles in cognitive function.

In this study, we have also found that the matrix proteinase MMP9 is a key downstream effector of microglial activity in the developing cortex. We find that microglial hyperactivity results in increased levels of MMP9, leading to cortical basement membrane degradation and neuronal ectopia and inhibiting MMP9 directly or indirectly suppresses the phenotype. This suggests that the regulation of MMP9 may be a key mechanism by which glial cells regulate brain development and plasticity. Indeed, independent studies have shown that, in the visual cortex, MMP9 is also a pivotal mediator of TNFα-dependent homeostatic upscaling of central synapses following monocular deprivation (Akol et al., 2022; Kaneko et al., 2008; Kelly et al., 2015; Spolidoro et al., 2012). In the Xenopus tectum, MMP9 has similarly been found to be induced by neural activity and promote visual activity-induced dendritic growth (Gore et al., 2021). Importantly, in both wildtype and amblyopic animals, light reintroduction after dark exposure has been found to reactivate plasticity in the adult visual cortex via MMP9, uncovering a potential treatment for common visual conditions (Murase et al., 2017; Murase et al., 2019). These results therefore highlight a conserved glia/cytokine/MMP9-mediated mechanism that regulates brain development and plasticity from embryogenesis to adulthood. In ocular dominance plasticity, MMP9 is activated at perisynaptic regions (Murase et al., 2017; Murase et al., 2019). MMP9 mRNA translation has

been also observed in dendrites (Dziembowska et al., 2012). In the *ric8a* mutant cortex, we find that MMP9 activity is increased. Further studies are required to determine the cellular sources of MMP9 and how its activity is regulated.

Aβ monomer anti-inflammatory activity and Alzheimer's disease

Aβ is well known as a component of the amyloid plaques in the Alzheimer's disease brain. It is a unique amphipathic peptide that can, dependent on concentration and other conditions, remain as monomers or form oligomers. Studies on Aβ have historically focused on the neurotoxic effects of Aβ oligomers and their proinflammatory effects on glia (Gulisano et al., 2018; Halle et al., 2008; He et al., 2019; Huang, 2023; Kim et al., 2013; Lauren et al., 2009; Lazarevic et al., 2017; Lorton et al., 1996; Muehlhauser et al., 2001; Parodi et al., 2010; Puzzo et al., 2008; Shankar et al., 2008; Tan et al., 1999; Walsh et al., 2002; Yang et al., 2015; Zott et al., 2019). In this study, we have found that, in contrary to Aβ oligomers, Aβ monomers instead possess a previously unknown anti-inflammatory activity that acts through a unique microglial pathway. We have further found that genetic disruption of this pathway in corticogenesis results microglial hyperactivity, leading to neuronal ectopia and large disruption of cortical structural organization. To our knowledge, ours is the first study to uncover this overlooked anti-inflammatory activity of Aβ monomers. It is in alignment with recent studies showing that Aβ monomers are also directly protective to neurons and positively regulate synapse development and function (Galanis et al., 2021; Giuffrida et al., 2009; Plant et al., 2003; Ramsden et al., 2002; Zhou et al., 2022). Assuming a set amount of Aβ peptides, the formation of Aβ oligomers and aggregates in the brain would, by chemical law, be predicted to result in the depletion of Aβ monomers (Dear et al., 2020; Michaels et al., 2020). Thus, in the Alzheimer's disease brain, besides the obvious formation of Aβ aggregates, there may also be a less visible depletion of Aβ monomers taking place at the same time, which may, like Aβ oligomers, also contribute to the development of neuroinflammation and neuronal damage (Huang, 2023). In support of this interpretation, high soluble brain Aβ42, which likely also means high levels of Aβ monomers in the brain, have been found in clinical studies to preserve cognition in patients of both familial and sporadic Alzheimer's disease, in spite of increasing amyloidosis detected in their brains (Espay et al., 2021; Sturchio et al., 2022; Sturchio et al., 2021). In our study, we have also found that the effects of microglial disinhibition are mediated by MMP9. Importantly, in neurodegenerative diseases, MMP9 has been similarly found to be a key determinant regulatings the selective degeneration of neuronal cell types (Kaplan et al., 2014; Tran et al., 2019). MMP9 levels are also upregulated in the plasma in both mild cognitive impairment and Alzheimer's disease patients (Bruno et al., 2009; Lorenzl et al., 2008; Tsiknia et al., 2022). In addition, in several motor neuron disease models, reducing MMP9 has been found to protect neurons and delay the loss of motor function (Kaplan et al., 2014; Spiller et al., 2019). Thus, our study has not only uncovered a potentially overlooked role of Aβ monomer depletion in the development of Alzheimer's disease but also identified downstream effectors. Elucidating the roles these factors play may reveal new insight into the pathogenesis of Alzheimer's disease.

Methods

Generation of *ric8a* conditional allele

Standard molecular biology techniques were employed for generating the conditional *ric-8a* allele. Briefly, genomic fragments, of 4.5 and 2.5 kilobases and flanking exons 2-4 of the *ric-8a* locus at the 5' and 3' side, respectively, were isolated by PCR using high fidelity polymerases. Targeting plasmid was constructed by flanking the genomic fragment containing exons 2-4 with two loxP sites together with a *neomycin* positive selection cassette, followed by 5' and 3' genomic fragments as homologous recombination arms and a *pgk-DTA* gene as a negative selection cassette. ES cell clones were screened by Southern blot analysis using external probes at 5' and 3' sides. For derivation of conditional allele, the *neomycin* cassette was removed by crossing to an *actin-flpe*

transgenic line after blastocyst injection and germ line transmission. The primer set for genotyping *ric-8a* conditional allele, which produces a wildtype band of ~110bp and a mutant band of ~200bp, is: 5'-cctagttgtgaatcagaagcacttg-3' and 5'-gccatacctgagttacctaggc-3'. Animals homozygous for the conditional *ric-8a* allele are viable and fertile, without obvious phenotypes.

Mouse breeding and pharmacology

emx1-cre, nestin-cre, foxg1-cre, cx3cr1-cre, floxed app as well as the *BAT-lacZ* reporter mouse lines were purchased from the Jackson Lab. *nex-cre* and *wnt3a-cre* were as published (Goebbels et al., 2006; Yoshida et al., 2006). *cre* transgenes were introduced individually into the *ric8a or app* conditional mutant background for phenotypic analyses and *ric8a or app* homozygotes without *cre* as well as heterozygotes with *cre* (littermates) were both analyzed as controls. For BB94 and MMP9/13 inhibitor injection, pregnant females were treated daily from E12.5 to E14.5 at 30 μg (BB94) or 37.5 μg (MMP9/13 inhibitor) per g of body weight. For dorsomorphin and S3I-201 injection, pregnant females were treated on E12.5 at 7.5 and 25 μg per g of body weight, respectively. For sham treatment, pregnant females were treated on E12.5 with 100 μls of DMSO. BrdU was injected at 100 μg per g of body weight, and embryos were collected 4 hours later for cell proliferation analysis, or alternatively, pups were sacrificed at P5 for neuronal migration analysis and at P17 for other analysis. For LPS treatment, pregnant females were injected intraperitoneally with 400ng (*ric8a* genetic background) or 150ng (*app* genetic background) LPS per g of body weight on both E11.5 and E12.5. Animal use was in accordance with institutional guidelines.

Immunohistochemistry

Vibratome sections from brains fixed in 4% paraformaldehyde were used. The following primary antibodies were used at respective dilutions/concentrations: mouse anti-BrdU supernatant (clone G3G4, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA; 1:40), mouse anti-RC2 supernatant (DSHB; 1:10), mouse anti-Nestin supernatant (DSHB; 1:20), mouse anti-Vimentin supernatant (DSHB; 1:10), mouse anti-Pax6 supernatant (DSHB; 1:20), moue anti-Reelin (Millipore, 1:500), mouse anti-chondroitin sulfate (CS-56, Sigma, 1:100), rat anti-Ctip2 (Abcam, 1:500), rabbit anti-phospho Histone H3 (Ser10) (Millipore; 1:400), rabbit anti-Cux1 (CDP) (Santa Cruz; 1:100), rabbit anti-laminin (Sigma; 1:2000), rabbit anti-GFAP (Dako;1:1000), rabbit anti-ALDH1L1 (Abcam, 1:500), rabbit anti-MMP9 (Abcam, 1:1000), goat anti-MMP2 (R&D Systems; 5 μg/ml), rabbit anti-Calretinin (Chemicon, 1:2000), mouse anti-S100β (Thermo Scientific; 1:100), rabbit anti-S100β (Thermo Scientific; 1:200), and rabbit anti-phospho-Smad1/5 (Ser463/465) (41D10; Cell Signaling, 1:200). FITC and Cy3 conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Peroxidase conjugated secondary antibodies were purchased from Santa Cruz Biotech. Staining procedures were performed as described previously (Huang et al., 2006), except for anti-Ric-8a, MMP9, and phospho-Smad1/5 staining, in which a tyramide signal amplification (TSA) plus Cy3 kit (PerkinElmer, Waltham, MA) was used per manufacturer's instruction. Sections were mounted with Fluoromount G medium (Southern Biotech, Birmingham, AB) and analyzed under a Nikon *eclipse* Ti microscope or an Olympus confocal microscope.

Microglia culture and assay

Cerebral hemispheres were dissected from individual neonates, mechanically dissociated, split into 3-4 wells each and cultured in DMEM-F12 (Lonza) containing 10% fetal bovine serum (FBS) (Invitrogen). Microglial cells were harvested by light trypsinization that removes astroglial sheet on day 13-15. For experiments other than assaying IL-1β secretion, microglia were treated with LPS at 20ng/ml for 3 hours or at 5ng/ml overnight and, if applicable, DMSO or Aβ40 (ApexBio and Genscript) was applied at the same time as LPS. For assaying IL-1β secretion, microglia were primed with LPS at 200ng/ml for 5-6 hours before treatment with 3mM ATP for 15 minutes. In these experiments, DMSO or Aβ40 was applied at the same time as ATP if applicable. Supernatants were collected and used for cytokine ELISA assays per manufacturer's instructions (Biolegend).

Total RNAs were prepared from collected cells using Trizol (Invitrogen) and cDNAs were synthesized using a High-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR was performed using a GoTaq qPCR master mix per manufacturer's instructions (Promega). All gene expression levels were normalized against that of GAPDH.

Quantitative and statistical analysis

The sample size was estimated to be 3-9 animals each genotype (every 4^{th} of 50 μm coronal sections, 7-10 sections each animal) for ectopia analysis, 3-5 animals each genotype (3-4 sections each animal) for immunohistochemical analysis, and 4-6 animals each genotype for gel zymography and Western blot analysis, as has been demonstrated by previous publications to be adequate for similar animal studies. Matching sections were used between controls and mutants. NIS-Elements BR 3.0 software (Nikon) was used for quantifying the numbers and sizes of neuronal ectopia, the numbers of laminin positive debris, as well as the numbers of astrocytes. ImageJ software (NIH) was used for quantifying the intensity of immunostainings. Statistics was performed using Student's *t* test when comparing two conditions, or one-way ANOVA followed by Tukey's post hoc test when comparing three or more conditions. All data are represented as means ± s.e.m.

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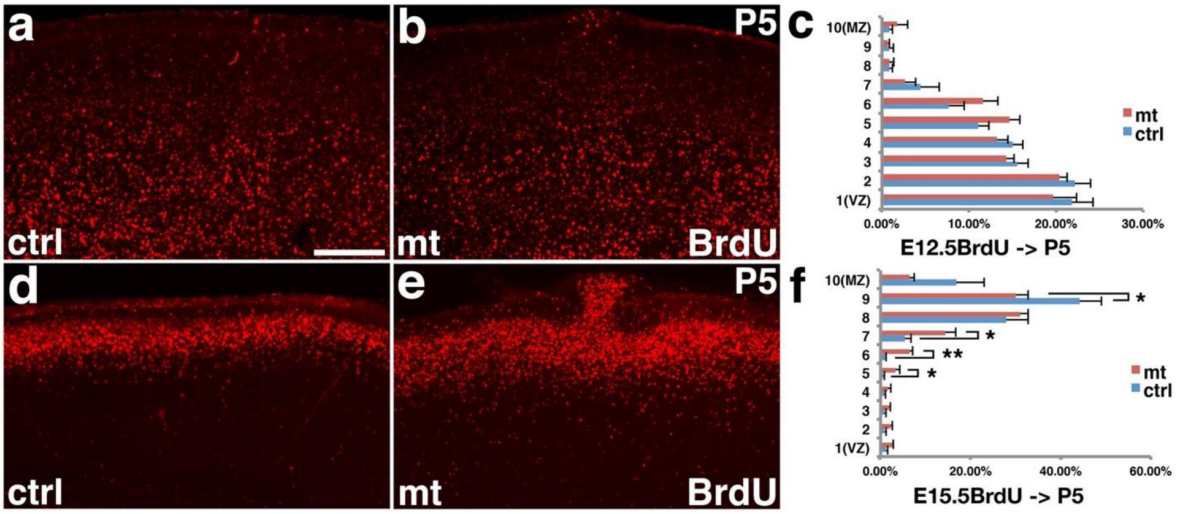
Author information

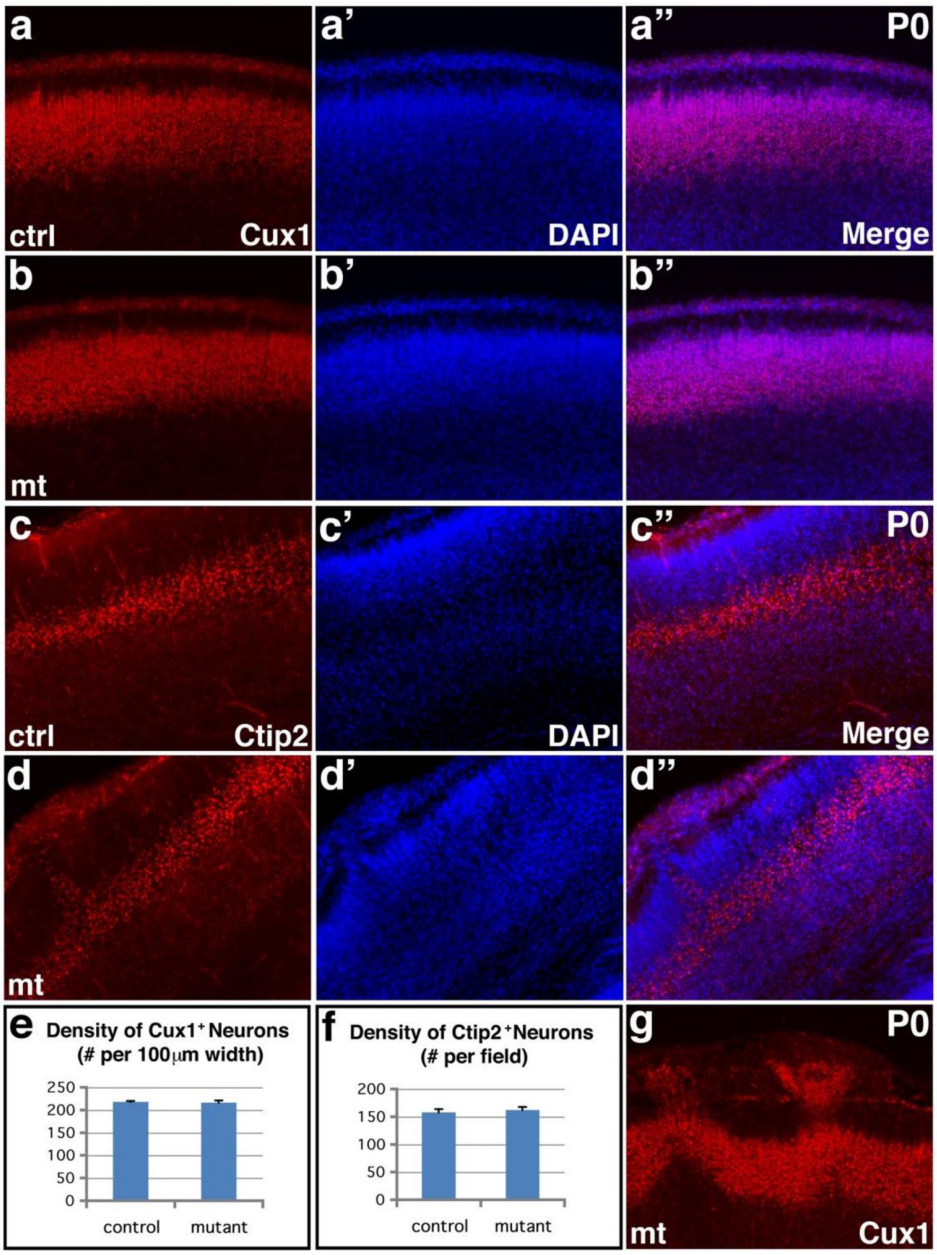
Z. H. designed experiments, generated *ric8a* conditional ES cells, performed microglial and related experiments, and wrote the manuscript. H. J. K., D. S., and Z. H. performed other experiments and analyzed data.



Supplemental Fig. 1.

Birth-dating of early and late-born neurons in *ric8a-emx1-cre* mutant cortices.

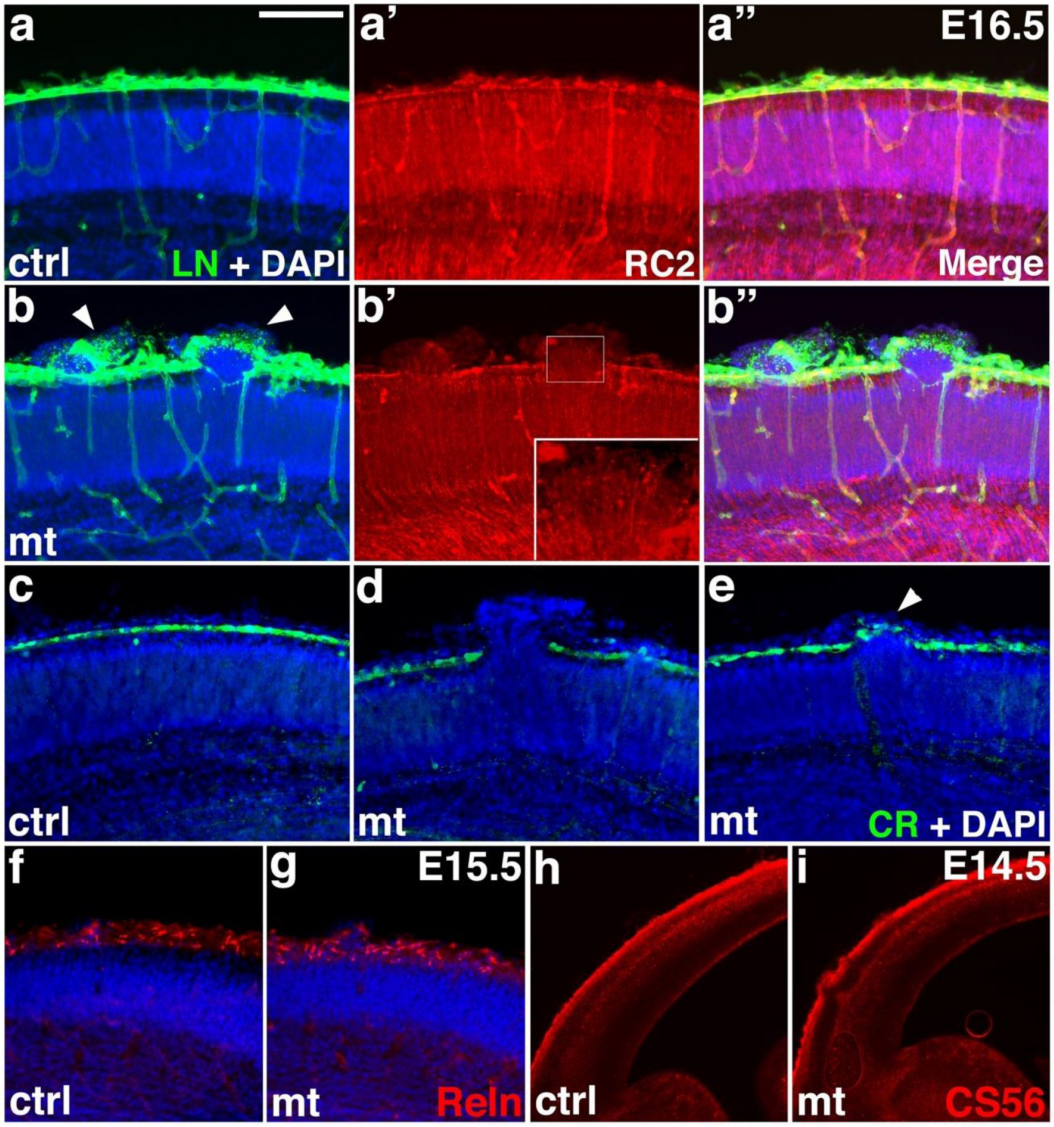
(**a-c**) BrdU (in red) staining in control (**a**) and mutant (**b**) cortices at P5 after administration at E12.5. Quantification is shown in (**c**). No statistically significant differences were observed between control and mutant neurons in regions without ectopia. (**d-f**) BrdU staining in control (**d**) and mutant (**e**) cortices at P5 after administration at E15.5. Quantification is shown in (**f**). Neuronal migration appears slightly delayed in mutants as compared to controls. *, $P < 0.05$; **, $P < 0.01$; n = 5.



Supplemental Fig. 2.

Lamina-specific neuronal markers are normal outside ectopia in *ric8a-emx1-cre* mutant cortices.

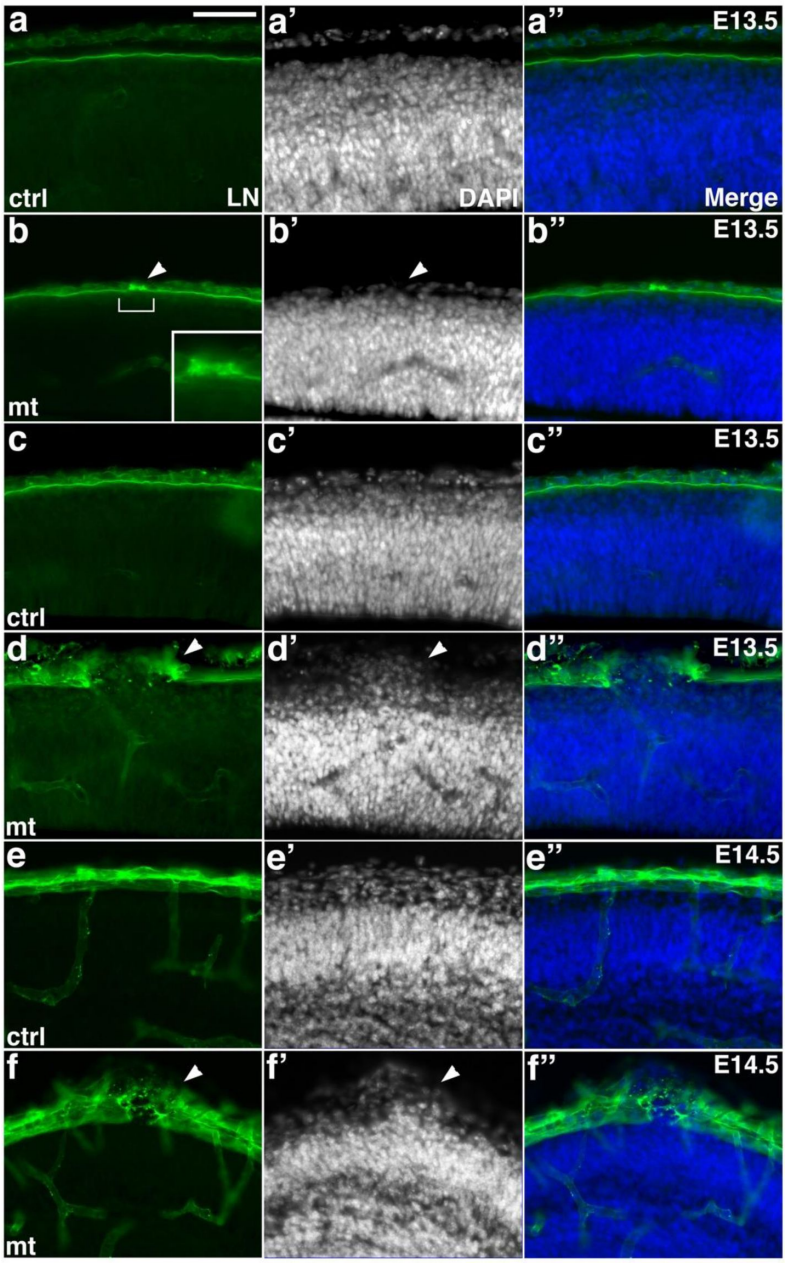
(**a**-**b**") Cux1 (in red) and nuclear (DAPI, in blue) staining of control (**a-a"**) and mutant (**b-b"**) cortices at P0 in a region without ectopia. No obvious changes in the expression pattern of Cux1, an upper layer neuronal marker, were observed in the mutant cortex, except in areas with ectopia (see panel (**g**)).
(**c**-**d**") Ctip2 (in red) and nuclear (DAPI, in blue) staining of control (**c**-**c**") and mutant (**d**-**d**") cortices at P0. No obvious changes in the expression pattern of Ctip2, a deep layer neuronal marker, were observed in the mutant cortex, except in areas with ectopia.
(**e-f**) Quantification of cortical neurons positive for Cux1 (**e**) and Ctip2 (**f**) in matching cortical regions at P0. No significant differences were observed in the density of Cux1 (control, 218.1 ± 1.7 per 100 μm cortical width; mutant, 216.4 ± 4.3 per 100 μm cortical width; $P = 0.36$, n = 12) or Ctip2 (control, 157.8 ± 5.0 per field; mutant, 161.9 ± 5.9 per field; $P = 0.31$, n = 12) positive neurons between controls and mutants.
(**g**) Cux1 (in red) staining of mutant cortices at P0 in a region with ectopia.
Scale bar in (**a**), 200 μm for all panels.



Supplemental Fig. 3.

Neuronal ectopia in *ric8a-emx1-cre* mutants result from pial basement membrane breach during embryogenesis.

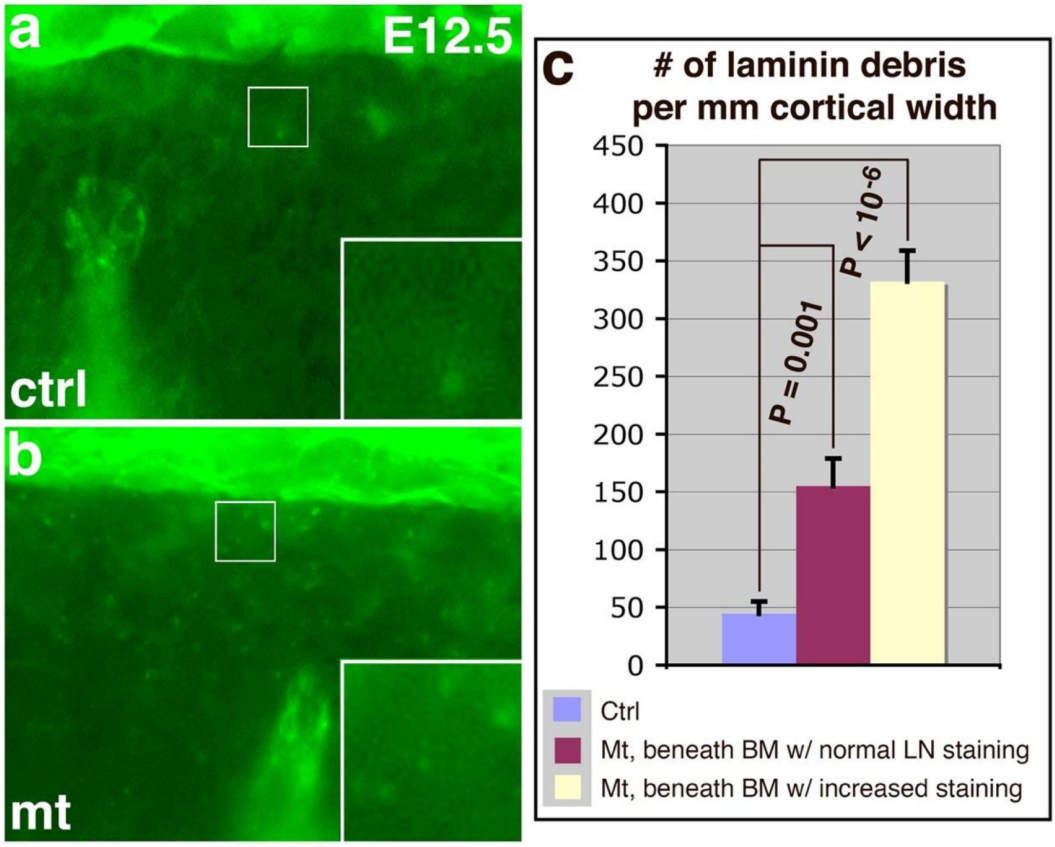
(a-a") Laminin (LN, in green), radial glial marker RC2 (in red), and nuclear (DAPI, in blue) staining of control cortices at E16.5. A continuous basement membrane is observed at the pia, where radial glial endfeet are anchored.
(**b-b"**) Laminin, RC2, and nuclear staining of *ric8a/emx1-cre* mutant cortices at E16.5. Neuronal ectopias are consistently observed at sites of basement membrane breakage (arrowheads in **b**). Radial glial fibers at these sites extend beyond the pia (inset in **b'**).
(**c-e**) Calretinin (CR, in green) and nuclear (DAPI, in blue) staining of control (**c**) and mutant (**d-e**) cortices at E16.5. A continuous row of Calretinin positive Cajal-Retzius cells is observed in the marginal zone of control cortices (**c**). By contrast, in mutants, Cajal-Retzius cells are absent at large ectopias (**d**). However, they appear passively displaced by over-migrating neurons at small ectopias (arrowhead in **e**).
(f-g) Reelin (Reln, in red) and nuclear (DAPI, in blue) staining of control (**f**) and *ric8a/emx1-cre* mutant (**g**) cortices at E15.5. Strong Reelin expression is observed in Cajal-Retzius cells in the marginal zone of both control and mutant cortices.
(h-i) Chondroitin sulfate proteoglycan (CS56, in red) staining of control (**h**) and mutant (**i**) cortices at E14.5. Normal preplate splitting is observed in mutants.
Scale bar in (**a**), 200μm for (**a-g)** and 500μm for (**h-i**).



Supplemental Fig. 4.

Basement membrane breaches precede neuronal ectopia in *ric8a-emx1-cre* mutant cortices.

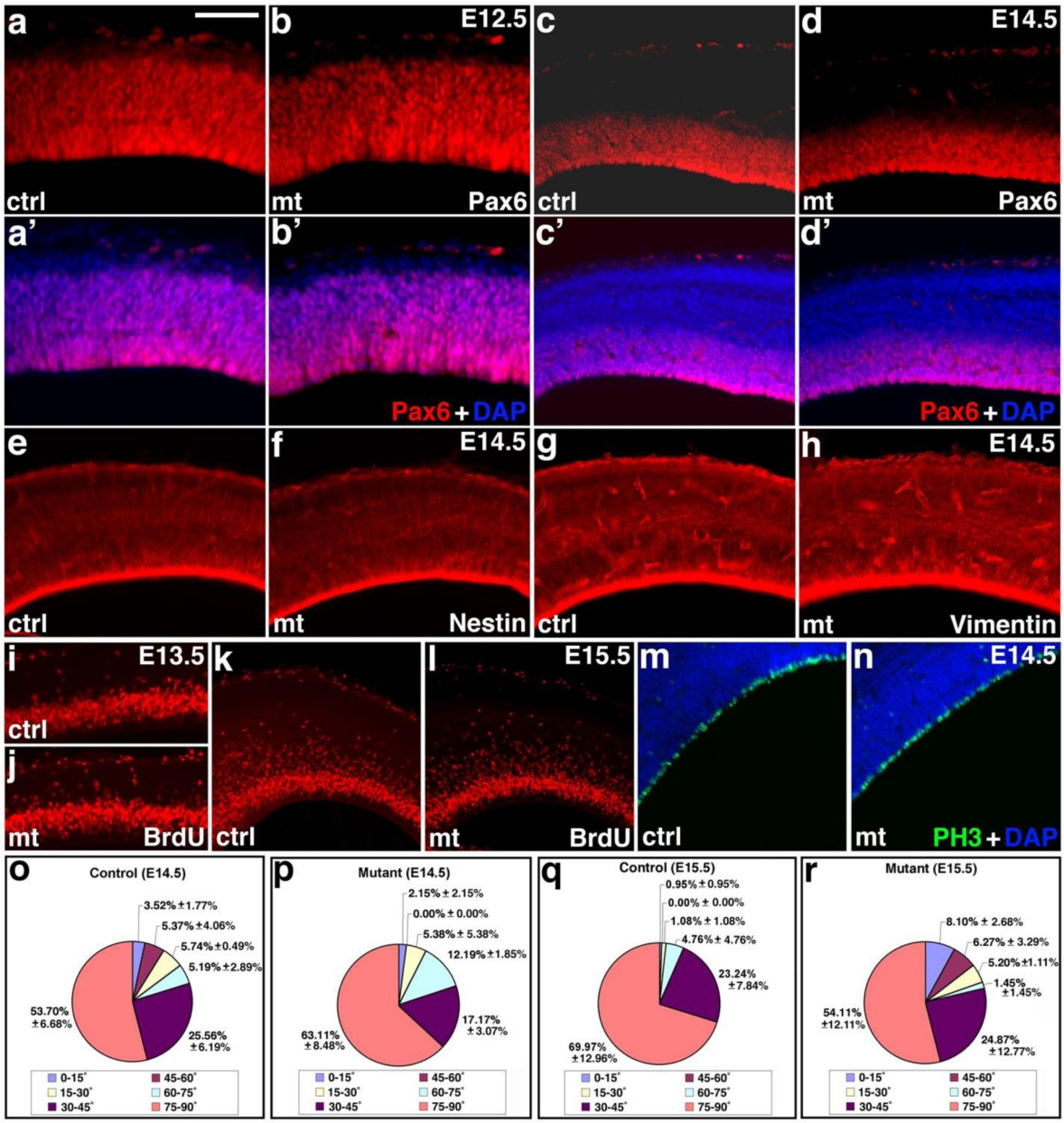
(**a**-**a”**) Laminin (LN, in green) and nuclear (DAPI, in blue) staining of control cortices at E13.5. A continuous basement membrane is observed at the pia, beneath which cells are well organized in the cortical wall.
(**b**-**b”**) Laminin and nuclear staining of *ric8a-emx1-cre* mutant cortices at E13.5. In a subset of mutants, a small disruption of basement membrane is observed (bracket and inset in **b**), but not yet associated with ectopia (arrowhead in **b’**).
(c-**c”**) Laminin (LN, in green) and nuclear (DAPI, in blue) staining of control cortices at E13.5.
(**d**-**d”**) Laminin and nuclear staining of *ric8a-emx1-cre* mutant cortices at E13.5. Although at E13.5 we observe basement membrane defects in the absence of neuronal ectopia (see **b**-**b”**), when there are neuronal ectopia, they are always associated with basement membrane breakage.
(**e**-**e”**) Laminin and nuclear staining of control cortices at E14.5.
(**f**-**f”**) Laminin and nuclear staining of *ric-8a-emx1-cre* mutant cortices at E14.5. Neuronal ectopia at E14.5 are also always associated with basement membrane breakage.
Scale bar in (**a**), 100 μm for all panels.



Supplemental Fig. 5.

Signs of basement membrane degradation before breach formation at E12.5.

(**a-b**) Laminin (in green) staining of control (**a**) and *ric8a-emx1-cre* mutant (**b**) cortices at E12.5. Increased numbers of laminin positive debris were observed in mutants (compare insets), even though breaches had yet to form.
(**c**) Quantitative analysis shows significant increases.



Supplemental Fig. 6.

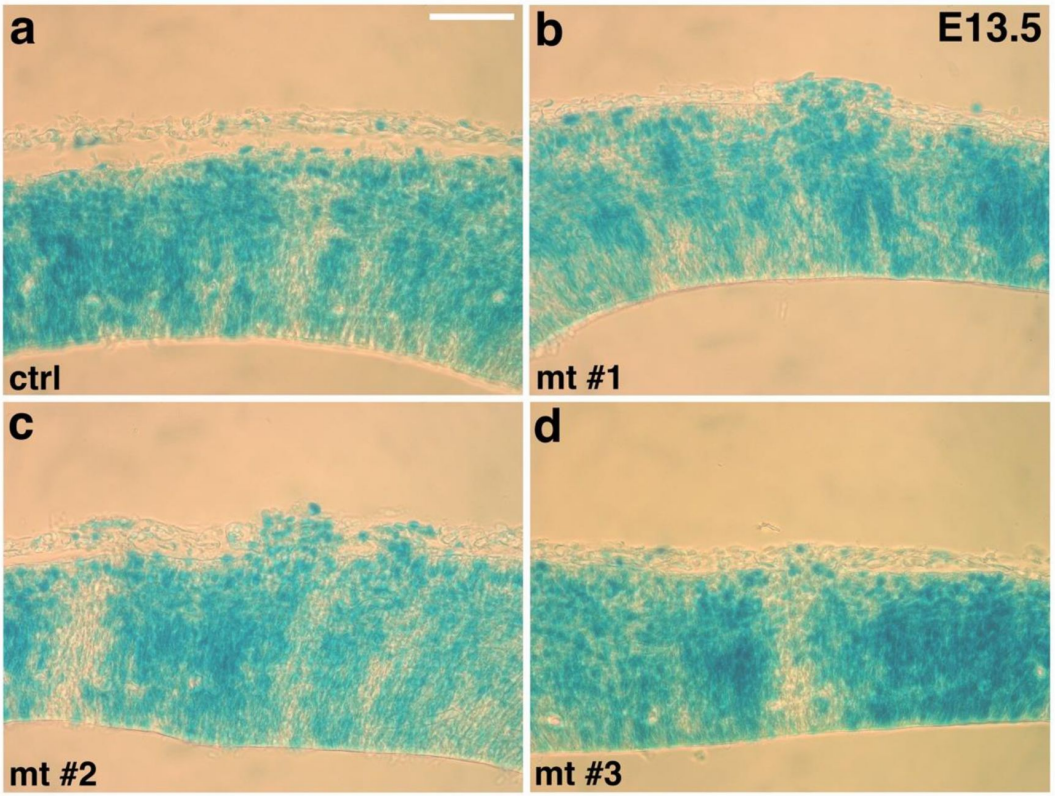
Cortical radial glial identity and proliferation are unaffected in *ric8a-emx1-cre* mutants.

(a-b') Pax6 (in red) and nuclear (DAPI, in blue) staining of control (**a**&**a'**) and mutant (**b**&**b'**) cortices at E12.5.
(**c-d'**) Pax6 (in red) and nuclear (DAPI, in blue) staining of control (**c**&**c'**) and mutant (**d**&**d'**) cortices at E14.5. No ectopic Pax6 positive cells were observed at either E12.5 or E14.5.
(e-f) Nestin (in red) and nuclear (DAPI, in blue) staining of control (**e**) and mutant (**f**) cortices at E14.5.
(**g-h**) Vimentin (in red) and nuclear (DAPI, in blue) staining of control (**g**) and mutant (**h**) cortices at E14.5.
(i-j) BrdU staining (in red) in control (**i**) and mutant (**j**) cortices at E13.5. (**k-l**) BrdU staining (in red) in control (**k**) and mutant (**l**) cortices at E15.5.
(**m-n**) Phospho-histone 3 (PH3, in green) and nuclear (DAPI, in blue) staining of control (**m**) and mutant (**n**) cortices at E14.5.
(o-p) Cleavage plane distribution of radial glial mitosis in control (**o**) and mutant (**p**) cortices at E14.5. No significant differences were observed ($P > 0.4$, n = 3 animals each genotype; 73 cells for controls and 76 cells for mutants).
(**q-r**) Cleavage plane distribution of radial glial mitosis in control (**q**) and mutant (**r**) cortices at E15.5. No significant differences were observed ($P > 0.1$, n = 3 animals each genotype; 70 cells for controls and 59 cells for mutants).
Scale bar in (**a**), 100 μm for (**a-b'**) and 200 μm for (**c-n**).

Supplemental Fig. 7.

Wnt pathway activity is normal in *ric8a-emx1-cre* mutant cortices.

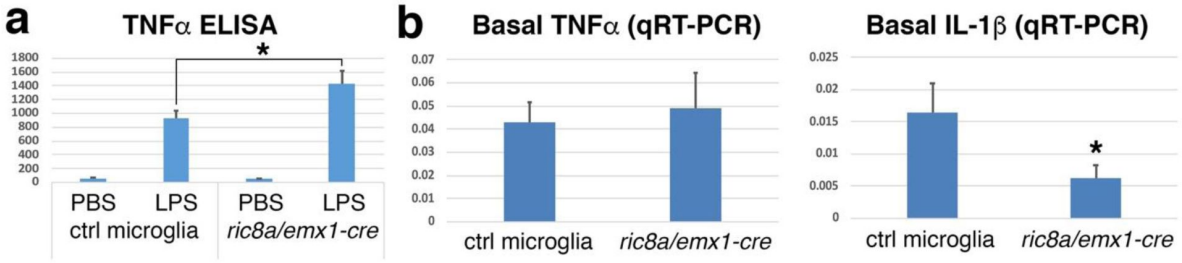
X-gal staining of BAT-lacZ expression in *ric8a-emx1-cre* control (**a**) and mutant (**b**-**d**) cortices at E13.5. No obvious differences are observed between controls and three different mutants at this stage.

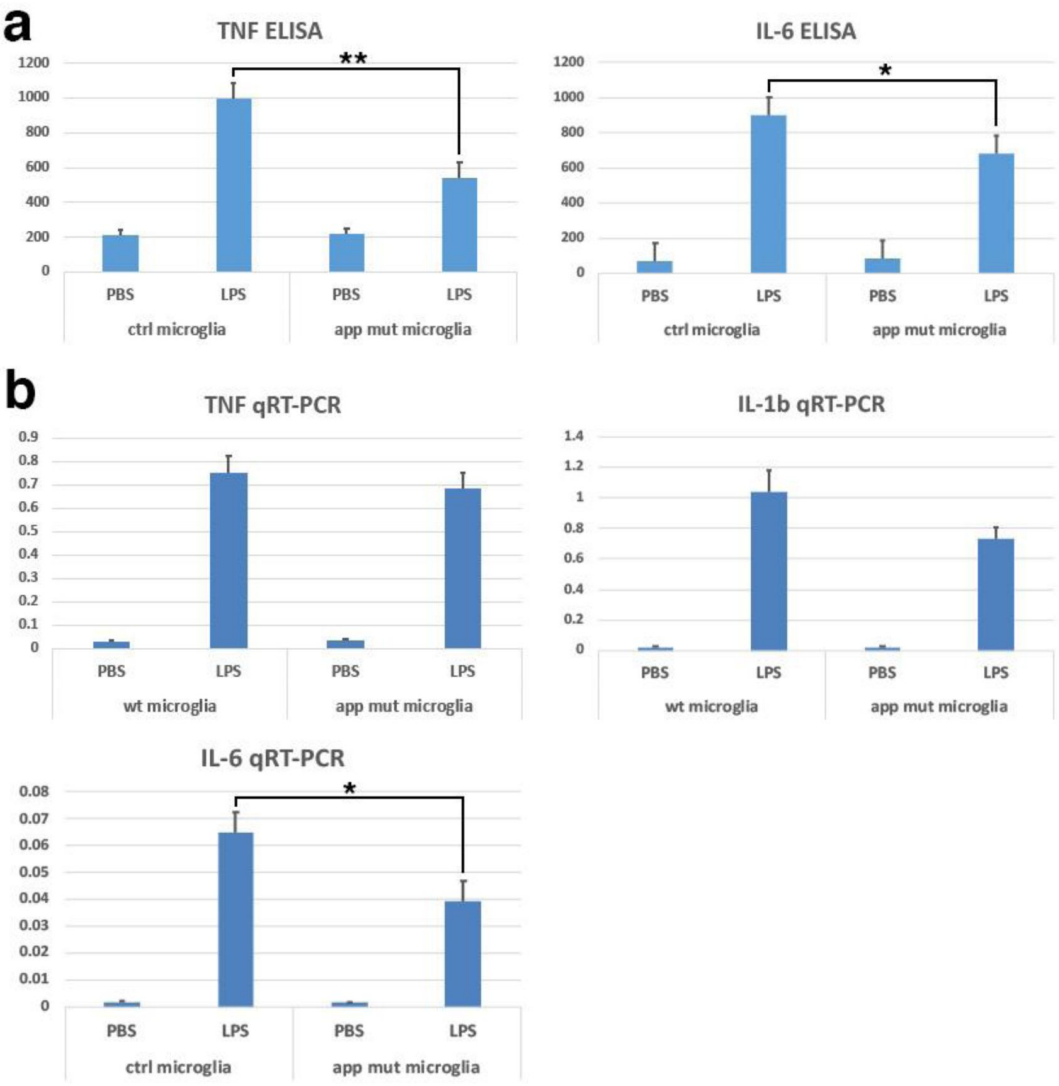


Supplemental Fig. 8.

emx1-cre is active in microglia.

TNFα secretion (pg/ml) (**a**) and basal TNFα and IL-1β mRNA expression (**b**) in control and *ric8a-emx1-cre* mutant microglia. *, $P < 0.05$; n = 5-8 each group.

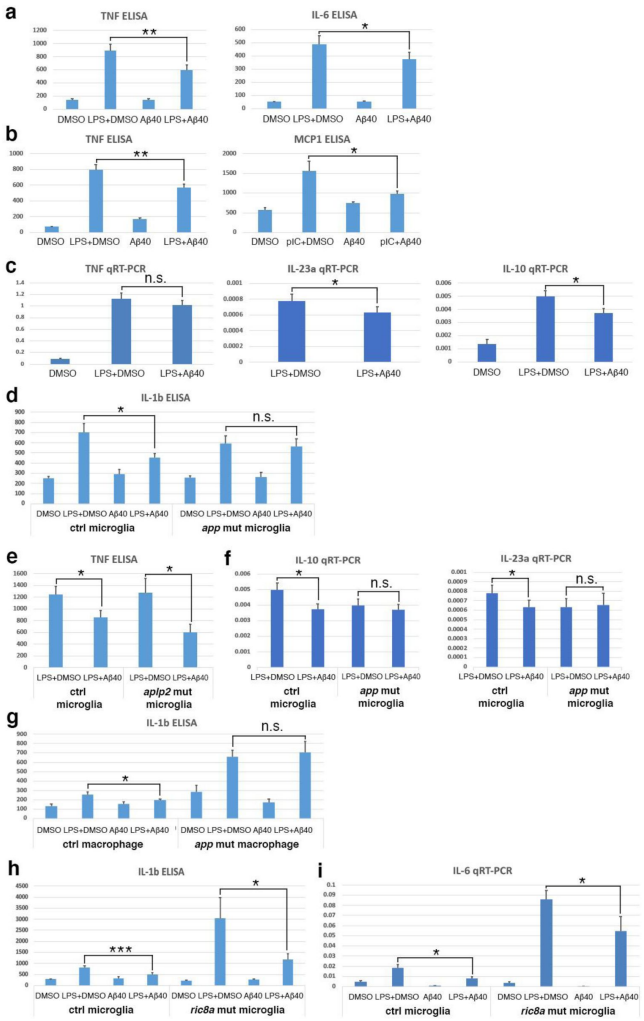




Supplemental Fig. 9.

Cytokine secretion and transcriptional induction in *app-cx3cr1-cre* mutant microglia.

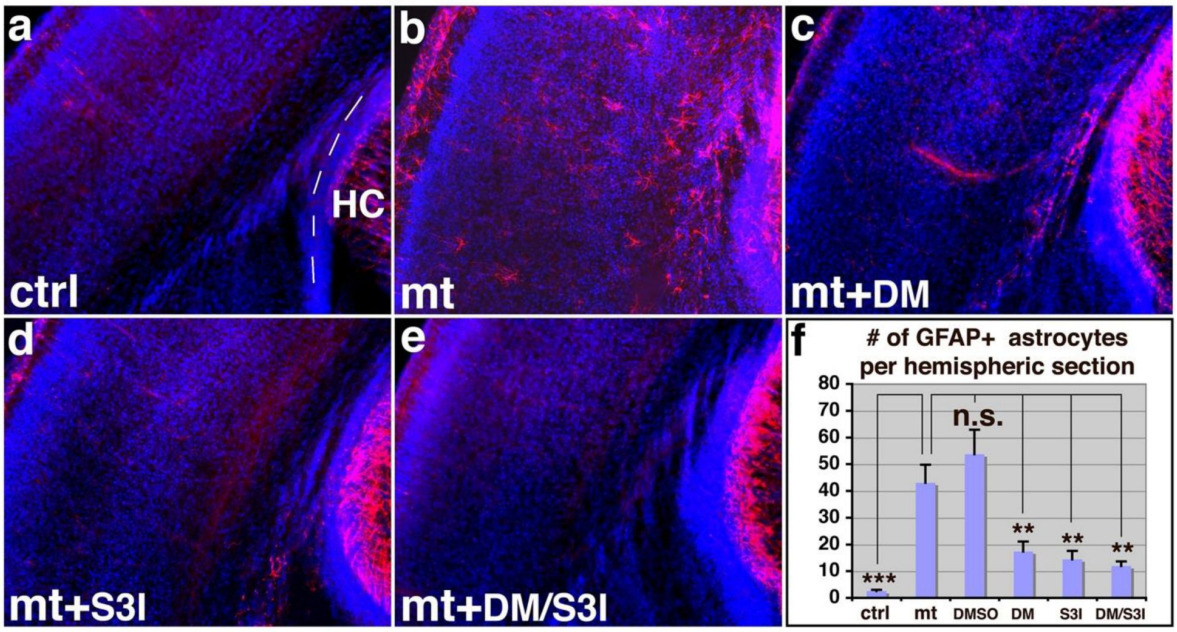
(a) TNFα and IL-6 secretion (pg/ml) in control and *app-cx3cr1-cre* mutant microglia following overnight LPS stimulation. *, $P < 0.05$; **, $P < 0.01$; n = 9-13 each group.
(b) TNFα, IL-1β and IL-6 mRNA expression in control and *app-cx3cr1-cre* mutant microglia following overnight 3-hr LPS stimulation. *, $P < 0.05$; n = 6-7 each group.



Supplemental Fig. 10.

Effects of monomeric Aβ on cytokine secretion and transcription in control and mutant microglial lineage cells.

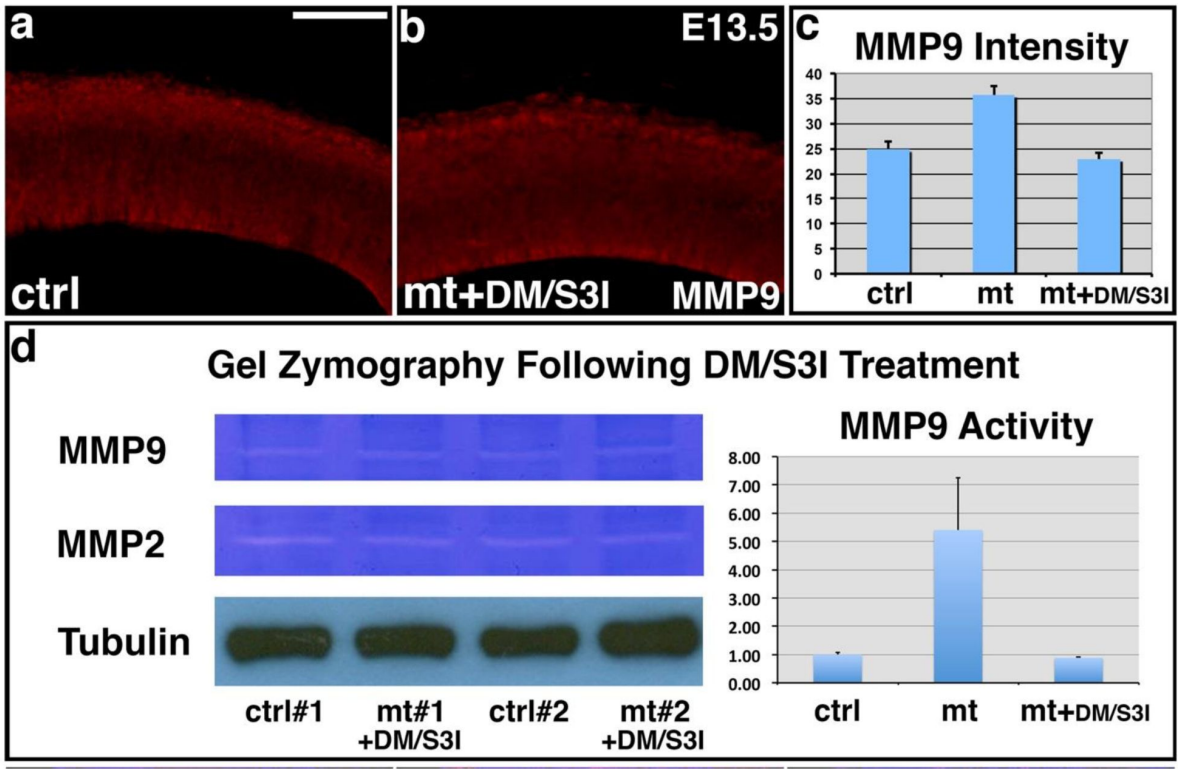
(a) TNFα and IL-6 secretion (pg/ml) in wildtype microglia following LPS stimulation in the absence or presence of Aβ40 (50nM). *, $P < 0.05$; **, $P < 0.01$; n = 25 each group for TNFα and 11 each group for IL-6.
(b) TNFα and MCP1 secretion (pg/ml) in wildtype microglia following LPS stimulation in the absence or presence of Aβ40 (500nM) from Genscript. Effects on IL-1β secretion in **Fig. 4b** was also performed with Genscript Aβ40. All other experiments in Fig. 7 were performed with ApexBio Aβ40. *, $P < 0.05$; **, $P < 0.01$; n = 5-7 each group.
(c) TNFα, IL-23, and IL-10 mRNA expression in wildtype microglia following LPS stimulation in the absence or presence of Aβ40 (400nM). *, $P < 0.05$; n = 6 each group
(d) IL-1β secretion (pg/ml) in control and *app/cx3cr1-cre* mutant microglia following LPS stimulation in the absence or presence of Aβ40. *, $P < 0.05$; n = 8-12 each group.
(e) TNFα (pg/ml) in control or *aplp2/cx3cr1-cre* mutant microglia following LPS stimulation in the absence or presence of Aβ40 (400nM). *, $P < 0.05$; n = 9-13 each group.
(f) IL-10 and IL-23 mRNA expression in control and *app/cx3cr1-cre* mutant microglia following LPS stimulation in the absence or presence of Aβ40 (400nM). *, $P < 0.05$; n = 6 each group.
(g) IL-1β secretion (pg/ml) in fresh unelicited control and *app/cx3cr1-cre* mutant peritoneal macrophages following LPS stimulation in the absence or presence of Aβ40 (400nM). *, $P < 0.05$; n = 12 each group.
(h) IL-1β secretion (pg/ml) in f control and *ric8a/cx3cr1-cre* mutant microglia following LPS stimulation in the absence or presence of Aβ40 (500nM). *, $P < 0.05$; ***, $P < 0.001$; n = 7-8 each group.
(i) IL-6 mRNA expression in control and *ric8a/cx3cr1-cre* mutant microglia following LPS stimulation in the absence or presence of Aβ40 (200nM). *, $P < 0.05$; n = 6 each group.



Supplemental Fig. 11.

Suppression of astrogliosis in *ric8a-emx1-cre* mutant cortices by anti-inflammatory drugs, dorsomorphin (DM) and S3I-201 (S3I).

(a-e) GFAP (in red) and nuclear (DAPI, in blue) staining of neonatal control (**E**) and mutant cortices without treatment (**F**) or mutant cortices after dorsomorphin (DM, **G**), S3I-201 (S3I, **H**), or dual (DM+S3I, **I**) treatment at E12.5. Note, GFAP is normally expressed in the neonatal hippocampus (HC) (dashed line in (a)).
(f) Quantitative analysis of GFAP-positive astrocyte numbers in the neonatal mutant cortex after treatment at E12.5.



Supplemental Fig. 12.

Suppression of MMP9 expression in *ric8a-emx1-cre* mutant cortices by anti-inflammatory drugs, dorsomorphin (DM) and S3I-201 (S3I).

(a) MMP9 (in red) staining in control cortices at E13.5.
(b) MMP9 (in red) staining in mutant cortices at E13.5 after DM and S3I dual treatment at E12.5.
(c) Quantitative analysis of MMP9 expression. No significant differences are observed in mutants after inhibitor treatment in comparison to controls (P = 0.44, n = 6).
(d) Gel zymography of E13.5 control and mutant cortical lysates following DM/S3I treatment at E12.5.
Similar levels of MMP9 are observed between controls and mutants. Quantification also showed no significant differences in normalized MMP9 levels (P = 0.46, n = 4).

References

Afshar K., Willard F.S., Colombo K., Johnston C.A., McCudden C.R., Siderovski D.P., Gonczy P. (2004) **RIC-8 is required for GPR-1/2-dependent Galpha function during asymmetric division of C. elegans embryos** *Cell* **119**:219–230

Akol I., Kalogeraki E., Pielecka-Fortuna J., Fricke M., Lowel S. (2022) **MMP2 and MMP9 Activity Is Crucial for Adult Visual Cortex Plasticity in Healthy and Stroke-Affected Mice** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **42**:16–32

Barnes S.J., Franzoni E., Jacobsen R.I., Erdelyi F., Szabo G., Clopath C., Keller G.B., Keck T. (2017) **Deprivation-Induced Homeostatic Spine Scaling In Vivo Is Localized to Dendritic Branches that Have Undergone Recent Spine Loss** *Neuron* **96**:871–882

Barres B.A. (2008) **The mystery and magic of glia: a perspective on their roles in health and disease** *Neuron* **60**:430–440

Beggs H.E., Schahin-Reed D., Zang K., Goebbels S., Nave K.A., Gorski J., Jones K.R., Sretavan D., Reichardt L.F. (2003) **FAK deficiency in cells contributing to the basal lamina results in cortical abnormalities resembling congenital muscular dystrophies** *Neuron* **40**:501–514

Belvindrah R., Nalbant P., Ding S., Wu C., Bokoch G.M., Muller U. (2006) **Integrin-linked kinase regulates Bergmann glial differentiation during cerebellar development** *Molecular and cellular neurosciences* **33**:109–125

Bruno M.A., Mufson E.J., Wuu J., Cuello A.C. (2009) **Increased matrix metalloproteinase 9 activity in mild cognitive impairment** *J Neuropathol Exp Neurol* **68**:1309–1318

Chamberlain L.M., Holt-Casper D., Gonzalez-Juarrero M., Grainger D.W. (2015) **Extended culture of macrophages from different sources and maturation results in a common M2 phenotype** *J Biomed Mater Res A* **103**:2864–2874

Cirrito J.R., Yamada K.A., Finn M.B., Sloviter R.S., Bales K.R., May P.C., Schoepp D.D., Paul S.M., Mennerick S., Holtzman D.M. (2005) **Synaptic activity regulates interstitial fluid amyloid-beta levels in vivo** *Neuron* **48**:913–922

Colonna M., Butovsky O. (2017) **Microglia Function in the Central Nervous System During Health and Neurodegeneration** *Annu Rev Immunol* **35**:441–468

Couwenbergs C., Spilker A.C., Gotta M. (2004) **Control of embryonic spindle positioning and Galpha activity by C. elegans RIC-8** *Current biology : CB* **14**:1871–1876

Cunningham C.L., Martinez-Cerdeno V., Noctor S.C. (2013) **Microglia regulate the number of neural precursor cells in the developing cerebral cortex** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **33**:4216–4233

David N.B., Martin C.A., Segalen M., Rosenfeld F., Schweisguth F., Bellaiche Y. (2005) **Drosophila Ric-8 regulates Galphai cortical localization to promote Galphai-dependent planar orientation of the mitotic spindle during asymmetric cell division** *Nature cell biology* **7**:1083–1090

Dear A.J., Michaels T.C.T., Meisl G., Klenerman D., Wu S., Perrett S., Linse S., Dobson C.M., Knowles T.P.J. (2020) **Kinetic diversity of amyloid oligomers** *Proceedings of the National Academy of Sciences of the United States of America* **117**:12087–12094

Dziembowska M., Milek J., Janusz A., Rejmak E., Romanowska E., Gorkiewicz T., Tiron A., Bramham C.R., Kaczmarek L. (2012) **Activity-dependent local translation of matrix metalloproteinase-9** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **32**:14538–14547

Eichler A., Kleidonas D., Turi Z., Fliegauf M., Kirsch M., Pfeifer D., Masuda T., Prinz M., Lenz M., Vlachos A. (2023) **Microglial Cytokines Mediate Plasticity Induced by 10 Hz Repetitive Magnetic Stimulation** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **43**:3042–3060

Espay A.J., Sturchio A., Schneider L.S., Ezzat K. (2021) **Soluble Amyloid-beta Consumption in Alzheimer's Disease** *J Alzheimers Dis* **82**:1403–1415

Fogel H. *et al.* (2014) **APP homodimers transduce an amyloid-beta-mediated increase in release probability at excitatory synapses** *Cell reports* **7**:1560–1576

Gabay M., Pinter M.E., Wright F.A., Chan P., Murphy A.J., Valenzuela D.M., Yancopoulos G.D., Tall G.G. (2011) **Ric-8 proteins are molecular chaperones that direct nascent G protein alpha subunit membrane association** *Science signaling* **4**

Galanis C., Fellenz M., Becker D., Bold C., Lichtenthaler S.F., Muller U.C., Deller T., Vlachos A. (2021) **Amyloid-Beta Mediates Homeostatic Synaptic Plasticity** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **41**:5157–5172

Garcia-Osta A., Alberini C.M. (2009) **Amyloid beta mediates memory formation** *Learn Mem* **16**:267–272

Ginhoux F. *et al.* (2010) **Fate mapping analysis reveals that adult microglia derive from primitive macrophages** *Science* **330**:841–845

Giuffrida M.L. *et al.* (2009) **Beta-amyloid monomers are neuroprotective** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**:10582–10587

Goebbels S., Bormuth I., Bode U., Hermanson O., Schwab M.H., Nave K.A. (2006) **Genetic targeting of principal neurons in neocortex and hippocampus of NEX-Cre mice** *Genesis* **44**:611–621

Gore S.V., James E.J., Huang L.C., Park J.J., Berghella A., Thompson A.C., Cline H.T., Aizenman C.D. (2021) **Role of matrix metalloproteinase-9 in neurodevelopmental deficits and experience-dependent plasticity in Xenopus laevis** *eLife* **10**

Gorski J.A., Talley T., Qiu M., Puelles L., Rubenstein J.L., Jones K.R. (2002) **Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **22**:6309–6314

Grant J.L., Ghosn E.E., Axtell R.C., Herges K., Kuipers H.F., Woodling N.S., Andreasson K., Herzenberg L.A., Herzenberg L.A., Steinman L. (2012) **Reversal of paralysis and reduced inflammation from peripheral administration of beta-amyloid in TH1 and TH17 versions of experimental autoimmune encephalomyelitis** *Sci Transl Med* **4**

Graus-Porta D., Blaess S., Senften M., Littlewood-Evans A., Damsky C., Huang Z., Orban P., Klein R., Schittny J.C., Muller U. (2001) **Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex** *Neuron* **31**:367–379

Guenette S., Chang Y., Hiesberger T., Richardson J.A., Eckman C.B., Eckman E.A., Hammer R.E., Herz J. (2006) **Essential roles for the FE65 amyloid precursor protein-interacting proteins in brain development** *The EMBO journal* **25**:420–431

Gulisano W., Melone M., Li Puma D.D., Tropea M.R., Palmeri A., Arancio O., Grassi C., Conti F., Puzzo D. (2018) **The effect of amyloid-beta peptide on synaptic plasticity and memory is influenced by different isoforms, concentrations, and aggregation status** *Neurobiol Aging* **71**:51–60

Gulisano W. *et al.* (2019) **Neuromodulatory Action of Picomolar Extracellular Abeta42 Oligomers on Presynaptic and Postsynaptic Mechanisms Underlying Synaptic Function and Memory** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **39**:5986–6000

Halle A., Hornung V., Petzold G.C., Stewart C.R., Monks B.G., Reinheckel T., Fitzgerald K.A., Latz E., Moore K.J., Golenbock D.T. (2008) **The NALP3 inflammasome is involved in the innate immune response to amyloid-beta** *Nat Immunol* **9**:857–865

Hampoelz B., Hoeller O., Bowman S.K., Dunican D., Knoblich J.A. (2005) **Drosophila Ric-8 is essential for plasma-membrane localization of heterotrimeric G proteins** *Nat Cell Biol* **7**:1099–1105

Hattori Y. *et al.* (2023) **CD206(+) macrophages transventricularly infiltrate the early embryonic cerebral wall to differentiate into microglia** *Cell reports* **42**

He D. *et al.* (2022) **Disruption of the IL-33-ST2-AKT signaling axis impairs neurodevelopment by inhibiting microglial metabolic adaptation and phagocytic function** *Immunity* **55**:159–173

He Y. *et al.* (2019) **Amyloid beta oligomers suppress excitatory transmitter release via presynaptic depletion of phosphatidylinositol-4,5-bisphosphate** *Nat Commun* **10**

Hebert J.M., McConnell S.K. (2000) **Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures** *Developmental biology* **222**:296–306

Heir R., Abbasi Z., Komal P., Altimimi H.F., Franquin M., Moschou D., Chambon J., Stellwagen D. (2024) **Astrocytes Are the Source of TNF Mediating Homeostatic Synaptic Plasticity** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **44**

Herms J., Anliker B., Heber S., Ring S., Fuhrmann M., Kretzschmar H., Sisodia S., Muller U. (2004) **Cortical dysplasia resembling human type 2 lissencephaly in mice lacking all three APP family members** *The EMBO journal* **23**:4106–4115

Huang Z. (2023) **A Function of Amyloid-beta in Mediating Activity-Dependent Axon/Synapse Competition May Unify Its Roles in Brain Physiology and Pathology** *J Alzheimers Dis* **92**:29–57

Huang Z. (2024) **Evidence that Alzheimer's Disease Is a Disease of Competitive Synaptic Plasticity Gone Awry** *J Alzheimers Dis* **99**:447–470

Huang Z., Shimazu K., Woo N.H., Zang K., Muller U., Lu B., Reichardt L.F. (2006) **Distinct roles of the beta 1-class integrins at the developing and the mature hippocampal excitatory synapse** *J Neurosci* **26**:11208–11219

Iyer H., Shen K., Meireles A.M., Talbot W.S. (2022) **A lysosomal regulatory circuit essential for the development and function of microglia** *Sci Adv* **8**

Kamenetz F., Tomita T., Hsieh H., Seabrook G., Borchelt D., Iwatsubo T., Sisodia S., Malinow R. (2003) **APP processing and synaptic function** *Neuron* **37**:925–937

Kaneko M., Stellwagen D., Malenka R.C., Stryker M.P. (2008) **Tumor necrosis factor-alpha mediates one component of competitive, experience-dependent plasticity in developing visual cortex** *Neuron* **58**:673–680

Kaplan A., Spiller K.J., Towne C., Kanning K.C., Choe G.T., Geber A., Akay T., Aebischer P., Henderson C.E. (2014) **Neuronal matrix metalloproteinase-9 is a determinant of selective neurodegeneration** *Neuron* **81**:333–348

Kelly E.A., Russo A.S., Jackson C.D., Lamantia C.E., Majewska A.K. (2015) **Proteolytic regulation of synaptic plasticity in the mouse primary visual cortex: analysis of matrix metalloproteinase 9 deficient mice** *Front Cell Neurosci* **9**

Kim T., Vidal G.S., Djurisic M., William C.M., Birnbaum M.E., Garcia K.C., Hyman B.T., Shatz C.J. (2013) **Human LilrB2 is a beta-amyloid receptor and its murine homolog PirB regulates synaptic plasticity in an Alzheimer's model** *Science* **341**:1399–1404

Lauren J., Gimbel D.A., Nygaard H.B., Gilbert J.W., Strittmatter S.M. (2009) **Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers** *Nature* **457**:1128–1132

Lazarevic V., Fienko S., Andres-Alonso M., Anni D., Ivanova D., Montenegro-Venegas C., Gundelfinger E.D., Cousin M.A., Fejtova A. (2017) **Physiological Concentrations of Amyloid Beta Regulate Recycling of Synaptic Vesicles via Alpha7 Acetylcholine Receptor and CDK5/Calcineurin Signaling** *Front Mol Neurosci* **10**

Leathers T.A., Rogers C.D. (2022) **Time to go: neural crest cell epithelial-to-mesenchymal transition** *Development* **149**

Lee Y., Park B.H., Bae E.J. (2016) **Compound C inhibits macrophage chemotaxis through an AMPK-independent mechanism** *Biochemical and biophysical research communications* **469**:515–520

LeVine H. (2004) **Alzheimer's beta-peptide oligomer formation at physiologic concentrations** *Anal Biochem* **335**:81–90

Lewitus G.M., Konefal S.C., Greenhalgh A.D., Pribiag H., Augereau K., Stellwagen D. (2016) **Microglial TNF-alpha Suppresses Cocaine-Induced Plasticity and Behavioral Sensitization** *Neuron* **90**:483–491

Liu W., Yan M., Liu Y., Wang R., Li C., Deng C., Singh A., Coleman W.G., Rodgers G.P. (2010) **Olfactomedin 4 down-regulates innate immunity against Helicobacter pylori infection** *Proceedings of the National Academy of Sciences of the United States of America* **107**:11056–11061

Lorenzl S., Buerger K., Hampel H., Beal M.F. (2008) **Profiles of matrix metalloproteinases and their inhibitors in plasma of patients with dementia** *Int Psychogeriatr* **20**:67–76

Lorton D., Kocsis J.M., King L., Madden K., Brunden K.R. (1996) **beta-Amyloid induces increased release of interleukin-1 beta from lipopolysaccharide-activated human monocytes** *Journal of neuroimmunology* **67**:21–29

Ma S., Kwon H.J., Huang Z. (2012) **Ric-8a, a guanine nucleotide exchange factor for heterotrimeric G proteins, regulates bergmann glia-basement membrane adhesion during cerebellar foliation** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **32**:14979–14993

Ma S., Santhosh D., Kumar T.P., Huang Z. (2017) **A Brain-Region-Specific Neural Pathway Regulating Germinal Matrix Angiogenesis** *Developmental cell* **41**:366–381

Michaels T.C.T. *et al.* (2020) **Dynamics of oligomer populations formed during the aggregation of Alzheimer's Abeta42 peptide** *Nat Chem* **12**:445–451

Milosch N. *et al.* (2014) **Holo-APP and G-protein-mediated signaling are required for sAPPalpha-induced activation of the Akt survival pathway** *Cell death & disease* **5**

Moore S.A. *et al.* (2002) **Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy** *Nature* **418**:422–425

Morley J.E., Farr S.A., Banks W.A., Johnson S.N., Yamada K.A., Xu L. (2010) **A physiological role for amyloid-beta protein:enhancement of learning and memory** *J Alzheimers Dis* **19**:441–449

Muehlhauser F., Liebl U., Kuehl S., Walter S., Bertsch T., Fassbender K. (2001) **Aggregation-Dependent interaction of the Alzheimer's beta-amyloid and microglia** *Clin Chem Lab Med* **39**:313–316

Murase S., Lantz C.L., Quinlan E.M. (2017) **Light reintroduction after dark exposure reactivates plasticity in adults via perisynaptic activation of MMP-9** *eLife* **6**

Murase S., Winkowski D., Liu J., Kanold P.O., Quinlan E.M. (2019) **Homeostatic regulation of perisynaptic matrix metalloproteinase 9 (MMP9) activity in the amblyopic visual cortex** *eLife* **8**

Niewmierzycka A., Mills J., St-Arnaud R., Dedhar S., Reichardt L.F. (2005) **Integrin-linked kinase deletion from mouse cortex results in cortical lamination defects resembling cobblestone lissencephaly** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **25**:7022–7031

Nishimoto I., Okamoto T., Matsuura Y., Takahashi S., Okamoto T., Murayama Y., Ogata E. (1993) **Alzheimer amyloid protein precursor complexes with brain GTP-binding protein G(o)** *Nature* **362**:75–79

Pagenstecher A., Stalder A.K., Kincaid C.L., Shapiro S.D., Campbell I.L. (1998) **Differential expression of matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase genes in the mouse central nervous system in normal and inflammatory states** *Am J Pathol* **152**:729–741

Palmeri A. *et al.* (2017) **Amyloid-beta Peptide Is Needed for cGMP-Induced Long-Term Potentiation and Memory** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **37**:6926–6937

Parodi J., Sepulveda F.J., Roa J., Opazo C., Inestrosa N.C., Aguayo L.G. (2010) **Beta-amyloid causes depletion of synaptic vesicles leading to neurotransmission failure** *The Journal of biological chemistry* **285**:2506–2514

Patani R., Hardingham G.E., Liddelow S.A. (2023) **Functional roles of reactive astrocytes in neuroinflammation and neurodegeneration** *Nat Rev Neurol* **19**:395–409

Plant L.D., Boyle J.P., Smith I.F., Peers C., Pearson H.A. (2003) **The production of amyloid beta peptide is a critical requirement for the viability of central neurons** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23**:5531–5535

Puzzo D., Privitera L., Leznik E., Fa M., Staniszewski A., Palmeri A., Arancio O. (2008) **Picomolar amyloid-beta positively modulates synaptic plasticity and memory in hippocampus** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**:14537–14545

Qin H. *et al.* (2012) **Signal transducer and activator of transcription-3/suppressor of cytokine signaling-3 (STAT3/SOCS3) axis in myeloid cells regulates neuroinflammation** *Proceedings of the National Academy of Sciences of the United States of America* **109**:5004–5009

Ramaker J.M., Swanson T.L., Copenhaver P.F. (2013) **Amyloid precursor proteins interact with the heterotrimeric G protein Go in the control of neuronal migration** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **33**:10165–10181

Ramsden M., Henderson Z., Pearson H.A. (2002) **Modulation of Ca2+ channel currents in primary cultures of rat cortical neurones by amyloid beta protein (1-40) is dependent on solubility status** *Brain research* **956**:254–261

Rice H.C., Townsend M., Bai J., Suth S., Cavanaugh W., Selkoe D.J., Young-Pearse T.L. (2012) **Pancortins interact with amyloid precursor protein and modulate cortical cell migration** *Development* **139**:3986–3996

Satz J.S. *et al.* (2010) **Distinct functions of glial and neuronal dystroglycan in the developing and adult mouse brain** *J Neurosci* **30**:14560–14572

Sayed F.A. *et al.* (2018) **Differential effects of partial and complete loss of TREM2 on microglial injury response and tauopathy** *Proceedings of the National Academy of Sciences of the United States of America* **115**:10172–10177

Schafer D.P., Stevens B. (2015) **Microglia Function in Central Nervous System Development and Plasticity** *Cold Spring Harb Perspect Biol* **7**

Shaked G.M., Kummer M.P., Lu D.C., Galvan V., Bredesen D.E., Koo E.H. (2006) **Abeta induces cell death by direct interaction with its cognate extracellular domain on APP (APP 597-624)** *FASEB J* **20**:1254–1256

Shankar G.M. *et al.* (2008) **Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory** *Nat Med* **14**:837–842

Shigemoto-Mogami Y., Hoshikawa K., Goldman J.E., Sekino Y., Sato K. (2014) **Microglia enhance neurogenesis and oligodendrogenesis in the early postnatal subventricular zone** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **34**:2231–2243

Spiller K.J. *et al.* (2019) **Reduction of matrix metalloproteinase 9 (MMP-9) protects motor neurons from TDP-43-triggered death in rNLS8 mice** *Neurobiol Dis* **124**:133–140

Spolidoro M., Putignano E., Munafo C., Maffei L., Pizzorusso T. (2012) **Inhibition of matrix metalloproteinases prevents the potentiation of nondeprived-eye responses after monocular deprivation in juvenile rats** *Cereb Cortex* **22**:725–734

Stellwagen D., Malenka R.C. (2006) **Synaptic scaling mediated by glial TNF-alpha** *Nature* **440**:1054–1059

Stenman J., Toresson H., Campbell K. (2003) **Identification of two distinct progenitor populations in the lateral ganglionic eminence: implications for striatal and olfactory bulb neurogenesis** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23**:167–174

Stine W.B., Jungbauer L., Yu C., LaDu M.J. (2011) **Preparing synthetic Abeta in different aggregation states** *Methods Mol Biol* **670**:13–32

Sturchio A. *et al.* (2022) **High Soluble Amyloid-beta42 Predicts Normal Cognition in Amyloid-Positive Individuals with Alzheimer's Disease-Causing Mutations** *J Alzheimers Dis*

Sturchio A. *et al.* (2021) **High cerebrospinal amyloid-beta 42 is associated with normal cognition in individuals with brain amyloidosis** *EClinicalMedicine* **38**

Tall G.G., Krumins A.M., Gilman A.G. (2003) **Mammalian Ric-8A (synembryn) is a heterotrimeric Galpha protein guanine nucleotide exchange factor** *The Journal of biological chemistry* **278**:8356–8362

Tan J., Town T., Paris D., Mori T., Suo Z., Crawford F., Mattson M.P., Flavell R.A., Mullan M. (1999) **Microglial activation resulting from CD40-CD40L interaction after beta-amyloid stimulation** *Science* **286**:2352–2355

Tran N.M. *et al.* (2019) **Single-Cell Profiles of Retinal Ganglion Cells Differing in Resilience to Injury Reveal Neuroprotective Genes** *Neuron* **104**:1039–1055

Tsiknia A.A., Sundermann E.E., Reas E.T., Edland S.D., Brewer J.B., Galasko D., Banks S.J., Alzheimer's Disease Neuroimaging, I. (2022) **Sex differences in Alzheimer's disease: plasma MMP-9 and markers of disease severity** *Alzheimers Res Ther* **14**

Vainchtein I.D. *et al.* (2018) **Astrocyte-derived interleukin-33 promotes microglial synapse engulfment and neural circuit development** *Science* **359**:1269–1273

Walsh D.M., Klyubin I., Fadeeva J.V., Cullen W.K., Anwyl R., Wolfe M.S., Rowan M.J., Selkoe D.J. (2002) **Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo** *Nature* **416**:535–539

Wang H., Ng K.H., Qian H., Siderovski D.P., Chia W., Yu F. (2005) **Ric-8 controls Drosophila neural progenitor asymmetric division by regulating heterotrimeric G proteins** *Nat Cell Biol* **7**:1091–1098

Wang X., Jung J., Asahi M., Chwang W., Russo L., Moskowitz M.A., Dixon C.E., Fini M.E., Lo E.H. (2000) **Effects of matrix metalloproteinase-9 gene knock-out on morphological and motor outcomes after traumatic brain injury** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **20**:7037–7042

Wang Y., Fu W.Y., Cheung K., Hung K.W., Chen C., Geng H., Yung W.H., Qu J.Y., Fu A.K.Y., Ip N.Y. (2021) **Astrocyte-secreted IL-33 mediates homeostatic synaptic plasticity in the adult hippocampus** *Proceedings of the National Academy of Sciences of the United States of America* **118**

Yang Y., Kim J., Kim H.Y., Ryoo N., Lee S., Kim Y., Rhim H., Shin Y.K. (2015) **Amyloid-beta Oligomers May Impair SNARE-Mediated Exocytosis by Direct Binding to Syntaxin 1a** *Cell reports* **12**:1244–1251

Yona S. *et al.* (2013) **Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis** *Immunity* **38**:79–91

Yoshida M., Assimacopoulos S., Jones K.R., Grove E.A. (2006) **Massive loss of Cajal-Retzius cells does not disrupt neocortical layer order** *Development* **133**:537–545

Young-Pearse T.L., Bai J., Chang R., Zheng J.B., LoTurco J.J., Selkoe D.J. (2007) **A critical function for beta-amyloid precursor protein in neuronal migration revealed by in utero RNA interference** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **27**:14459–14469

Yu D. *et al.* (2022) **Microglial GPR56 is the molecular target of maternal immune activation-induced parvalbumin-positive interneuron deficits** *Sci Adv* **8**

Zhang Y. *et al.* (2014) **An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex** *The Journal of neuroscience : the official journal of the Society for Neuroscience* **34**:11929–11947

Zhou B., Lu J.G., Siddu A., Wernig M., Sudhof T.C. (2022) **Synaptogenic effect of APP-Swedish mutation in familial Alzheimer's disease** *Sci Transl Med* **14**

Zipp F., Bittner S., Schafer D.P. (2023) **Cytokines as emerging regulators of central nervous system synapses** *Immunity* **56**:914–925

Zott B., Simon M.M., Hong W., Unger F., Chen-Engerer H.J., Frosch M.P., Sakmann B., Walsh D.M., Konnerth A. (2019) **A vicious cycle of beta amyloid-dependent neuronal hyperactivation** *Science* **365**:559–565

Editors

Senior Editor
Jonathan Cooper
Fred Hutchinson Cancer Research Center, Seattle, United States of America

Reviewer #1 (Public Review):

Summary:

The authors want to elucidate which are the mechanisms that regulate the immune response in physiological conditions in cortical development. To achieve this goal, authors used a wide range of mutant mice to analyse the consequences of immune activation in the formation of cortical ectopia in mice.

Strengths:

The authors demonstrated that Abeta monomers are anti-inflammatory and inhibit microglial activation. This is a novel result that demonstrates the physiological role of APP in cortical development.

Weaknesses:

-On the other hand, cortical ectopia has been already described in mouse models in which the amyloid signalling has been disrupted (Herms et al., 2004; Guenette et al., 2006), making the current study less novel.

One of the molecules analysed is Ric8a, a GTPase activator involved in neuronal development. Authors used the conditional mutant mice Emx1-Ric8a to delete Ric8a from early progenitors and glutamatergic neurons in the pallium. Emx1-Ric8a mutant mice present cortical ectopias and authors attributed this malformation to the increase in inflammatory response due to Ric8a deletion in microglia. Several discordances do not fit this interpretation:

-The role of Ric8a in cortical development and function has been already described in several papers, but none of them has been cited in the current manuscript (Kask et al., 2015, 2018; Ruisu et al., 2013; Tonissoo et al., 2006).

-Ectopia formation in the cortex has been already described in Nestin-Ric8a cKO mice (Kask et al., 2015). In the current manuscript, authors analyzed the same mutant mice (Nestin-Ric8a), but they did not detect any ectopia. Authors should discuss this discordance.

-Authors claim that microglia express Emx1, and therefore, Ric8a is deleted in microglia cells. However, the arguments for this assumption are very weak and the evidence suggests that this is not the case. This is an important point considering that authors want to emphasise the role of Ric8a in microglia activation, and therefore, additional experiments should demonstrate that Ric8a is deleted in microglia in Emx1-Ric8a mutant mice.

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Reviewer #2 (Public Review):

Kwon et al. used several conditional KO mice for the deletion of ric8a or app in different cell types. Some of them exhibited pial basement membrane breaches leading to neuronal ectopia in the neocortex.

They first investigated ric8a, a Guanine Nucleotide Exchange Factor for Heterotrimeric G Proteins. They observed the above-mentioned phenotype when ric8a is deleted from microglia and neural cells (ric8a-emx1-cre or dual deletion with cre combination cx3cr1 (in

microglia) and nestin (in neural cells)) but not in microglia alone or neural cells alone (whether it is in CR cells (ric8a-Wnt3a-cre), post-mitotic neurons (nex-cre or dlx5/6-cre), or in progenitors and their progeny (nestin-cre or foxg1-cre). They also show that ric8a KO mutant microglia cells stimulated in vitro by LPS exhibit an increased TNFa, IL6 and IL1b secretion compared to controls (Fig 2). They therefore injected LPS in vivo and observed the neuronal ectopia phenotype in the ric8a-cx3cr1-cre (microglial deletion) cortices at P0 (Fig 2). They suggest that ric8a KO in neuronal cells mimics immune stimulation (but we have no clue how ric8a KO in neural cells would induce immune stimulation).

The authors then turned their attention on APP. They observed neuronal ectopia into the marginal zone when APP is deleted in microglia (app-cxcr3-cre) + intraperitoneal LPS injection (they did not show it, but we have to assume there would not be a phenotype without the injection of LPS) (Fig 3). (The phenotype is similar but not identical to ric8a-cx3cr1-cre + LPS. They suggest that the reason is because they had to inject 3 times less LPS due to enhanced immune sensitivity in this genetic background but it is only a hypothesis). After in vitro stimulation by LPS, app mutant microglia show a reduced secretion of TNFa and IL6 but not IL1b (this is the opposite to ric8a-cx3cr1-cre microglia cells) while peritoneal macrophages in culture show increased secretion of TNFa, IL1, IL6 and IL23 (fig 3 and Suppl. Fig 9).

Amyloid beta (Ab) being one of the molecules binding to APP, the authors showed that Ab40 monomers (they did not test Ab40 oligomers) partially inhibit cytokines (TNFa, IL6, IL1b, MCP-1, IL23a, IL10) secretion in vitro by microglia stimulated by LPS but does not affect secretion by microglia from app-cx3cr1-cre (tested for TNFa, IL6, IL1b, IL23a, IL10) (Fig 4, Suppl fig 10) (but still does it in aplp2-cx3cr1-cre) and does not affect secretion by ric8a-cx3cr1-cre microglia (tested for TNFa and IL6 but still suppress IL1b) (Therefore here is another difference between app and ric8a KO microglia).

The authors injected inhibitors of Akt or Stat3 in the ric8a-emx1-cre cortex and found it suppressed neuronal ectopia (Fig 5, Suppl fig 11). It is not clear whether it suppresses immune stimulation from neuronal cells or immune reaction from microglia cells.

Finally, the authors examined the activities of MMP2 and MMP9 in the developing cortex using gelatin gel zymography. The activity and protein levels of MMP9 but not MMP2 in the ric8a-emx1-cre cortex were claimed significantly increased (Fig 5, Suppl fig 12). Unfortunately, they did not show it in the app-cx3cr1-cre +LPS mouse. They make a connection between ric8a deletion and MMP9 but unfortunately do not make the connection between app deletion and MMP9, which is at the center of the pathway claimed to be important here). Then they injected BB94, a broad-spectrum inhibitor of MMPs or an inhibitor specific for MMP9 and 13. They both significantly suppress the number and the size of the ectopia in ric8a mutants (Fig5).

After reading the manuscript, I still do not know how ric8a in neural cells is involved in the immune inhibition. Is it through the control of Ab monomers? In addition, the authors did not show in vivo data supporting that Ab monomers are the key players here. As the authors said, this is not the only APP interactor. Finally, I still do not know how ric8a is linked to APP in microglia in the model.

While several of the findings presented in this manuscript are of potential interest, there are a number of shortcomings. Here are some suggestions that could improve the manuscript and help substantiate the conclusions:

(1) As the title suggests it, the focus is on Ab and APP functions in microglia. However, the analysis is more focused on ric8a. The connection between ric8a and APP in this study is not investigated, besides the fact that their deletion induces somewhat similar but not identical phenotypes. Showing a similar phenotype is not enough to conclude that they are working on

the same pathway. The authors should find a way to make that connection between ric8a and app in the cells investigated here.

(2) This would help to show the appearance of breaches in the pial basement membrane leading to neuronal ectopia; to investigate laminin debris, cell identity, Wnt pathway for app-cxcr3-cre + LPS injection as you did for ric8a-emx1-cre.

(3) As a control, this would help to show that app-cxcr3-cre without the LPS injection does not display the phenotype.

(4) This would help to show the activity and protein levels of MMP9 and MMP2 and perform the rescue experiments with the inhibitors in the app-cx3cr1-cre cortex +LPS.

(5) Is MMP9 secreted by microglia cells or neural cells?

(6) The in vitro evidence indicates that one of the multiple APP interactors, ie Ab40 monomers, is less effective in suppressing the expression of some cytokines by microglia cells mutants for ric8a (TNFa and IL6 but still suppress IL1b) or APP (TNFa, IL6, IL1b, IL23a, IL10) when compared to WT. But there are other interactors for APP. In order to support the claim, it seems crucial to have in vivo data to show that Ab40 monomers are the molecules involved in preventing the breach in the pial basement membrane.

(7) In order to claim that this is specific to Ab40 monomers and not oligomers, it is necessary to show that the Ab40 oligomers do not have the same effect in vitro and in vivo. Also, an assay should be done to show that your Ab preparations are pure monomers or oligomers.

(8) Most of the cytokine secretion assays used microglia cells in culture. Two results draw my attention. Ric8a deletion increases TNFa and IL6 secretion after LPS stimulation in vitro on microglia cells while app deletion decreases their secretion. Then later, papers show that the decrease in IL1b induced by Ab on microglia cells is prevented by APP deletion but not ric8a deletion. Those two pieces of data suggest that ric8a and APP might not be in the same pathway. In addition, the phenotype from app-cxcr3-cre + LPS injection and ric8a-cxcr3-cre + LPS injection are not exactly the same. It could be due to the level of LPS as the author suggests or it might not be. More experiments are needed to prove they are in the same pathway.

(9) How do the authors reconcile the reduced TNFa and IL6 secretion upon stimulation of app mutant microglia with the model where app is attenuating immune response in vivo? Line 213 says that microglia exhibit attenuated immune response following chronic stimulation but I don't know if 3 hours of LPS in vitro is a chronic stimulation.

(10) Line 119: In their model, the authors suggest that there is a breach in pial basement membrane but that the phenotype is different from the retraction of the radial fibers due to reduced adhesion. So, could the author discuss to what substrate the radial fibers are attached to, in their model where the pial surface is destroyed?

(11) The authors should show that the increased cytokine secretion observed in vitro is also happening in vivo in ric8a-emx1-cre compared to WT mice and compared to ric8a-nestin-cre mice. Or when app is deleted in microglia (app-cxcr3-cre) + LPS injection compared to WT mice +LPS.

(12) The authors injected inhibitors of Akt or Stat3 in the ric8a-emx1-cre cortex and found that it suppressed neuronal ectopia (Fig 5, Suppl fig 11). Does it suppress immune stimulation from neuronal cells or immune reaction from microglia cells?

(13) Fig 5 and Supplementary fig 12: Please show a tubulin loading control in Fig 5i as you did in suppl fig 12 d (gel zymography). Please provide a gel zymography showing side by side

Control, mutant and mutant +DM/S3I treatment. The same request for the MMP9 staining. Please provide statistics for control vs mutant for suppl fig 12c and d.

(14) Please provide the name and the source of the MMP9/13 inhibitor used in this study.

(15) The results show that deletion of ric8a in microglia and neural cells induced pia membrane breaches but no phenotype is apparent in ric8a deletion in microglia or neural cells alone. Then, the results showed that intraperitoneal injection of LPS induced the phenotype in ric8a-cxcr3-cre mutants. It would be beneficial as a control supporting the model to show that the insult induced by LPS injection does not induce the phenotype in the ric8a-foxg1-cre mice.

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Author response:

> ***Reviewer #1 (Public Review):***
>
> *Summary:*
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> *The authors want to elucidate which are the mechanisms that regulate the immune response in physiological conditions in cortical development. To achieve this goal, authors used a wide range of mutant mice to analyse the consequences of immune activation in the formation of cortical ectopia in mice.*
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> *Strengths:*
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> *The authors demonstrated that Abeta monomers are anti-inflammatory and inhibit microglial activation. This is a novel result that demonstrates the physiological role of APP in cortical development.*
>
> *Weaknesses:*
>
> *-On the other hand, cortical ectopia has been already described in mouse models in which the amyloid signalling has been disrupted (Herms et al., 2004; Guenette et al., 2006), making the current study less novel.*

We agree these previous studies have implicated amyloid precursor protein in cortical ectopia. However, since these studies use whole-body knockouts, they have not implicated the functional roles of specific cell types. Nor have they identified the specific mechanisms underlying the formation of this unique class of cortical ectopia. In contrast, our studies show that the disruption of a novel Abeta-regulated signaling pathway in microglia is the primary cause of ectopia formation in this class of ectopia mutants. This is the first time that microglia have been specifically implicated in the development of cortical ectopia. We further show that elevated MMP activity and resulting cortical basement membrane degradation is the underlying mechanism leading to ectopia formation. This is also the first time that MMP activity and basement membrane degradation (instead of maintenance) have been implicated in cortical ectopia development. As such, our results have provided novel insights into the diverse mechanisms underlying cortical ectopia formation in developmental brain disorders.

> *One of the molecules analysed is Ric8a, a GTPase activator involved in neuronal development. Authors used the conditional mutant mice Emx1-Ric8a to delete Ric8a from early progenitors and glutamatergic neurons in the pallium. Emx1-Ric8a mutant mice present cortical ectopias and authors attributed this malformation to the increase in*

> *inflammatory response due to Ric8a deletion in microglia. Several discordances do not fit this interpretation:*
>
> *-The role of Ric8a in cortical development and function has been already described in several papers, but none of them has been cited in the current manuscript (Kask et al., 2015, 2018; Ruisu et al., 2013; Tonissoo et al., 2006).*

We will include reference to these publications in revision.

> *-Ectopia formation in the cortex has been already described in Nestin-Ric8a cKO mice (Kask et al., 2015). In the current manuscript, authors analyzed the same mutant mice (Nestin-Ric8a), but they did not detect any ectopia. Authors should discuss this discordance.*

The expression pattern of nestin-cre is known to vary dependent on factors including transgene insertion site, genetic background, and sex. Early studies show, for example, that the nestin gene promoter drives cre expression in many non-neural tissues in another transgenic line in the FVB/N genetic background (Dubois et al Genesis. 2006 Aug;44(8):355-60. doi: 10.1002/dvg.20226). The specific nestin-cre line used in Kask et al 2015 has also been shown to be active in brain microglia and lead to increased microglia pro-inflammatory activity upon breeding to a conditional allele of a cholesterol transporter gene (Karasinska et al., Neurobiol Dis. 2013 Jun:54:445-55; Karasinska et al., J Neurosci. 2009 Mar 18; 29(11): 3579–3589). The ectopia reported in Kask et al 2015 are also significantly more subtle than what we have observed and apparently not observed in all mutant animals (we observe severe ectopia in every single emx1-cre mutant). We presume the ectopia reported in Kask et al 2015 may result from a combined deletion of ric8a gene from microglia and neural cells due to unique combinations of factors affecting nestin-cre expression in a subset of mutants.

> *-Authors claim that microglia express Emx1, and therefore, Ric8a is deleted in microglia cells. However, the arguments for this assumption are very weak and the evidence suggests that this is not the case. This is an important point considering that authors want to emphasise the role of Ric8a in microglia activation, and therefore, additional experiments should demonstrate that Ric8a is deleted in microglia in Emx1-Ric8a mutant mice.*

We have observed altered mRNA expression of several genes in purified microglia cultured from the emx1-cre mutants (Supplemental Fig. 8), which indicates that ric8a is deleted from microglia and suggests a role of microglial ric8a deficiency in ectopia formation. This interpretation is further strengthened by the observation that deletion of ric8a from microglia using a microglia-specific cx3cr1-cre results in similar ectopia (Fig. 2). We also have other data supporting this interpretation, including data showing induction of the expression of a cre reporter in brain microglia by emx1-cre and loss of ric8a gene expression in microglia cells isolated from emx1-cre mutants. We will include these data in revision.

> ***Reviewer #2 (Public Review):***
>
> *Kwon et al. used several conditional KO mice for the deletion of ric8a or app in different cell types. Some of them exhibited pial basement membrane breaches leading to neuronal ectopia in the neocortex.*
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> *They first investigated ric8a, a Guanine Nucleotide Exchange Factor for Heterotrimeric G Proteins. They observed the above-mentioned phenotype when ric8a is deleted from microglia and neural cells (ric8a-emx1-cre or dual deletion with cre combination cx3cr1 (in microglia) and nestin (in neural cells)) but not in microglia alone or neural cells alone (whether it is in CR cells (ric8a-Wnt3a-cre), post-mitotic neurons (nex-cre or dlx5/6-cre), or*

> *in progenitors and their progeny (nestin-cre or foxg1-cre). They also show that ric8a KO mutant microglia cells stimulated in vitro by LPS exhibit an increased TNFa, IL6 and IL1b secretion compared to controls (Fig 2). They therefore injected LPS in vivo and observed the neuronal ectopia phenotype in the ric8a-cx3cr1-cre (microglial deletion) cortices at P0 (Fig 2). They suggest that ric8a KO in neuronal cells mimics immune stimulation (but we have no clue how ric8a KO in neural cells would induce immune stimulation).*

We agree we do not currently know the precise mechanisms by which mutant microglia are activated in the mutant brain. However, this does not affect the conclusion that deficiency in the Abeta monomer-regulated APP/Ric8a pathway in microglia is the primary cause of cortical ectopia in these mutants, since we have shown that genetic disruption of this pathway in microglia alone by different means targeting different pathway components, using cell type specific cre, all results in similar cortical ectopia phenotypes. Regarding the source of the immunogens, there are several possibilities which we plan to investigate in future studies. For example, the clearance of apoptotic cells and associated cellular debris is an important physiological process and deficits in this process have been linked to inflammatory diseases throughout life (Doran et al., Nat Rev Immunol. 2020 Apr;20(4):254-267; Boada-Romero et al., Nat Rev Mol Cell Biol. 2020 Jul;21(7):398-414.). In the embryonic cortex, studies have shown that large numbers of cell death take place starting as early as E12 (Blaschke et al., Development. 1996 Apr;122(4):1165-74; Blaschke et al., J Comp Neurol. 1998 Jun 22;396(1):39-50). Studies have also shown that radial glia and neuronal progenitors play critical roles in the clearance of apoptotic cells and associated cellular debris in the brain (Lu et al., Nat Cell Biol. 2011 Jul 31;13(9):1076-83; Ginisty et al., Stem Cells. 2015 Feb;33(2):515-25; Amaya et al., J Comp Neurol. 2015 Feb 1;523(2):183-96). Moreover, Ric8a-dependent heterotrimeric G proteins have been found to specifically promote the phagocytic activity of both professional and non-professional phagocytic cells (Billings et al., Sci Signal. 2016 Feb 2;9(413):ra14; Preissler et al., Glia. 2015 Feb;63(2):206-15; Pan et al. Dev Cell. 2016 Feb 22;36(4):428-39; Flak et al. J Clin Invest. 2020 Jan 2;130(1):359-373; Zhang et al., Nat Commun. 2023 Sep 14;14(1):5706). Thus, it is likely that the failure to promptly clear up apoptotic cells and debris by radial glia may play a role in the triggering of microglial activation in ric8a mutants. We have not included discussion of these possibilities since the precise mechanisms remain to be determined. Moreover, they also do not impact the conclusion of the current study.

> *The authors then turned their attention on APP. They observed neuronal ectopia into the marginal zone when APP is deleted in microglia (app-cxcr3-cre) + intraperitoneal LPS injection (they did not show it, but we have to assume there would not be a phenotype without the injection of LPS) (Fig 3). (The phenotype is similar but not identical to ric8a-cx3cr1-cre + LPS. They suggest that the reason is because they had to inject 3 times less LPS due to enhanced immune sensitivity in this genetic background but it is only a hypothesis). After in vitro stimulation by LPS, app mutant microglia show a reduced secretion of TNFa and IL6 but not IL1b (this is the opposite to ric8a-cx3cr1-cre microglia cells) while peritoneal macrophages in culture show increased secretion of TNFa, IL1, IL6 and IL23 (fig 3 and Suppl. Fig 9).*

We have data showing that that app-cxcr3-cre mutants without LPS injection do not show ectopia and will include them in revision. The reason we employ LPS injection is, in the first place, we do not see a phenotype without the injection. We agree, and have also stated in the text, that the phenotype of the app mutants is not as severe as that of the ric8a mutant. Besides the low LPS dosage used, we also suggest that other app family members may compensate since the ectopia in the app family gene mutants reported previously were only observed in app/aplp1/2 triple knockouts, not even in any of the double knockouts (Herms et al., 2004). These potential causes are also not mutually exclusive. Nonetheless, the microglia

specific app mutants clearly show ectopia upon immune stimulation, implicating a role of microglial APP in cortical ectopia formation.

The distinct response of ric8a and app microglia to LPS results from in vitro culturing of microglia. Indeed, we have shown that, when acutely isolated macrophages are used, these mutants show changes in the same direction (both increased cytokine secretion). The microglia used for analysis in this study have all been cultured in vitro for two weeks before assay. They have thus been under chronic stimulation exposing to dead cells and debris in the culture dish through this period. Dependent on the degree of perturbation to inflammation-regulating pathways, such exposures are known to significantly change microglial cytokine expression, sometimes in an opposite direction from expected. For example, under chronic immune stimulation, while the trem2+/- microglia, which are heterozygous mutant for the anti-inflammatory Trem2, show elevated pro-inflammatory cytokine expression as expected, trem2-/- (null) microglia under the same conditions instead not only do not show increases but for some pro-inflammatory cytokines, actually show decreases in expression (Sayed et al.,, Proc Natl Acad Sci U S A. 2018 Oct 2;115(40):10172-10177). In several systems, Ric8a-dependent heterotrimeric G proteins have been shown to act downstream of APP and mediate one of the branches of the signaling activated by APP (Milosch et al., Cell Death Dis. 2014 Aug 28;5(8):e1391; Fogel et al,, Cell Rep. 2014 Jun 12;7(5):1560-1576; Ramaker et al., J Neurosci. 2013 Jun 12;33(24):10165-81; Nishimoto et al., Nature. 1993 Mar 4;362(6415):75-9). It is likely that in microglia Ric8a-dependent heterotrimeric G proteins may also mediate only a subset of the signaling downstream of APP. As such, app knockout in microglia may have more severe effects than ric8a knockout on microglial immune activation and lead to changes in the opposite direction compared to ric8a knockout, as has been observed for trem2 null mutation vs heterozygosity discussed above. This may explain the subdued TNF and IL6 secretion by cultured app mutant microglia.

> *Amyloid beta (Ab) being one of the molecules binding to APP, the authors showed that Ab40 monomers (they did not test Ab40 oligomers) partially inhibit cytokines (TNFa, IL6, IL1b, MCP-1, IL23a, IL10) secretion in vitro by microglia stimulated by LPS but does not affect secretion by microglia from app-cx3cr1-cre (tested for TNFa, IL6, IL1b, IL23a, IL10) (Fig 4, Suppl fig 10) (but still does it in aplp2-cx3cr1-cre) and does not affect secretion by ric8a-cx3cr1-cre microglia (tested for TNFa and IL6 but still suppress IL1b) (Therefore here is another difference between app and ric8a KO microglia).*

We have tested the effects of Abeta40 oligomers, which induce instead of suppressing microglial cytokine secretion, and will include the data in revision. As mentioned above, in several systems, Ric8a-dependent heterotrimeric G proteins have been shown to act downstream of APP and mediate one of the branches of the signaling activated by APP (Milosch et al., Cell Death Dis. 2014 Aug 28;5(8):e1391; Fogel et al,, Cell Rep. 2014 Jun 12;7(5):1560-1576; Ramaker et al., J Neurosci. 2013 Jun 12;33(24):10165-81; Nishimoto et al., Nature. 1993 Mar 4;362(6415):75-9). We assume that this is likely also true in microglia and that Ric8a-dependent heterotrimeric G proteins may mediate only a subset of the signaling downstream of APP. This may explain the difference in the effects of APP and ric8a knockout mutation in abolishing the anti-inflammatory effects of Abeta monomers on IL-1b vs TNF/IL-6. It also suggests that TNF/IL-6 and IL-1b secretion must be regulated by different mechanisms. Indeed, it is well established in immunology that the secretion of IL1b, but not of TNF or IL6, is regulated by inflammasome-dependent mechanisms (see, for example, Proz & Dixit. Nat Rev Immunol. 2016 Jul;16(7):407-20. doi: 10.1038/nri.2016.58).

> *The authors injected inhibitors of Akt or Stat3 in the ric8a-emx1-cre cortex and found it suppressed neuronal ectopia (Fig 5, Suppl fig 11). It is not clear whether it suppresses immune stimulation from neuronal cells or immune reaction from microglia cells.*

We agree at present the pharmacological approaches we have taken are not able to distinguish these possibilities. However, whichever of these possibilities turns out to be the case would still implicate a role of excessive microglial activation in the formation of cortical ectopia and support the conclusion of the study. Thus, while potentially worthwhile of further investigation, this question does not impact the conclusion of this study. Furthermore, as mentioned, we plan to determine the mechanisms of how ric8a mutation in neural cells induces immune activation in future studies. These results will likely enable us to adopt more specific approaches to address this question.

> *Finally, the authors examined the activities of MMP2 and MMP9 in the developing cortex using gelatin gel zymography. The activity and protein levels of MMP9 but not MMP2 in the ric8a-emx1-cre cortex were claimed significantly increased (Fig 5, Suppl fig 12). Unfortunately, they did not show it in the app-cx3cr1-cre +LPS mouse. They make a connection between ric8a deletion and MMP9 but unfortunately do not make the connection between app deletion and MMP9, which is at the center of the pathway claimed to be important here). Then they injected BB94, a broad-spectrum inhibitor of MMPs or an inhibitor specific for MMP9 and 13. They both significantly suppress the number and the size of the ectopia in ric8a mutants (Fig5).*

For all the gelatin gel zymography analysis, we quantify protein concentrations in the cortical lysates using the Bio-Rad Bradford assay kit and load the same amounts of proteins per lane. The results across lanes are thus directly comparable. From the quantification, our results clearly show that MMP9, but not MMP2, levels are increased in the mutants (supplemental Figure 12). The data on MMP2 also provide an internal control further supporting the observation of a specific change in MMP9. For this analysis, we focus on the ric8a-emx1-cre mutants since the app-cx3cr1-cre +LPS animals show less severe, more localized ectopia and in most cases only in one of the hemispheres. Any changes in MMP9 are therefore likely to be masked and the experiments unlikely to yield meaningful results. On the other hand, we have clearly shown that the administration of different classes of MMP inhibitors significantly eliminate ectopia in ric8a-emx1-cre mutants. This has strongly implicated a functional contribution of MMPs.

> *After reading the manuscript, I still do not know how ric8a in neural cells is involved in the immune inhibition. Is it through the control of Ab monomers? In addition, the authors did not show in vivo data supporting that Ab monomers are the key players here. As the authors said, this is not the only APP interactor. Finally, I still do not know how ric8a is linked to APP in microglia in the model.*

As detailed above, there are several possibilities including potential deficits in the clearance of apoptotic cells and associated debris that may trigger microglial activation in ri8ca-emx1-cre mutants. We will investigate these possibilities in future studies. We have not included discussion since their roles remain to be determined. As for the role of Abeta monomers, we have indicated that we currently do not have evidence that in the developing cortex Abeta monomers play a role in inhibiting microglia. We have also indicated in the manuscript that our conclusion is that an Abeta monomer-activated microglial pathway regulates normal brain development, not that Abeta monomers themselves regulate brain development. Regarding the link between Ric8a and APP, the reviewer has missed several major lines of supporting evidence. For example, we have shown that Abeta monomers activates a pathway in microglia that inhibits the secretion of several proinflammatory cytokines including TNF, IL-6, IL-10, and IL-23 (Figure 4 and Supplemental Figures 8-10). This inhibition is abolished when either app or ric8a gene is deleted from microglia. This indicates that app and ric8a act in the same pathway activated by Abeta monomers in microglia. We also show that this Abeta monomer-activated pathway also inhibits the transcription of several cytokines in microglia.

This inhibition is also abolished when either app or ric8a gene is deleted from microglia. This reinforces the conclusion that app and ric8a act in the same pathway in microglia. Furthermore, cell type specific deletion of app or ric8a from microglia in vivo also results in similar phenotypes of cortical ectopia. Together, these results thus strongly support the conclusion that app and ric8a act in the same pathway activated by Abeta monomers in microglia. This conclusion is also consistent with published findings that Ric8a dependent heterotrimeric G proteins bind to APP and mediate subsets of APP signaling across different different species (Milosch et al., Cell Death Dis. 2014 Aug 28;5(8):e1391; Fogel et al,, Cell Rep. 2014 Jun 12;7(5):1560-1576; Ramaker et al., J Neurosci. 2013 Jun 12;33(24):10165-81; Nishimoto et al., Nature. 1993 Mar 4;362(6415):75-9).

> *While several of the findings presented in this manuscript are of potential interest, there are a number of shortcomings. Here are some suggestions that could improve the manuscript and help substantiate the conclusions:*
>
> *(1) As the title suggests it, the focus is on Ab and APP functions in microglia. However, the analysis is more focused on ric8a. The connection between ric8a and APP in this study is not investigated, besides the fact that their deletion induces somewhat similar but not identical phenotypes. Showing a similar phenotype is not enough to conclude that they are working on the same pathway. The authors should find a way to make that connection between ric8a and app in the cells investigated here.*

As discussed above, the reviewer misses several major lines of evidence showing that APP and Ric8a acts in the same pathway in microglia. For example, besides the similarity of the ectopia phenotypes, we have shown that Abeta monomers activates a pathway in microglia that inhibits the secretion of several proinflammatory cytokines including TNF, IL-6, IL-10, and IL-23 (Figure 4 and Supplemental Figures 8-10). These inhibitory effects are completely abolished when either app or ric8a gene is deleted from microglia. This indicates that app and ric8a act in the same pathway activated by Abeta monomers in microglia. We also show that this Abeta monomer-activated pathway inhibits the transcription of several cytokine genes in microglia. These effects are again completely abolished when either app or ric8a gene is deleted from microglia. This further reinforces the conclusion that app and ric8a act in the same pathway in microglia. Not only so we also show that the same results are true in macrophages. Together, these results therefore strongly support the conclusion that app and ric8a act in the same pathway in microglia. This conclusion is also consistent with published findings that Ric8a dependent heterotrimeric G proteins bind to APP and mediate APP signaling across different species (Milosch et al., Cell Death Dis. 2014 Aug 28;5(8):e1391; Fogel et al,, Cell Rep. 2014 Jun 12;7(5):1560-1576; Ramaker et al., J Neurosci. 2013 Jun 12;33(24):10165-81; Nishimoto et al., Nature. 1993 Mar 4;362(6415):75-9).

> *(2) This would help to show the appearance of breaches in the pial basement membrane leading to neuronal ectopia; to investigate laminin debris, cell identity, Wnt pathway for app-cxcr3-cre + LPS injection as you did for ric8a-emx1-cre.*

We will provide further data on the breaches in the pial basement membrane. We have not observed any changes in cell identity or Wnt pathway activity in ric8a-emx1-cre mutants. The ectopia phenotype in the app-cxcr3-cre + LPS animals is also less severe. It is therefore likely of limited value to examine potential changes in these areas.

> *(3) As a control, this would help to show that app-cxcr3-cre without the LPS injection does not display the phenotype.*

We have the data on app-cx3cr1-cre mutants without LPS injection, which show no ectopia, and will include the data in revision.

> *(4) This would help to show the activity and protein levels of MMP9 and MMP2 and perform the rescue experiments with the inhibitors in the app-cx3cr1-cre cortex +LPS.*

As discussed above, we focus analysis on the ric8a-emx1-cre mutants since app-cx3cr1-cre +LPS animals show less severe, more localized ectopia and in most cases only in one of the hemispheres. Determining potential changes in MMP9 levels and effects of MMP inhibitors are therefore not likely to yield useful data. On the other hand, we have shown that MMP9 levels are increased and administration of different classes of MMP inhibitors eliminate cortical ectopia in ric8a-emx1-cre mutants. This has strongly implicated a functional contribution of MMPs.

> *(5) Is MMP9 secreted by microglia cells or neural cells?*

Our in situ hybridization data show MMP9 is most highly expressed in macrophage-like cells in the embryonic cortex, suggesting that microglia may be a major source of MMP9. We will incorporate these data in revision.

> *(6) The in vitro evidence indicates that one of the multiple APP interactors, ie Ab40 monomers, is less effective in suppressing the expression of some cytokines by microglia cells mutants for ric8a (TNFa and IL6 but still suppress IL1b) or APP (TNFa, IL6, IL1b, IL23a, IL10) when compared to WT. But there are other interactors for APP. In order to support the claim, it seems crucial to have in vivo data to show that Ab40 monomers are the molecules involved in preventing the breach in the pial basement membrane.*

As addressed in detail above, we have indicated that our conclusion is that an Abeta monomer-activated microglial pathway regulates normal brain development, not that Abeta monomers themselves regulate brain development. We currently do not have evidence that the Abeta monomers play a role in inhibiting microglia in the developing cortex. There are candidate ligands for the pathway in the developing cortex, the functional study of which, however, is a major undertaking and beyond the scope of the current study.

> *(7) In order to claim that this is specific to Ab40 monomers and not oligomers, it is necessary to show that the Ab40 oligomers do not have the same effect in vitro and in vivo. Also, an assay should be done to show that your Ab preparations are pure monomers or oligomers.*

We have tested the effects of Abeta40 oligomers, which induce instead of suppressing microglial cytokine secretion, and will include the data in revision. The protocols we use in preparing the monomers and oligomers are standard protocols employed in the field of Alzheimer's disease research and have been optimized and validated repeatedly over the past several decades.

> *(8) Most of the cytokine secretion assays used microglia cells in culture. Two results draw my attention. Ric8a deletion increases TNFa and IL6 secretion after LPS stimulation in vitro on microglia cells while app deletion decreases their secretion. Then later, papers show that the decrease in IL1b induced by Ab on microglia cells is prevented by APP deletion but not ric8a deletion. Those two pieces of data suggest that ric8a and APP might not be in the same pathway. In addition, the phenotype from app-cxcr3-cre + LPS injection and ric8a-cxcr3-cre + LPS injection are not exactly the same. It could be due to the level of LPS as the author suggests or it might not be. More experiments are needed to prove they are in the same pathway.*

As discussed above, the reviewer misses several major lines of evidence, which strongly support the conclusion that APP and Ric8a act in the same pathway activated by Abeta monomers in microglia (see detailed discussion in point 1). The differential response of app and ric8a mutant microglia likely results from chronic immune stimulation during in vitro culturing, which is known to alter microglia cytokine expression (see detailed discussion in point 9 below on how chronic immune stimulation changes microglial cytokine expression). We have demonstrated this by showing that, without culturing, acutely isolated app and ric8a mutant macrophages both display elevated cytokine secretion (Figure 4). Regarding the distinct regulation of TNF/IL-6 and IL-1b by APP and Ric8a, as discussed above, in several systems, Ric8a-dependent heterotrimeric G proteins have been shown to act downstream of APP and mediate one of the branches of the signaling activated by APP (Milosch et al., Cell Death Dis. 2014 Aug 28;5(8):e1391; Fogel et al,, Cell Rep. 2014 Jun 12;7(5):1560-1576; Ramaker et al., J Neurosci. 2013 Jun 12;33(24):10165-81; Nishimoto et al., Nature. 1993 Mar 4;362(6415):75-9). It is likely this is also the case in microglia and Ric8a-dependent heterotrimeric G proteins may mediate only a subset of the anti-inflammatory signaling activated by APP. As such, this may explain why app, but ric8a, mutation abolishes the inhibitory effects of Abeta monomers on IL-1b. This also suggests that the secretion of TNF/IL-6 and IL-1b must be regulated by different mechanisms. Indeed, it is well established in immunology that the secretion of IL1b, but not that of TNF or IL6, is regulated by inflammasome-dependent mechanisms (see, for example, Proz & Dixit. Nat Rev Immunol. 2016 Jul;16(7):407-20. doi: 10.1038/nri.2016.58).

> *(9) How do the authors reconcile the reduced TNFa and IL6 secretion upon stimulation of app mutant microglia with the model where app is attenuating immune response in vivo? Line 213 says that microglia exhibit attenuated immune response following chronic stimulation but I don't know if 3 hours of LPS in vitro is a chronic stimulation.*

The reviewer has misunderstood. The microglia used in this study have all been cultured in vitro for approximately two weeks before assay. They have thus been under chronic stimulation exposing to dead cells and debris in the culture dish throughout this period. Dependent on the degree of perturbation to inflammation-regulating pathways, such exposures are known to significantly change microglial cytokine expression, sometimes in an opposite direction than expected. For example, under chronic immune stimulation, while the trem2+/- microglia, which are heterozygous mutant for the anti-inflammatory Trem2, show elevated pro-inflammatory cytokine expression as expected, trem2-/- (null) microglia under the same conditions instead not only do not show increases but for some pro-inflammatory cytokines, actually show decreases in expression (Sayed et al.,, Proc Natl Acad Sci U S A. 2018 Oct 2;115(40):10172-10177). As mentioned, in several systems, Ric8a-dependent heterotrimeric G proteins have also been shown to bind to APP and mediate one of the branches of the signaling activated by APP (Milosch et al., Cell Death Dis. 2014 Aug 28;5(8):e1391; Fogel et al,, Cell Rep. 2014 Jun 12;7(5):1560-1576; Ramaker et al., J Neurosci. 2013 Jun 12;33(24):10165-81; Nishimoto et al., Nature. 1993 Mar 4;362(6415):75-9). It is likely that Ric8a-dependent heterotrimeric G proteins also mediate only a subset of the anti-inflammatory signaling activated by APP in microglia. As such, app knockout in microglia may have more severe effects than ric8a knockout on microglial immune activation, similar to the relationship between trem2 null mutation vs heterozygosity discussed above. This likely explains why TNF and IL6 secretion by cultured app mutant microglia is subdued. In contrast, we find that acutely isolated app mutant macrophages show increased cytokine secretion. This is likely more representative of the response of app mutant microglia in the absence of chronic stimulation.

> *(10) Line 119: In their model, the authors suggest that there is a breach in pial basement membrane but that the phenotype is different from the retraction of the radial fibers due*

> *to reduced adhesion. So, could the author discuss to what substrate the radial fibers are attached to, in their model where the pial surface is destroyed?*

Radial glial endfeet normally bind to the basement membrane via cell surface receptors including the integrin and the dystroglycan protein complexes. We observe free radial glial endfeet at the breach sites, apparently without attachment to any basement membrane. However, we cannot exclude the possibility that there may be residual basement components not detected by the methodology employed.

> *(11) The authors should show that the increased cytokine secretion observed in vitro is also happening in vivo in ric8a-emx1-cre compared to WT mice and compared to ric8a-nestin-cre mice. Or when app is deleted in microglia (app-cxcr3-cre) + LPS injection compared to WT mice +LPS.*

Unfortunately, this is not technically feasible since it is impossible to extract the extracellular (secreted) fractions of cytokines from an embryonic brain without causing cell lysis and the release of the intracellular pool. This, however, does not affect our conclusion that the Abeta monomer-regulated microglia pathway plays a key role in regulates normal brain development since its genetic disruption, by different approaches, clearly results in brain malformation.

> *(12) The authors injected inhibitors of Akt or Stat3 in the ric8a-emx1-cre cortex and found that it suppressed neuronal ectopia (Fig 5, Suppl fig 11). Does it suppress immune stimulation from neuronal cells or immune reaction from microglia cells?*

As discussed above, we agree at present the pharmacological approaches we have taken are not able to distinguish these two possibilities. However, no matter which possibility is true, it does not affect our conclusion. Furthermore, we also plan to determine the mechanisms of how ric8a mutation in neural cells induce immune activation in future studies. These results will likely enable us to adopt specific approaches to address this question.

> *(13) Fig 5 and Supplementary fig 12: Please show a tubulin loading control in Fig 5i as you did in suppl fig 12 d (gel zymography). Please provide a gel zymography showing side by side Control, mutant and mutant +DM/S3I treatment. The same request for the MMP9 staining. Please provide statistics for control vs mutant for suppl fig 12c and d.*

For all experiments of the gelatin gel zymography analysis, we quantify protein concentrations in the cortical lysates using the Bio-Rad Bradford assay kit and load the same amounts of proteins per lane. The results across lanes are thus all comparable. These experiments were also performed several years ago before the pandemic and we unfortunately no longer have the samples. We will, however, provide the protein quantification information in revision. The MMP9 staining images for the controls and mutants have also all been taken with the same parameters on the microscope and can be directly compared. The statistics will be provided as suggested.

> *(14) Please provide the name and the source of the MMP9/13 inhibitor used in this study.*

This inhibitor is MMP-9/MMP-13 inhibitor I (CAS 204140-01-2), from Santa Cruz Biotechnology. This information will be included in revision.

> *(15) The results show that deletion of ric8a in microglia and neural cells induced pia membrane breaches but no phenotype is apparent in ric8a deletion in microglia or neural cells alone. Then, the results showed that intraperitoneal injection of LPS induced the phenotype in ric8a-cxcr3-cre mutants. It would be beneficial as a control supporting*

> *the model to show that the insult induced by LPS injection does not induce the phenotype in the ric8a-foxg1-cre mice.*

We agree it may potentially be useful to show that LPS injection does not induce ectopia in ric8a-foxg1-cre mice. Unfortunately, since the ric8a-foxg1-cre mutation shows no phenotype, we are no longer in possession of this line.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *-The information in the abstract and the introduction is only related to app. So, it is very abrupt how authors start the manuscript studying the role of Ric8a, with no information at all about this protein and why the authors want to investigate this role in microglial activation. Later in the manuscript, the authors tried to link Ric8a with app to study the role of app in the inflammatory response and ectopia formation. This link is quite weak as well.*

In the last paragraph of the Introduction, we explain the use of the ric8a mutant and how it leads to discovery of the Abeta monomer-regulated pathway. We will improve the writing in revision to make these points clearer. We will also improve the writing of the potential link of Ric8a to APP by highlighting, especially, the fact that ric8a and app pathway mutants are among a unique group of only three mouse mutants (ric8a, app/aplp1/2, and apbb1/2) that show cortical ectopia exclusively in the lateral cortex, while all other cortical ectopia mutants show the most severe ectopia are at the midline.

> *-In order to validate the mouse model, double immunofluorescence or immunofluorescence+in situ hybridization should be performed to show that microglia express ric8a and that is eliminated in the Emx1-Ric8a mutant mice.*

As mentioned above, we have additional lines of evidence showing that ric8a is deleted from microglia in emx1-cre mutants. This includes data showing induction of the expression of a cre reporter in brain microglia by emx1-cre and loss of ric8a gene expression in microglia cells isolated from emx1-cre mutants. We will include these data in revision.

> *-In Supplemental Fig. 6, the authors claimed that cell proliferation is normal in Ric8a mutant mice without doing any quantification. They also quantified the angle of mitotic division of progenitors in the ventricular zone, but there are no images for the spindle orientation quantification, and no description of how they did it. In addition, this data is contrary to what has already been published in conditional Ric8a mutant mice (Kask et al., 2015). The Vimentin staining should be improved.*

We will provide quantification of cell proliferation in revision. We will also provide details on the quantification on mitotic spindle orientation. We are not sure why the results are different from the other study. We were indeed anticipating deficits in mitotic spindle orientation and spent major efforts in the analysis. However, based on the data, we could not draw the conclusion.

> *-Analysis of the MMP9 expression should be done by western blot and not by immunofluorescence. In fact, the MMP9 expression shown in Figure 5g,h, does not correspond with RNA expression shown in gene expression atlas like genepaint or the allen atlas, doubting the specificity of the antibody. The expression of Mmp9 is quite low or absent in the cortex at E13.5-E14.5, making this protein very unlikely to be responsible for laminin degradation during development.*

We perform gelatin gel zymography on MMP2/9, which shows increased MMP9 activity levels in the mutant cortex. This is similar to Western blot analysis (all lanes are loaded with the same amounts of cortical lysates). The immunofluorescence staining, a different type, of analysis, was designed as a complementary approach. Regarding RNA expression, please also note that MMP9 is a secreted protein and the protein expression pattern is expected to be different from that of RNA. We also have in situ data showing that, while MMP9 mRNA is indeed low, it is strongly expressed in macrophage-like cells most prominently in cortical blood vessels at E12-E13 (we will include these data in revision). We suspect that these cells are microglial lineage cells populating the embryonic cortex at this stage (see, for example, Squarzoni et al., Cell Rep. 2014 Sep 11;8(5):1271-9. doi: 10.1016/j.celrep.2014.07.042.) and may be a major source of cortical MMP9. As for functional contributions, we agree that we cannot rule roles played by other MMPs. However, based on the ectopia suppression data, our results clearly indicate a key functional contribution by MMP9/13.

> *For MMP9 activity, authors should show the whole membrane with a minimum of three control and three mutant individual samples and with the quantification.*
> *-The graphs should be improved, including individual values and titles of the Y axes.*

We will include these data in revision (the quantification of MMP9 activity is provided in Supplemental Figure 12d) and improve the graphs as suggested.

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