

Synaptic Pruning by Microglia Is Necessary for Normal Brain Development

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Microglia are highly motile phagocytic cells that infiltrate and take up residence in the developing brain, where they are thought to provide a surveillance and scavenging function. However, although microglia have been shown to engulf and clear damaged cellular debris after brain insult, it remains less clear what role microglia play in the uninjured brain. Here, we show that microglia actively engulf synaptic material and play a major role in synaptic pruning during postnatal development in mice. These findings link microglia surveillance to synaptic maturation and suggest that deficits in microglia function may contribute to synaptic abnormalities seen in some neurodevelopmental disorders.

Microglia are abundant nonneuronal cells derived from myeloid progenitors that take up residence in the brain of vertebrates during development (1, 2). Their heavily branched morphology, response to pathological tissue changes, and similarity to macrophages have led researchers to propose that they might have a surveillance or maintenance role in brain function (3, 4). Microglia actively move toward the site of damage, including ischemic, excitotoxic, and neurodegenerative insults, and engulf and eliminate neuronal debris after cell death (3, 5–7). However, emerging evidence suggests that microglia might also play a crucial role in monitoring and maintaining synapses in the uninjured brain. Time-lapse imaging has shown that microglia processes are highly motile even in the uninjured brain (8–10) and that they make frequent, but transient, contact with synapses (10). These and other observations have led to the hypothesis that microglia monitor synaptic function and are involved in synapse maturation or elimination (4, 11–15). Moreover, neurons during this period up-regulate the expression of the chemokine fractalkine, Cx3cl1 (16), whose receptor in the central nervous system is exclusively expressed by microglia (17, 18) and is essential for microglia migration (19–21). If, in fact, microglia are involved in scavenging synapses, then this activity is likely to be particularly important during synaptic maturation when synaptic turnover is highest (22). However, until now, no direct evidence existed to support a role of microglia in synaptic pruning during development.

We analyzed whether microglia engulf synapses during the period of synaptic maturation in the mouse. Immunohistochemistry against PSD95, a marker of excitatory postsynaptic density, re-

vealed abundant PSD95-immunoreactive puncta and green fluorescent protein (GFP)-labeled microglial processes in the mouse hippocampus (Fig. 1A). A small number of PSD95 puncta colocalized with GFP (fig. S1A). Colocalization of PSD95 immunoreactivity and GFP was confirmed using stimulated emission depletion (STED) microscopy in which PSD95 puncta could be resolved down to ~80 nanometers in the focal plane (Fig. 1B and fig. S2). Three-dimensional reconstruction demonstrated that many PSD95 puncta that colocalized with GFP were surrounded by GFP-labeled microglial cytoplasm (Fig. 1C),

consistent with the intracellular localization of PSD95. Finally, PSD95-immunoreactive electron-dense material was observed inside both clathrin-coated and nonclathrin-coated vesicles within GFP-positive microglial processes using electron microscopy and double immuno-gold labeling (Fig. 1D and fig. S3). Immunoreactivity against the presynaptic protein SNAP25 was also found within GFP-labeled microglia, suggesting engulfment of both pre- and postsynaptic components (fig. S1B). Together, these data show that in the uninjured brain microglia engulf synaptic material.

Next, we examined synaptic engulfment by microglia in mice lacking the fractalkine receptor (*Cx3cr1*) (18, 23). Similar colocalization of PSD95 immunoreactivity and GFP was seen in *Cx3cr1*^{KO} mice and control littermates (Fig. 1E). However, quantification of total PSD95 puncta revealed a significantly higher density in *Cx3cr1* knockout mice than in control littermates (Fig. 1F). Moreover, dendritic spine densities on CA1 pyramidal neurons were significantly higher in *Cx3cr1* knockout mice than in wild-type (WT) littermates during the second and third postnatal weeks (Fig. 2, A to C), suggesting a transient deficit in synaptic pruning in *Cx3cr1* knockout mice. Single-cell recordings of spontaneous excitatory postsynaptic current (sEPSC) and miniature synaptic activity (mEPSC) in CA1 pyramidal neurons from *Cx3cr1* knockout and WT littermates at postnatal days 13 to 16 (P13 to P16)

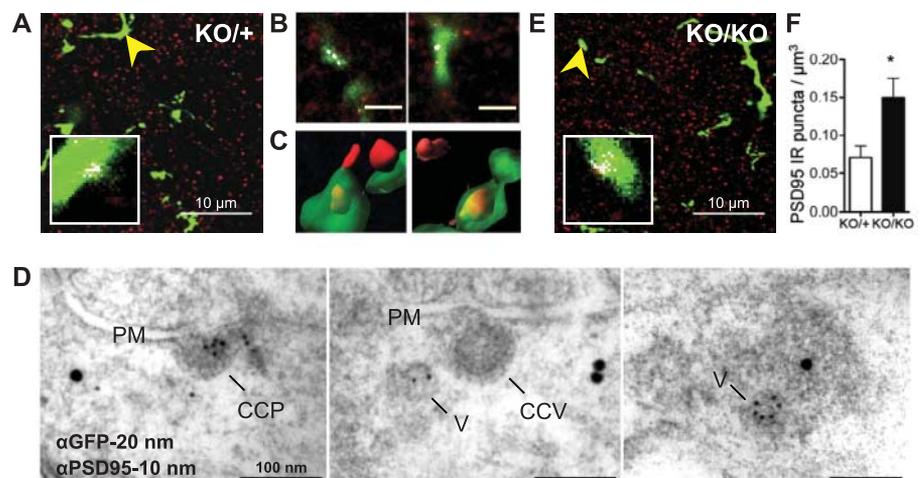


Fig. 1. Engulfment of synaptic material by microglia. (A) Representative confocal z-stack projection of CA1 stratum radiatum taken from the hippocampus of a *Cx3cr1*^{GFP/+} reporter mouse (KO/+) at P15 revealed abundant PSD95-immunoreactive puncta and sparse GFP-positive microglia processes. Colocalization of PSD95 immunoreactivity and GFP fluorescence [yellow arrowhead points to colocalization (white color) shown in inset] was determined in single consecutive confocal planes. (B) Representative STED microscopic single-plane images confirming colocalization of PSD95 puncta and GFP immunoreactivity in stratum radiatum of control mice. Scale bar, 1 μ m. (C) Three-dimensional reconstructions showing PSD95-immunoreactivity completely and partially inside small-diameter microglia processes. (D) Representative electron microscopic images of stratum radiatum of control mice showing PSD95-immunoreactive material (10-nm gold particles) within GFP-positive microglia processes (20-nm gold particles). PM, plasma membrane; CCP, clathrin-coated pit; CCV, clathrin-coated vesicle; V, vesicle. Scale bar, 100 nm. (E) Representative confocal z-stack projection of hippocampal CA1 stratum radiatum of a *Cx3cr1*^{GFP/Cx3cr1}^{KO} reporter knockout mouse (KO/KO) at P15 revealed a similar colocalization of PSD95 immunoreactivity and GFP fluorescence as seen in controls (yellow arrowhead points to colocalization shown in inset). (F) Quantification of absolute density of PSD95 puncta revealed a significant increase in *Cx3cr1* knockout mice compared with littermate controls (KO/+; $n = 3$ mice, sections = 14; KO/KO; $n = 3$ mice, sections = 14). *, $P < 0.05$.

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revealed a decreased frequency of sEPSCs in knockout mice (Fig. 2, D and E). Although sEPSCs reflect both action potential-dependent and spontaneous neurotransmitter release, mEPSCs reveal only spontaneous vesicle release. The sEPSC/mEPSC amplitude ratio increases during development and reflects increased connectivity and redundancy of afferent synaptic inputs (24). These data suggest immature synaptic connectivity in the knockout animals (Fig. 2F). A small, but significant, increase in mEPSC amplitude seen in the knockout was also consistent with immature synapse function (24) (Fig. 2G). Finally, the frequency of mEPSC events in knock-

out mice was significantly increased compared with control littermates (Fig. 2H), confirming an increase in synaptic release sites. Next, we examined synaptic plasticity in Schaffer collateral inputs to CA1. Long-term depression (LTD) was significantly enhanced in slices from knockout mice when compared with control littermates at P13 (Fig. 2I), but not at P40 (Fig. 2J), consistent with a delay in the maturation of synaptic plasticity in this pathway (25). Finally, we measured susceptibility to seizures after administration of the proconvulsant drug pentylenetetrazole (PTZ) in P17 to P18 mice. Susceptibility to PTZ is significantly lower during the early postnatal pe-

riod than in adulthood (26), and administration of PTZ revealed a significant reduction in seizure frequency and duration in knockout mice when compared with WT littermates at P13 (Fig. 2, K to M) but not in adulthood (Fig. 2N), consistent with a delay in brain circuit development at the whole-animal level.

Two different mechanisms could explain how deficient fractalkine signaling in microglia leads to reduced synaptic pruning. On the one hand, soluble fractalkine might act to promote microglia migration into the brain or proliferation during development. In this case, the density of microglia would be reduced in the brain of *Cx3cr1* knockout mice and their capacity for synaptic pruning compromised. On the other hand, tethered or locally released fractalkine might be critical for microglia recognition of synapses before or during engulfment, in which case the density of microglia might be normal but the efficiency of engulfment might be reduced. Consistent with the first hypothesis, we found a significant reduction in microglia density in the brains of *Cx3cr1* knockout mice compared with littermate controls during the first through fourth postnatal weeks (Fig. 3, A to C). This finding suggests that reduced synaptic pruning and circuit maturation in these mice was probably due to a transient reduction in microglia surveillance (Fig. 2A).

It remains possible that synaptic deficits in *Cx3cr1* knockout mice are due to an effect of this chemokine receptor on general brain maturation rather than a specific deficit in microglia-mediated synaptic pruning. However, some data argue against such a general effect. First, the synaptic deficits we observe are not consistent with a simple delay in brain maturation. *Cx3cr1* knockout mice show increased spine density and functional excitatory synapses (Fig. 2H), features that are normally seen later in development (Fig. 2A) (24). At the same time, synaptic connectivity (Fig. 2F), strength (Fig. 2G), plasticity (Fig. 2I), and excitability (Fig. 2, K and M) all point to more immature brain circuitry. This phenotype of exuberant immature synapses is best explained by a deficit in synaptic pruning. Second, the transient decrease in microglia density observed in *Cx3cr1* knockout mice (Fig. 3C) significantly precedes the transient increase in spine density in these animals (Fig. 2A) and suggests that pruning does occur in the knockout mice, but at a later developmental stage. Third, although fractalkine is expressed both by neurons and vascular endothelial cells in the brain (27) and microglia are known to have a role in the formation of cerebral blood vessels (28), the absence of its receptor on microglia does not lead to remodeling of brain vasculature (fig. S4), ruling out a general effect of fractalkine signaling on cerebral blood supply.

What signals guide microglia-dependent pruning? Although our data are consistent with fractalkine signaling being primarily important for controlling the abundance of microglia in the brain (Fig. 3), it remains possible that fractalkine also has a role in local neuron-microglia signal-

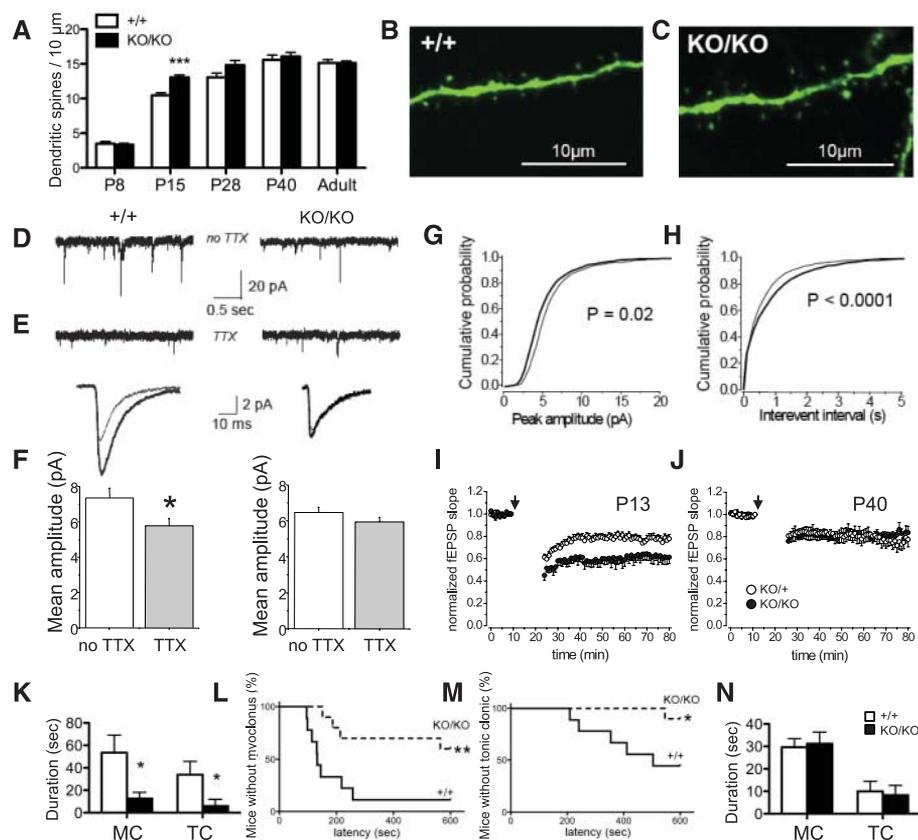


Fig. 2. Increased dendritic spines and immature synapses in *Cx3cr1*^{KO} mice. **(A)** A transient increase in dendritic spine density was observed in *Cx3cr1* knockout (KO/KO) mice when compared with WT (+/+) littermates during the second and third postnatal weeks (WT, segments = 15 to 38; KO, segments = 20 to 46; ***, $P < 0.0001$). **(B and C)** Representative images of secondary apical dendrites of hippocampal CA1 pyramidal neurons from *Cx3cr1* (B) WT or (C) knockout littermates at P15 visualized with the *Thy1::GFP* transgene. **(D and E)** Representative traces of sEPSCs [no tetrodotoxin (TTX), thick line] and mEPSCs (0.5 μ M TTX, thin line) of whole-cell recordings from CA1 pyramidal cells of P13 to P16 WT and *Cx3cr1* knockout littermates. **(F)** In WT mice, but not knockout mice, sEPSC amplitudes were significantly greater than mEPSC amplitudes (WT, $n = 11$; KO, $n = 13$; *, $P < 0.05$). **(G and H)** Cumulative mEPSC (G) amplitude and (H) interevent interval distributions showed a significant increase in amplitude and frequency in *Cx3cr1* knockout mice (thin lines) compared with WT littermates (thick lines) (WT, $n = 20$; KO, $n = 26$). **(I and J)** LTD was induced in acute hippocampal slices from P13 and adult *Cx3cr1*^{GFP/+} (KO/+) and *Cx3cr1*^{GFP/GFP} (KO/KO) mice, using a standard low-frequency stimulation (LFS) protocol (one 15-min train; 1 Hz; 900 pulses per train, as indicated by the arrows). **(I)** LTD was significantly enhanced in the knockout ($42 \pm 6\%$, $n = 13$ slices, 8 mice; closed circles) compared with control ($22 \pm 4\%$, $n = 15$ slices, 6 mice; open circles; $P = 0.006$) mice at P13, but not **(J)** at P40 (control: $17 \pm 6\%$, $n = 7$ slices, 4 mice, open circles; KO: $16 \pm 5\%$, $n = 9$ slices, 4 mice, closed circles; $P = 0.91$). **(K and N)** A significant reduction in the (K) duration of and (L and M) latency to myoclonic (MC) and tonic-clonic (TC) seizure response after administration of PTZ was found in knockout mice when compared with WT littermates (WT, $n = 9$; KO, $n = 10$) at P17 and P18 but not in **(N)** adulthood (WT, $n = 14$; KO, $n = 12$, *, $P < 0.05$; **, $P < 0.005$).

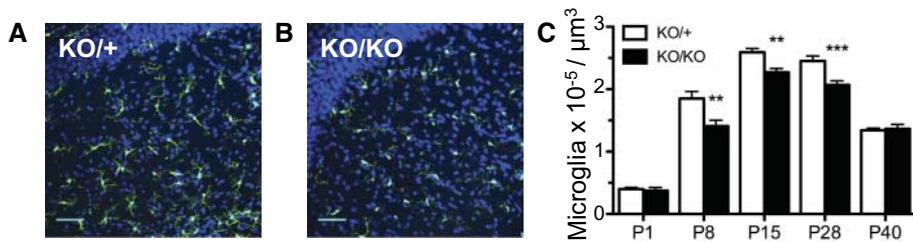


Fig. 3. Transient reduction in microglia in *Cx3cr1*^{KO} mice. Representative confocal images of CA1 stratum radiatum from the hippocampus of (A) a *Cx3cr1*^{GFP/+} reporter mouse (KO/+) and (B) a *Cx3cr1*^{GFP}/*Cx3cr1*^{KO} reporter knockout mouse (KO/KO) at P8 revealed extensive arborization of microglia across the brain parenchyma. (C) Quantification of microglia nuclei (4',6'-diamidino-2-phenylindole⁺, GFP⁺) revealed a transient decrease in microglia density in *Cx3cr1* knockout mice (sections = 5 to 28) at P8, P15, and P28 compared with control littermates (sections = 4 to 36; **, *P* < 0.005).

ing necessary for synaptic pruning. In particular, tethered and released fractalkine may serve different roles in neuron-microglia communication. Other candidates for such signaling include the complement cascade components C1q and C3 that were shown to be necessary for pruning of retinohalamic axons during development (11). C1q knockout mice show reduced pruning of excitatory cortical synapses (29), a phenotype reminiscent of that of *Cx3cr1* knockout mice.

In conclusion, we show that microglia engulf and eliminate synapses during development. In mice lacking *Cx3cr1*, a chemokine receptor expressed by microglia in the brain, microglia numbers were transiently reduced in the developing brain and synaptic pruning was delayed. Deficient synaptic pruning resulted in an excess of dendritic spines and immature synapses and was associated with a persistence of electrophysiological and pharmacological hallmarks of immature brain circuitry. Genetic variation in *Cx3cr1*, along with environmental pathogens that affect microglia function, may contribute to susceptibility to developmental disorders associated with al-

tered synapse number. Understanding microglia-mediated synaptic pruning is likely to lead to a better understanding of synaptic homeostasis and an appreciation of interactions between the brain and immune system.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1202529/DC1
Materials and Methods
Figs. S1 to S4
References

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Light-Induced Structural and Functional Plasticity in *Drosophila* Larval Visual System

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How to build and maintain a reliable yet flexible circuit is a fundamental question in neurobiology. The nervous system has the capacity for undergoing modifications to adapt to the changing environment while maintaining its stability through compensatory mechanisms, such as synaptic homeostasis. Here, we describe our findings in the *Drosophila* larval visual system, where the variation of sensory inputs induced substantial structural plasticity in dendritic arbors of the postsynaptic neuron and concomitant changes to its physiological output. Furthermore, our genetic analyses have identified the cyclic adenosine monophosphate (cAMP) pathway and a previously uncharacterized cell surface molecule as critical components in regulating experience-dependent modification of the postsynaptic dendrite morphology in *Drosophila*.

Proper functions of neuronal circuits rely on their fidelity, as well as plasticity, in responding to experience or changing environment, including the Hebbian form of plasticity, such as long-term potentiation, and the

homeostatic plasticity important for stabilizing the circuit (1, 2). Activity-dependent modification of neuronal circuits helps to establish and refine the nervous system and provides the cellular correlate for cognitive functions, such as learn-

ing and memory (3, 4). Multiple studies have examined synaptic strength regulation by neuronal activity, whereas to what extent and how the dendritic morphology may be modified by neuronal activity remain open questions (1, 5).

The model organism *Drosophila melanogaster* has facilitated genetic studies of nervous system development and remodeling (6). Notwithstanding the relatively stereotyped circuitry, flies exhibit experience-induced alterations in neuronal structures and behaviors such as learning and memory (7–10). In our study of experience-dependent modifications of the *Drosophila* larval CNS, we found that different light exposures dramatically altered dendritic arbors of ventral lateral neurons [LN(v)s], which are postsynaptic to the photoreceptors. Unlike the visual activity-induced

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