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## Microglia enable mature perineuronal nets disassembly upon anesthetic ketamine exposure or 60-Hz light entrainment in the healthy brain

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### Summary

Perineuronal nets (PNN) are a component of the extracellular matrix that preferentially coat parvalbumin-positive interneurons and constrain critical period plasticity in the adult cerebral cortex. Current strategies to remove PNN are long-lasting, invasive, and trigger neuropsychiatric symptoms. Here, we apply repeated anesthetic ketamine as a method with minimal behavior impact. We find that this paradigm strongly reduces PNN-coating in the healthy adult brain and promotes juvenile-like plasticity. Microglia are critically involved in PNN loss as they engage with parvalbumin-positive neurons in their defined cortical layer. We identify external 60-Hz light flickering entrainment to recapitulate microglia-mediated PNN removal. Importantly, 40-Hz frequency, which is known to remove amyloid plaques, does not induce PNN-loss suggesting microglia might functionally tune to distinct brain frequencies. Thus, our 60-Hz light entrainment strategy provides an alternative form of PNN intervention in the healthy adult brain.

### Introduction

Sensory processing is established through experience during the critical period of brain development (Hensch, 2005). The end of this phase coincides with increased inhibition from fast-spiking parvalbumin-positive (Pvalb<sup>+</sup>) neurons and the formation of the perineuronal net (PNN), a defined extracellular matrix (ECM) compartment consisting of chondroitin sulfate

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Author Contributions

Conceptualization, A.V., S.S.; Investigation, validation, and methodology, A.V., M.M., G.C., B.N., F.S.U., S.S.; Formal Analysis and software, R.S., R.J.A.C.; Writing – Original Draft: A.V., H.D.J.-C., M.F.B., S.S.; Writing – Review & Editing, A.V., M.M., B.N., H.D.J.-C., S.S.; Supervision and funding acquisition: S.S.; For OD plasticity: Conceptualization, A.V., H.D.J.-C., M.F.B., and S.S.; Investigation and formal analysis, H.D.J.-C. and F.R.-A.; Supervision and funding acquisition: M.F.B.

Declaration of Interests

The authors declare no competing interests. A.V. and S.S. disclose an international patent application (PCT/EP2020/079365).

Inclusion and diversity

We worked to ensure sex balance in the selection of non-human subjects.

proteoglycans and various ECM and cell-adhesion molecules (Ueno et al., 2017a, 2019). Approximately 80% of Pvalb<sup>+</sup> neurons in the primary somatosensory cortex are coated with PNN (Härtig et al., 1992; Ueno et al., 2019; Wen et al., 2018). PNN sterically restricts synapse formation and receptor mobility and thus prevents adaptation of juvenile plasticity in adulthood (Celio and Blumcke, 1994; Frischknecht and Gundelfinger, 2012; Kuhlman et al., 2013; Lensjø et al., 2017; Pizzorusso et al., 2002). Disruptive environmental interferences during this sensitive period can cause long-lasting circuit impairment resulting in neurological disorders like loss of visual acuity (Fong et al., 2016). Enzymatic digestion of the ECM with intracerebral application of chondroitinase-ABC can reinstate juvenile-like plasticity in the adult cortex, but the enzyme uncontrollably degrades the ECM for several weeks and is not suitable for therapeutic interventions (Hensch, 2005; Pizzorusso et al., 2002). An alternative approach surfaced in the form of the rapidly-acting dissociative drug ketamine that preferentially acts on NMDA receptors of cortical inhibitory neurons (Brown et al., 2011; Kaplan et al., 2016; Li and Vlisides, 2016; Picard et al., 2019). Recent studies in rats suggested that repeated exposure of low dosage of ketamine mildly reduces PNN-coverage (Kaushik et al., 2020; Matuszko et al., 2017). At the same time, this dosage regime provokes schizophrenia-like phenotypes limiting its therapeutic use. We decided to circumvent this constraint by applying ketamine at an anesthetic dosage. Ketamine complies to the principles of general anesthesia, which requires a drug-inducible fully-reversible change of consciousness and cognition (Brown et al., 2011). Mice exposed to repeated anesthetic ketamine showed only minimal effects in their well-being and behavior (Hohlbaum et al., 2018). Furthermore, the application of ketamine at anesthetic dosage achieves the maximum pharmacological response with a consistent phenotypic readout across animals avoiding confounding effects of ketamine dosage, frequency and time interval. This allows us to address the underlying cellular mechanism involved in PNN reduction, which provides the fundamentals to find alternative non-invasive strategies to modify PNN in the healthy adult brain.

Here, we show that repeated ketamine exposure at anesthetic dosage results in massive PNN loss in various cortical brain regions and is sufficient to promote juvenile-like plasticity. We identified microglia, the brain-resident immune cells (Kettenmann et al., 2011), to be critically involved in this process as they engaged with Pvalb<sup>+</sup> neurons in a layer-specific manner and subsequently remodeled the PNN-coating. Since ketamine exposure influences the gamma frequency (Ahnaou et al., 2017; Castro-Zaballa et al., 2019; Pinault, 2008) and microglia are capable of removing extracellular amyloid plaque depositions upon stimulation in the gamma range (Iaccarino et al., 2016), we entrained the visual cortex with either low or high light flickering frequencies. With 60-Hz, we recapitulated the same microglia action towards PNN as under ketamine. Importantly, this only occurred at 60 Hz and not at 8 or 40 Hz suggesting an alternative, non-invasive strategy to reduce PNN coverage in the healthy adult cortex.

## Results

### Repeated ketamine exposure results in PNN loss.

To establish the consequences of ketamine exposure on PNNs, we subjected adult C57BL/6J animals to single or repeated ketamine anesthesia at 3-day intervals and collected the brains four hours after 1, 2, 3, or 6 injections (Figure 1A). Since ketamine at this dosage induces muscle rigidity, we combined it with xylazine and the phenothiazine tranquilizer acepromazine (KXA). The PNN was stained with *Wisteria floribunda* agglutinin (WFA), which selectively binds to the N-acetyl-galactosamine group of the chondroitin sulfate proteoglycans of the PNN (Härtig et al., 1992), and has the highest density in layer 4 in the mouse somatosensory cortex (Ueno et al., 2019; Wen et al., 2018). We confirmed that WFA is enriched in various brain regions (Figure S1A) and preferentially surrounds cortical parvalbumin-positive (Pvalb<sup>+</sup>) neurons in layer 4 (Figure S1B). Strikingly with increased ketamine repetitions, coronal overview images showed substantial PNN loss in the primary somatosensory cortex (S1, Figures 1B and S1C). To quantify the PNN loss, we took higher magnification tile images covering layer 3 to 5 and counted the number of WFA-stained cells in the S1. We confirmed the gradual decrease in the number of PNN-coated cells as predicted from the overview images (Figures 1C and S1D). This effect was not observed with the same number of repeated saline (Figures S2A and S2B) or xylazine-acepromazine (XA, Figures 1D and 1E, S1E and S1F) injections indicating that the effect is due to ketamine. Both sexes were similarly affected by ketamine (Figures 1B–E, S1C–S1F and S2C), excluding a long-term sex-specific difference as previously proposed (Carrier and Kabbaj, 2013; Thelen et al., 2016).

Excitotoxic cell death induced by ketamine could be an alternative cause for the PNN loss (Hauser et al., 2017; Schobel et al., 2013). Thus, we stained for caspase-3 and counted the number of Pvalb<sup>+</sup> neurons four hours after the last injection of the 6× KXA treatment in S1. We did not observe caspase-3-positive cells (Figure S2D and S2E) nor any changes in Pvalb<sup>+</sup> neuron density (Figure S2F). To further confirm PNN remodeling, we performed immunostaining for hyaluronic acid binding protein (HABP), a major ECM component and part of the PNN (Ueno et al., 2018). HABP colocalizes with WFA-coated cells in the deeper cortical layers of S1 (Ueno et al., 2018). After 3x KXA, HABP staining was evenly distributed throughout cortical layers 3–5 compared to 3x saline and the number of HABP-coated cells was reduced to levels comparable to WFA-coated cells, whereas the overall relative mean fluorescence intensity remained unaltered (Figures S2G–S2I). Taken together, our data demonstrate that repeated ketamine exposure at anesthetic dosage reduces the number of PNN-coated cells in the cortex.

### Ketamine-mediated PNN loss promotes juvenile-like plasticity in adult mice.

Next, we were interested whether PNN removal would reinstate juvenile-like plasticity in the adult brain using the well-established ocular dominance (OD) plasticity paradigm in the primary visual cortex (V1) (Frenkel and Bear, 2004; Sawtell et al., 2003; Ye and Miao, 2013). Similar to S1, we found that 3× KXA results in PNN loss in V1 (Figures S3A and S3B) and occurs independently of XA (Figure S3C), mouse strain background (Figures S3D and S3E), or sex (Figure S3F).

In general, P40 marks the cut-off for OD shifts after short term (< 5 days) monocular deprivation (MD) (Gordon and Stryker, 1996; Sato and Stryker, 2008). Since PNN was still significantly reduced 3 days after recovery from 3× KXA (Figures S3A and S3B), we tested whether three days of MD initiated after the last KX dose was sufficient to induce OD plasticity in adult animals. Thus, we measured visually-evoked potentials (VEPs) before and after MD in binocular layer 4 of V1 (Figure 1F). In normally reared mice, the deprived contralateral eye-evoked response was 2–3 times greater than the non-deprived, ipsilateral, eye-evoked response, yielding a contralateral (C) to ipsilateral (I) eye ratio of ~2.5. In saline treated animals, neither the contralateral-to-ipsilateral (C/I) VEP ratio nor the absolute amplitude of VEPs evoked by the two eyes was affected by 3-day MD, as expected in adult mice (Figures 1G and 1H). Although ketamine treatment did not significantly affect the C/I ratio or VEP magnitudes at baseline, it did render the visual cortex vulnerable to brief MD. Following 3-day MD, the C/I ratio was shifted significantly to favor the non-deprived ipsilateral eye (Figure 1G), which was accounted for exclusively by a decrease in the amplitude of responses through the deprived contralateral eye (Figure 1I). Quantitatively and qualitatively, this OD shift is comparable to what is observed in young mice at ~P30 (Frenkel and Bear, 2004). In addition, we confirmed the OD shift with a significantly reduced C/I VEP ratio that persisted for at least a week after MD (Figure 1G). Our results show that PNN loss via repeated ketamine exposure promotes juvenile-like OD plasticity in adult animals.

### Microglia interact with Pvalb<sup>+</sup> neurons and remodel PNN.

Fast-spiking Pvalb<sup>+</sup> neurons as well as PNN-coating are a critical component for establishing OD plasticity during the visual critical period (Hensch, 2005; Ueno et al., 2017b, 2019; Wen et al., 2018). Since a recent study indicated a role for microglia in this context (Sipe et al., 2016), we investigated whether an interaction between Pvalb<sup>+</sup> neurons and microglia exist. The immunostaining for parvalbumin and the microglial marker Iba1 did not result in an obvious phenotype under 3× saline (Figure 2A). In contrast after 3× KXA, microglia showed a close interaction with Pvalb<sup>+</sup> neurons (Figure 2B). Next, we investigated the expression of matrix metalloproteinase 9 (MMP-9), which has been shown to play a critical role in juvenile and adult OD-plasticity and is released in a neuronal activity-dependent manner (Dziembowska et al., 2012; Fawcett et al., 2019; Kaczmarek, 2016; Murase et al., 2017; Pielecka-Fortuna et al., 2015; Reinhard et al., 2015; Tewari et al., 2018). Pvalb<sup>+</sup> neurons as well as microglia localized in cortical layers 3–5 significantly increased MMP-9 upon 3× KXA (Figures S4A and S4B, respectively), whereas microglia in cortical layer 1 and astrocytes lacked this effect (Figures S4C and S4D, respectively). We also quantified the percentage of Pvalb<sup>+</sup> neurons and microglia containing MMP-9 and found in both cases a three-fold increase after 3× KXA (Figure S4A and S4B).

To support microglial interaction with WFA-coated structures, we performed *ex-vivo* live imaging from coronal brain slices in the S1 of Cx3cr1-CreERT2<sup>het</sup>/Ai9<sup>het</sup> animals prepared two hours after the second KXA or saline injection and stained for WFA-FITC. We chose to image after 2× KXA because the number of PNN-coated cells is significantly decreased yet enough WFA-stained cells remain to allow reliable detection (Figure 1C). Under 2x KXA, microglia were in constant contact with the soma of WFA-labeled cells and accumulated

WFA-fragments to WFA-containing compartments within their cell soma over the course of 20 minutes (Figure 2C and 2D). Compared to saline-treated animals, the WFA accumulation was significantly higher under KXA. Consequently, the WFA-staining became unevenly distributed around the neuronal cell body. In contrast, the WFA-coated structures remained condensed after saline treatment, and the microglia were more ramified and acted in a surveying mode. Next, we analyzed the expression of the endosomal-lysosomal marker CD68, which increases when microglia transition from a surveillant to a reactive state (Bauer et al., 1994). In control saline-injected animals, CD68 levels were unaltered (Figure S5A) while KXA exposure led to elevated CD68 levels that stayed upregulated (Figures 2E and S5B). CD68 gradually started to decline only three days after the last injection of 3× KXA (Figures 2F, S5C and S5D). To substantiate whether CD68 contains WFA-signal, we quantified the volume of WFA-signal inside microglial CD68. The WFA-volume increased with the number of KXA exposure reaching the peak at 3× KXA (Figures 2G and 2H, S6A and S6B). Interestingly, the level of WFA-containing CD68 dropped precipitously three days after the last KXA injection (Figures S6C and S6D) in both S1 and V1 suggesting that without further KXA stimulation, microglia gradually revert to the surveillant modus permitting the recovery of the PNN-coating (Figures S3A and S3B).

Finally, to confirm that microglia are a critical component for PNN removal, we depleted microglia by feeding mice the colony-stimulating factor 1 receptor (CSF1R) antagonist PLX5622 for 1.5 weeks before and during 3× KXA treatment (Figure 3A). In contrast to PLX3397, PLX5622 has faster kinetics in depleting microglia and higher specificity to the CSF1R, with a potential caveat that it affects long-lived tissue macrophages in the body (Liu et al., 2019). The PLX5622 treatment caused an approximately 80% reduction in the microglial population (Figures S7A and S7B), which was sufficient to prevent a decrease in the number of PNN-coated cells after 3× KXA treatment (Figures 3B and 3C). The WFA staining maintained a condensed network-like structure and did not dissolve in fragments as noticed under repeated KXA (Figures 2H and S6A). Overall, this data strongly supports that microglia presence is necessary for PNN removal upon ketamine exposure and that microglia are actively involved.

### **Microglia-mediated PNN remodeling is inducible with 60 Hz light stimulation.**

*In vivo* electrophysiological recording of various species have found that ketamine alters brain oscillatory dynamics without inducing epileptic seizure bursts (Ahnaou et al., 2017; Castro-Zaballa et al., 2019; Pinault, 2008). Pvalb<sup>+</sup> neurons contribute to cortical gamma oscillation (Cardin et al., 2009; Sohal et al., 2009). Since we identified enhanced interaction of microglia with Pvalb<sup>+</sup> neurons after KXA treatment (Figure 2B), we sought to determine whether the effect of microglia-mediated PNN remodeling is triggered by neuronal activity.

Microglia are known to sense changes in ATP/ADP levels from action potentials or injuries (Davalos et al., 2005; Fields, 2011; Nimmerjahn et al., 2005) via various purinergic receptors like P2Y12, which upon activation induces a chemotactic response (Haynes et al., 2006; Sipe et al., 2016). Therefore, we determined whether impaired P2Y12-mediated detection of neuronal activity prevents microglia from removing PNN. After confirming that P2Y12 surface expression remained unaltered upon ketamine exposure (Figure S7C), we

injected clopidogrel to block one of the ATP/ADP detection mechanisms before KXA treatment (Figure 3D). Systemic application of clopidogrel has been shown to disrupt P2Y<sub>12</sub> signaling in resident microglia, and the active metabolite has blood-brain barrier permeability (Herbert et al., 1993; Sipe et al., 2016). Indeed, we found that this treatment regime prevented ketamine-mediated loss of PNN-coated cells (Figures 3E and 3F). Furthermore, the overall WFA-stained structure remained intact (Figure 3G) suggesting that changes in neuronal activity could be one of the driving forces of the microglial response.

Microglia respond to low gamma oscillation and can be stimulated with external light entrainment to remove extracellular amyloid plaque dispositions (Bolmont et al., 2008; Iaccarino et al., 2016). Based on ketamine's impact in the gamma oscillations ranging from 25- to 100-Hz (Ahnaou et al., 2017; Castro-Zaballa et al., 2019; Pinault, 2008), we hypothesized that light entrainment at this frequency range might trigger microglia-mediated PNN remodeling. Thus, we selected 40-Hz and 60-Hz as representatives for the lower or higher gamma frequency, respectively. As controls, we applied constant bright light for the same amount of time or theta (8 Hz) flickering frequency (Figure 4A). The power spectrum of the local field potentials across the V1 cortical region verified that our selected light entrainment frequency reaches the V1 (Figure S7D) and confirmed previous studies (Fries et al., 2007; Gray et al., 1989; Herrmann, 2001; Rager, 1998). Strikingly, we found that selective 60 Hz flickering reduced the number of PNN-coated cells in the V1, whereas the other frequencies or constant light did not cause a reduction (Figures 4B and S7E). This effect was not observed when microglia were depleted 1.5 weeks before the 60 Hz light flickering stimulation (Figures 4C and S7F). Similar to repeated ketamine exposure, the distance between Pvalb<sup>+</sup> neurons and microglia only decreased upon 60 Hz light flickering (Figures 4D and S8A), and MMP-9 levels increased in both Pvalb<sup>+</sup> neurons and microglia (Figures 4E, S8B and S8C). Importantly, although microglia showed a significant increase in CD68 volume after 40 and 60 Hz gamma light-flickering (Figure 4F), we only observed an enrichment of WFA-fragments within microglia upon 60 Hz stimulation (Figures 4G and 4H). On several occasions, microglia cell bodies sat on top of WFA-coated cell body-like structures and enabled PNN dismantling (Figures 4H and 4I). Also, we observed increased CD68 enrichment at the perforation site, which was not found at the other frequencies (Figures 4H and S9). This data provides an alternative, non-invasive strategy to remodel PNN under defined externally-driven brain activity entrainment.

## Discussion

### Ketamine induces microglia-mediated PNN remodeling

Throughout development, microglia have been shown to impact synapse formation and circuit connectivity (Kettenmann et al., 2013; Paolicelli et al., 2011; Schafer et al., 2012; Tremblay et al., 2010). During the visual critical period, monocular deprivation results in reactive microglia which interact with synaptic elements and impact ocular dominance plasticity (Sipe et al., 2016). This effect is usually only observed before the PNN formation reaches a plateau at around P42 (Ye and Miao, 2013). From then onwards, multiple studies have demonstrated that short-term monocular deprivation can only cause a shift in ocular dominance plasticity upon enzymatic digestion of the ECM (Frenkel and Bear, 2004; Lensjø

et al., 2017; Pizzorusso et al., 2002). Therefore, we were surprised that repeated anesthetic ketamine exposure alone resulted in a reduced coverage of PNN-coated cells (Figures 1B and S1C) associated with ocular dominance plasticity after brief monocular deprivation, resembling what is observed during the juvenile period (Figures 1F–1I). This shift was dependent on visual deprivation as the VEP recordings after 3× KXA and before monocular deprivation did not impact the contra-to-ipsilateral ratio.

Furthermore, our results provide extensive evidence that ketamine exposure initiates microglia to remodel PNN. Recent studies suggested that microglia are capable of removing PNN in various neurological disorders, and altering ECM in the adult hippocampus (Crapser et al., 2020; Nguyen et al., 2020). A similar effect was described for repeated exposure to low ketamine dosage, which is a common model to induce schizophrenia-like phenotypes in rats (Kaushik et al., 2020; Matuszko et al., 2017). Here, we decided to take advantage of ketamine's compliance to the principles of general anesthesia and that repeated exposure only caused mild behavior changes (Hohlbaum et al., 2018). Moreover, the systematically delivered ketamine at an anesthetic dosage achieves the maximum pharmacological response comparable across animals without running into conflicts such as dosage or injection frequency. Our ketamine dosage was 100 mg/Kg, which can only be provided in combination with xylazine. Since we excluded xylazine-acepromazine as an effector (Figures 1D and S1E), our results support a ketamine dosage-dependent response in PNN reduction, extending the repertoire of known dose-dependent ketamine actions (Lavender et al., 2020) with the caveat that anesthetic ketamine dosage has a plethora of effects, which is not straightforward to dissect.

Commonly, the PNN is composed of multiple extracellular molecules such as chondroitin sulfate proteoglycans (CSPGs), hyaluronic acid, and linker proteins. To label the PNN, we used the well-defined marker WFA, which selectively binds to the terminal ends of the N-acetyl-galactosamine groups of CSPGs (Fawcett et al., 2019; Härtig et al., 1992; Ueno et al., 2017a, 2019; Wen et al., 2018). We confirmed the high specificity of this staining in cortical layers 3–5 by demonstrating the selective WFA-coating around Pvalb<sup>+</sup> neurons (Figures S1A and S1B). Moreover, we identified that the distance of microglia to Pvalb<sup>+</sup> neurons was reduced under ketamine exposure (Figure 2B), and that microglia actively interacted with and contained WFA-coated structures (Figures 2C and D). A common misconception is that 'reactive' microglia must change into an amoeboid morphology containing multiple phagocytic cups (Wolf et al., 2017). However, this strong reaction is usually only observed in injury and disease environments, where substantial cell death occurs. We did not observe this phenotype with repeated ketamine exposure (Figure S2D). Instead, we found with our *ex-vivo* live imaging that microglia frequently screen the WFA-labeled structures, even in the control conditions, suggesting a constant exchange between microglia and PNN-coated neurons. One potential modulator of this interaction could be MMP-9 as it has been suggested to be involved in PNN degradation (Kelly et al., 2015; Murase et al., 2017). To this end, we observed increased MMP-9 level in both Pvalb<sup>+</sup> neurons and microglia upon ketamine exposure (Figure S4). However, the role of MMP-9 is not fully understood as it can directly target the CSPG core protein (Hadler-Olsen et al., 2011; Malla et al., 2008; Pielecka-Fortuna et al., 2015; Spolidoro et al., 2012; Tewari et al., 2018; De Vivo et al., 2013; Wen et al., 2018; Winberg et al., 2003) or indirectly, it can act as a signaling molecule

to modulate neurotrophins, other ECM-regulatory components, cell adhesion molecules, or synaptic receptors (Beroun et al., 2019; Dityatev et al., 2010; Dziembowska et al., 2012; Kaczmarek, 2016). Besides MMP-9, also alternative ECM peptidases could also be involved in the PNN loss. Nevertheless, microglia's action under ketamine led us to reveal that they are capable of remodeling the PNN upon externally introduced neuronal activity entrainment. This provided a foundation to identify alternative strategies to alter PNN, since repeated ketamine exposure can lead to adverse side effects including schizophrenia-like phenotypes and response to general anesthesia (Brown et al., 2011).

### **Gamma entrainment at 60-Hz stimulates microglia**

In addition to PNN preventing receptor mobility and synapse formation, the PNN composition fosters a negatively charged local environment supporting the fast-spiking properties of cortical Pvalb<sup>+</sup> neurons (Tewari et al., 2018) that modulate gamma brain network activity (Cardin et al., 2009; Sohal et al., 2009). Ketamine application transiently alters brain dynamics mainly in the 30–80 Hz gamma frequency range (Ahnaou et al., 2017; Castro-Zaballa et al., 2019; Pinault, 2008) suggesting that neuronal activity might trigger microglia to remodel PNN. Loss of the 40-Hz gamma frequency in a model of Alzheimer's disease has been indicated to cause extracellular amyloid-plaque aggregations. This study entrained the cortical circuits with externally applied light-flickering at 40-Hz and observed microglia phagocytosing amyloid plaques, reducing one of the hallmarks of the disease (Iaccarino et al., 2016). Multiple studies have shown that external light entrainment reaches and drives oscillation in the visual cortex (Fries et al., 2001; Gray et al., 1989; Herrmann, 2001; Rager, 1998), which we confirmed (Figure S7D). We also prove that microglia reacted to 40-Hz entrainment with increased CD68 expression (Figure 4F). However, only with 60-Hz entrainment did we replicate the ketamine-mediated effects, including WFA-staining inside microglial CD68 (Figure 4G), MMP-9 upregulation in both Pvalb<sup>+</sup> neurons and microglia (Figure 4E), and an overall reduced number of PNN-coated cells (Figure 4B). If we depleted microglia before the 60-Hz stimulation, the number of PNN-coated cells did not change (Figure 4C) suggesting that microglia show a selective response towards PNN in the healthy, adult cortex.

In summary, we demonstrate that repeated ketamine exposure at anesthetic dosage reduces PNN levels and promotes juvenile-like plasticity. This strong, microglia-dependent effect provided the fundamentals to identify and confirm 60-Hz light entrainment as an alternative, non-invasive strategy to modify PNN levels without potential side-effects of repetitive ketamine exposure.

### **Limitations of Study**

Microglia prove to be a critical cellular component for PNN loss upon repeated stimulation, however the amount of WFA material inside microglia does not directly reflect the substantial PNN loss. We suspect that MMP-9 mediated enzymatic degradation of the PNN prior to engulfment was responsible for this discrepancy, yet the molecular mechanism for MMP-9 involvement in either Pvalb<sup>+</sup> neuron-microglial communication and/or PNN disassembly remains limited. Further studies are required to determine cell type and cortical layer-specific MMP-9 expression patterns, and whether MMP-9 is released and



extracellularly active. Additional mechanistic insights could be obtained through a screen for alternative endopeptidases and ECM components. The latter would overcome one limitation of WFA, which also labels disaggregated CSPG in the ECM. Despite the orthogonal projections to confirm WFA inside microglial CD68, we cannot fully exclude that additional ECM components remain intact and are omitted from microglial uptake. Lastly, the anesthetic dosage of ketamine might limit mechanistic insights due to many brain region-specific effects that are likely not exclusive to Pvalb<sup>+</sup> neurons. Then again, when we entrained the visual cortex with 60-Hz light flickering, we were able to reproduce the ketamine-induced observation suggesting that alteration in neuronal activity is a driving force, which is a focus of ongoing investigation.

## STAR Methods text

### RESOURCE AVAILABILITY

**Lead Contact**—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sandra Siegert (sandra.siegert@ist.ac.at).

**Materials Availability**—The study did not generate new unique reagents.

**Data and Code Availability**—This study did not generate any unique datasets or code.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

**Animals**—If not otherwise indicated, adult mice (8–12 weeks) of both sexes were used. C57BL/6J (Cat#000664), C57BL/6N (Cat#005304), B6.129P2(C)-Cx3cr1tm2.1(cre/ERT2)Jung/J (Cat#020940, named here: Cx3Cr1<sup>CreERT2</sup>) and B6;129S6-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J (Cat#007909, named here: Ai9) were purchased from The Jackson Laboratories. Cx3Cr1<sup>CreERT2</sup> were heterozygous. All mice were housed in the IST Austria Preclinical Facility with a 12 hour light-dark cycle, food and water provided *ad libitum*. All animal procedures are approved by the Bundesministerium für Wissenschaft, Forschung und Wirtschaft (bmfwf) Tierversuchsgesetz 2012 (TVG 2012), BGBl. I Nr. 114/2012, idF BGBl. I Nr. 31/2018 under the numbers 66.018/0005-WF/V/3b/2016, 66.018/0010-WF/V/3b/2017, 66.018/0025-WF/V/3b/2017, 66.018/0001\_V/3b/2019, 2020–0.272.234.

Visually-evoked potential recordings were done in the lab of M.F.B. at MIT with adult C57BL/6N<sup>Cr1</sup> animals of both sexes, which were purchased from Charles River Laboratories (Cat#027) and maintained at the MIT animal facility. Animals were housed with a 12 hour light-dark cycle, food and water provided *ad libitum* and in groups of 3 or more. We used 34 animals from 10 litters for the final data set. All procedures were approved by the MIT Committee on Animal Care (CAC) and adhere to the guideline of the National Institute of Health (NIH).

### METHOD DETAILS

**Drug application**—If not otherwise indicated, C57BL/6J mice were given intraperitoneal (*i.p.*) injections of drug or saline in the morning. Deep anesthesia was confirmed based on

the following parameters: 1. Absence of the toe pinch reflex around 10 minutes after induction. 2. Decrease in the respiratory frequency. 3. No responses to noxious stimuli. 4. Flaccid paralysis. 5. Absence of whisker movement (Tsukamoto et al., 2015). To prevent corneal dehydration, eye ointment (Oleo Vital) was applied. During the procedure and recovery phase, animals were kept at 37°C.

**Ketamine-xylazine-acepromazine (KXA):** Ketamine (100 mg/kg, MSD Animal Health, Cat #A137A01), xylazine (10 mg/kg, AniMedica, Cat#7630517) and acepromazine (3 mg/kg, VANA GmbH, Cat#18F211) were solubilized in physiological saline solution containing 0.9% (w/v) NaCl (Fresenius Kabi Austria, Cat#19MIA700). Solution was always freshly prepared to avoid pH fluctuations.

**Ketamine-xylazine (KX):** For OD plasticity experiments (performed in the lab of M.F.B. at MIT), ketamine (Ketset, 100 mg/kg, Zoetis, Cat#1051) and xylazine (Pivetal/Anased, 10 mg/kg, Patterson Veterinary, Cat#04606–6750-02) were used at the same concentrations as described above. The protocol did not contain acepromazine because the most recent animal protocol did not contain the drug and acepromazine has no effect on PNN density in combination with xylazine (Figures 1D, 1E, S1E, S1F, S3C).

**Xylazine-Acepromazine (XA):** Xylazine and acepromazine, without ketamine, at the same concentration as described above.

**Control:** Saline (*i.p.*) volume corresponding to KXA cocktail concentration was injected.

**Repeated KXA:** Animals were exposed either to 1×, 2×, 3×, or 6× KXA injections with 2–4 days between the treatments (Hohlbaum et al., 2018).

**PLX5622:** The animals received chow containing 1200 mg/kg PLX5622 (Research diets, #113512) 1.5 weeks before the first KXA injection, and were kept on that diet for the whole duration of the experiment (Dagher et al., 2015; Spangenberg et al., 2019).

**Clopidogrel:** Clopidogrel (Tocris, Cat#2490) was dissolved in dimethyl sulfoxide (DMSO, Sigma, Cat#D8418) and diluted 1:4 in phosphate buffer saline (PBS) before the injection. 50 mg/kg Clopidogrel were injected (*i.p.*) 5 minutes before KXA, which reaches peak plasma levels within 1 min of intravenous administration (Savi et al., 2001; Zhang et al., 2016).

**Tamoxifen administration:** Adult Cx3Cr1<sup>CreERT2</sup> *het*/Ai9 *het* mice were injected intraperitoneally with 150 mg/kg tamoxifen (Sigma, Cat#T5648, Lot WXBD2299V, dissolved in corn oil, Sigma, Cat#MKCH1635) for three consecutive days. Mice were sacrificed for *ex-vivo* imaging at least four weeks after tamoxifen administration to ensure that the Ai9 reporter is only visible in resident microglia (Parkhurst et al., 2013).

**Light flicker stimulation**—The stimulation box consists of a black plastic box with a lid (OBI Tauro 62 Liters, 60×40×32 cm). A strip of light emitting diodes (LEDs, RS Components, Part. N. LSWW61210MIP20) was attached to the side walls of the box. The flicker frequency was set at 8, 40 or 60 Hz with a square wave current pattern generated

using an Arduino system (Arduino UNO SMD). The frequency of flickering was verified using a photoresistor (5 mm GL5516 LDR Photo Resistance), controlled by an Arduino UNO, positioned 1 cm from the LED strip. Images for circuit diagram were generated with fritzing app (version 0.9.3b).  $3.9 \times 10^{18}$  photons/cm<sup>2</sup>/s were generated in the center of the stimulation box.

Mice were transported from the holding room of the animal facility to the laboratory. Before the cage was put in the center of the stimulation box, 8 cm away from the LEDs, all nesting materials and tools were removed. Animals were always exposed to the light stimulation protocols (2 hours for 5 consecutive days) in the morning. During the stimulation, mice were allowed to move freely inside their cage with access to food and water *ad libitum*. After the stimulation, the animals received nesting material and were transferred back to the facility.

Control animals underwent the same transport and lab environment, but were only exposed to constant light, same intensity as the one used for the stimulation protocol.

**Tissue Preparation**—All tissues were dissected 4h after the last post-drug treatment, passing the KXA half-life of 2–3 h (Zhang et al., 2018). For histological analysis, animals were quickly anesthetized with isoflurane (Zoetis, Cat#6089373) and secured to the perfusion plate. The chest was open to expose the heart. The left ventricle was cannulated and the *inferior vena cava* cut. The animals were initially perfused with 20 ml of phosphate-buffered saline (PBS) with heparin (100 mg/l, Sigma, Cat#H0878), followed by 20 ml of 4% (w/v) paraformaldehyde (PFA, Sigma, Cat#P6148) in PBS using a peristaltic pump (Behr, Cat#PLP 380, speed: 25 rpm). The animals were decapitated, the retina and brain explanted and post-fixed in 4% (w/v) PFA/PBS for 30 minutes and overnight (16h), respectively. Then the tissues were washed in PBS and stored at 4°C with 0.025% (w/v) sodium azide (VWR, Cat#786–299). For cryoprotection, the tissue was transferred to 30% (w/v) sucrose (Sigma, Cat#84097) in PBS and incubated overnight at 4°C. To increase antibody permeability, the brain slices were frozen over dry-ice and thawed at room temperature for three cycles. Then, the brain was sliced in 100 µm coronal slices on a vibratome (Leica VT 1200S), if not otherwise indicated. For parasagittal sections, the brain was divided along the midline and the same vibratome settings were used.

**Immunohistochemistry**—The tissue was incubated with blocking solution containing 1% (w/v) bovine serum albumin (Sigma, Cat#A9418), 5% (v/v) Triton X-100 (Sigma, Cat#T8787), 0.5% (w/v) sodium azide (VWR, Cat#786–299), and 10% (v/v) serum (either goat, Millipore, Cat#S26, or donkey, Millipore, Cat#S30) for 1 hour at room temperature on a shaker. Afterwards, the samples were immunostained with primary antibodies diluted in antibody solution containing 1% (w/v) bovine serum albumin, 5% (v/v) triton X-100, 0.5% (v/v) sodium azide, 3% (v/v) goat or donkey serum, and incubated for 48 hours on a shaker at room temperature. The following primary antibodies were used: rabbit α-Caspase 3 (Cell Signaling, Cat#9661S, Lot 45, 1:400); rat α-CD68 (AbD Serotec, Cat#MCA1957, clone FA-11, Lot 1807, 1:250); Biotinylated Hyaluronan Binding protein (Amsbio, Cat#AMS.HKD-BC41, Lot SA, 1:150); goat α-Iba1 (Abcam, Cat#ab5076, Lot FR3288145–1, 1:250); rabbit α-Iba1 (GeneTex, Cat#GTX100042, Lot 41556, 1:750); rabbit α-MMP-9 (Abcam, Cat#ab38898, Lot GR3204084–23, 1:500); guinea pig α-parvalbumin (Synaptic

Systems, Cat#195004, Lot 2505, 1:500); chicken  $\alpha$ -parvalbumin (Novus Biologicals, Cat#NBP2-50036, Lot 72715, 1:1000); rabbit  $\alpha$ -P2y12 (Sigma, Cat#HPA014518, Lot F119293, 1:200); chicken  $\alpha$ -S100 $\beta$  (Synaptic Systems, Cat#287006, Lot 1-3, 1:500); *Wisteria floribunda* lectin – fluorescein-labeled (Szabo-Scandic, Cat#VECFL-1351, Lot ZE0611, 1:200); *Wisteria floribunda* lectin – biotinylated (Szabo-Scandic, Cat#VECB-1355, Lot ZE0424, 1:200). The slices were then washed 3 times with PBS and incubated protected from light for 2 hours at room temperature on a shaker, with the secondary antibodies diluted in antibody solution. The secondary antibodies raised in goat or donkey were purchased from Thermo Fisher Scientific (Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 647, 1:2000). The slices were washed 3 times with PBS. The nuclei were labeled with Hoechst 33342 (Thermo Fisher Scientific, Cat#H3570, 1:5000) diluted in PBS for 15 minutes. The slices were mounted on microscope glass slides (Assistant, Cat#42406020) with coverslips (Menzel-Glaser #0) using an antifade solution [10% (v/v) mowiol (Sigma, Cat#81381), 26% (v/v) glycerol (Sigma, Cat#G7757), 0.2M tris buffer pH 8, 2.5% (w/v) Dabco (Sigma, Cat#D27802)].

**Confocal microscopy**—Images were acquired with a Zeiss LSM880 Airyscan upright or inverted, or with a Zeiss LSM800 upright. Tile scan overview images showing the cortical area were taken using a Plan-Apochromat 10 $\times$  / NA 0.45 Air objective. To quantify image details such as number of PNN-coated cells, we used a Plan-Apochromat 40 $\times$  oil immersion objective N.A. 1.4 and acquired 2 $\times$ 2 tile scan z-stacks with a resolution of 0.208 $\times$ 0.208 $\times$ 0.371  $\mu$ m.

**Image analysis**—Confocal images were loaded in Fiji 1.52e (<http://imagej.net/Fiji>). To remove the background, the rolling ball radius was set to 35 pixels, and images were filtered using a median 3D-filter with x, y, z radius set at 3. Image stacks were exported as .tif files, converted to .ims files using the Imaris converter, and imported into Imaris 8.4.2. (Bitplane Imaris).

**Orthogonal projection:** Images were created with the “Section” tool in Imaris 9.3.1. A cell-of-interest was cropped out from the 40 $\times$  2 $\times$ 2 tile scan image with a crop area of 50 $\times$ 50  $\mu$ m. Then, the “Normal view” option was selected in the “view” bar and a single plane orthogonal projection was created. The white cross indicates the focal plane of interest, which is projected on the XY, YZ and XZ plane. For *ex-vivo* imaging in Figure 2C, we show an extended orthogonal projection with the ROI dimension of 5 $\times$ 5 $\times$ 5  $\mu$ m between the yellow lines.

**Image location:** For the analysis of the primary somatosensory cortex and the primary visual cortex, we took the region-of-interest at the cortical layers 3 to 5 of the S1(BF) and the V1.

**Perineuronal net density per mm<sup>3</sup>:** Defined PNN-staining around neurons allowed directly counting PNN-coated neuronal cell bodies in each image using the spot-detection function of Imaris. The total cell count was normalized to the entire image volume and data are represented as number of cells surrounded by perineuronal nets per mm<sup>3</sup>.

**HABP intensity measurements:** In contrast to WFA, which is a defined PNN marker (Härtig et al., 1992), HABP is a major ECM component (Ueno et al., 2018). Thus, an intensity measurement of HABP was performed. 16-bit confocal z-stacks were acquired using a Zeiss LSM-800 upright with aPlan APOCHROMAT 20x/0.8 (Cat#420650–9901), WD=0.55 mm (D=0.17 mm). The dimension of the z- stacks, centered between S1 cortical layers III and V, was 638.9×638.9×30 μm. All the samples were acquired at the same day to avoid artifacts due to fluctuation of the laser power. The mean fluorescence intensity was calculated on the entire field of view using the mean grey value analysis function of Fiji, version 1.53.

**Distance between Pvalb<sup>+</sup> neurons and microglia:** Five Pvalb<sup>+</sup> neurons were analyzed per animal. The image area was cropped to 60×60×50 μm and the Pvalb<sup>+</sup>-neuron soma defined as a spot center for the Imaris spot function, which was then converted into a surface. Typically, the cropped image contained 1–4 surrounding microglia, which were also identified via spots but not converted into surfaces. A surface-spot distance Imaris extension was used to calculate the distance between the centers of surfaces/spots of neuronal and microglial cell bodies.

**CD68, MMP-9, WFA volume within cells:** Surface renderings were generated on microglia, CD68, MMP-9, and WFA/PNN z-stacks using the surface rendering module of Imaris 8.4.2. Surfaces were generated with the surface detail set to 0.2 μm. To obtain CD68 or MMP-9 surface within microglia, or the surface of WFA within the microglial CD68, the surface-surface coloc plugin was used. This analysis was performed on the entire image. The total percentage of perineuronal net volume within this microglial CD68 volume was calculated per image.

**% of MMP-9 puncta in cell population:** The 2×2 40x confocal tile scans were visually inspected. Cells that contain at least one MMP-9 puncta inside were considered as positive. For microglia, we considered the cell body and processes. For Pvalb<sup>+</sup> neurons, we counted the cell body.

**Cell density per mm<sup>3</sup>:** In each image, either Pvalb<sup>+</sup> neurons or Iba1<sup>+</sup> microglia were counted using the spot detection function of Imaris. The total cell count was normalized to the entire image volume and data are represented as number of cells per mm<sup>3</sup>.

**Adult ocular dominance plasticity—**In P60–67 animals of both sexes (8 male and 8 female for saline-treated, 9 male and 9 female for KX-treated), we implanted tapered tungsten microelectrodes (300–500 MΩ) bilaterally in the binocular zone of L4 in the V1 as previously described (Fong et al., 2016; Sawtell et al., 2003). Animals recovered for at least one week from surgery before the start of KX (or saline) treatment to minimize interference of micro- and astrogliosis around the implanted electrodes. 2 days prior to recording the first visual evoked potentials (VEPs), we habituated the animals to restraint and the grey screen with binocular viewing for 30 min (first day) and monocular viewing (second day) for 15 min each eye using an opaque paddle to restrict vision to one eye per session. For VEP recordings, we exposed animals to sinusoidal gratings at 0.2 cycles per degree and 100% contrast, and phase-reversed at 2 Hz as described (Fong et al., 2016; Sawtell et al., 2003).

Every day of recording we used a novel orientation stimulus with at least 30° offset from a previously shown orientation. We used custom software written in C++ and MATLAB to analyze the VEPs. Monocular deprivation (MD) was performed at P78 at 1–3 h after recording a baseline PreMD VEP. Animals were anesthetized using isoflurane (1–3% in oxygen) and maintained on a heated surface for the whole procedure. One eye of each animal was sutured shut for 3 days using 1–2 mattress stitches of sterile Prolene 6–0 (Ethicon). At P81, all eyes were opened and rinsed with sterile eye drops under the same anesthesia conditions. PostMD recordings on that day (day 0) were performed at least 1 hr after opening the eye to allow full recovery from anesthesia. For all recordings, animals were brought to the lab at least 30 min prior to the experiment.

For randomization and blind testing, we divided the animals in two groups that were randomly assigned to one investigator. Each investigator injected the assigned group and never worked with those animals again until the full experiment was done and unblinding was necessary to run statistics.

**Live imaging of WFA/microglia**—Adult  $Cx3Cr1^{CreERT2\text{ }het/Ai9\text{ }het}$  mice were briefly anesthetized with isoflurane (Zoetis) prior to decapitation. The brain was quickly removed and submerged in ice-cold pre-carbogenated artificial cerebrospinal fluid (aCSF, 125 mM NaCl, 25 mM  $\text{NaHCO}_3$ , 25 mM glucose, 2.5 mM KCl, 1.25 mM  $\text{NaH}_2\text{PO}_4$ , 2 mM  $\text{CaCl}_2$ , 1mM  $\text{MgCl}_2$ ). The tissue was sliced at 350  $\mu\text{m}$  using a Leica VT 1200S Vibratom and incubated in *Wisteria floribunda* lectin –fluorescein-labeled (Szabo-Scandic, Cat#VECFL-1351, Lot ZE0611, 1:50 dilution) for 30 mins in constantly carbogenated aCSF. One hemisphere was mounted on black filter paper (Millipore, Cat# HABP02500) which was fixed in a closed perfusion chamber (RC-30 Warner Instruments) and constantly perfused (0.3 ml/min) with carbogenated aCSF at 37°C throughout the acclimatization (1 h) and imaging acquisition periods.

Images were acquired using an Andor Dragonfly spinning disc microscope equipped with a 40× Nikon Apochromat water objective (40× W NA 1.15/WD 0.6 mm) and a temperature-controlled chamber (37°C). Z-stacked images were captured simultaneously every minute for 30 mins using the Zyla sCMOS and iXon EMCCD cameras. A 308×308  $\mu\text{m}$  field of view was imaged with 1024×1024 pixels, giving 0.301  $\mu\text{m}/\text{pixel}$  in XY and a Z-step size of 0.4  $\mu\text{m}$ . The same exposure time (50 ms) and electron multiplying gain settings (200 for EMCCD) were applied to all imaging sessions. Drifting was corrected using the translational and rotational drifting correction option of the Imaris tracking spot function.

### ***In-vivo* electrophysiology**

**Surgery:** C57BL/6J adult males were anesthetized with isoflurane (5% induction, 2.5% maintenance) in 0.6 l/min  $\text{O}_2$  and were kept at 37°C using a heating pad connected to a rectal probe during the surgical procedure. After fixing the animal to a stereotaxic apparatus (KOPF digital plus) and septic sterilization, the skin over the skull was cut. The exposed skull was treated with 3% (v/v)  $\text{H}_2\text{O}_2$  (Sigma, Cat#216763) in PBS to remove the periosteum and to ensure enhanced cement adhesion.

The craniotomy ( $\varnothing \approx 2$  mm) was performed with a micro drill centered at Bregma  $-3.4$  and interaural  $2.5$  mm. These stereotaxic coordinates for the primary visual cortex were calculated on the Paxinos mouse brain atlas.

To record LFPs through the entire cortical layers, a 2-shank-32-channel chronic silicone probe (Cambridge Neurotech L2 sharp), equipped with an Omnetics 32 connector, was inserted into the brain parenchyma. The probe was grounded on both cerebellar hemispheres using two bone screws (FST, Cat#19010-00). The skull was then covered with dental cement (Henry Schein, NX3, Cat#246367). The pain was controlled with Metamizol (Sanofi Aventis, Cat#Ay005, *s.c.* 200 mg/kg during surgery) and with Meloxicam (Boehringer-Ingelheim, Cat#KPOEH3R, *s.c.* 5 mg/kg after surgery every 24 h for 3 consecutive days). The animals recovered at least for 2 weeks before used for experiments and did not develop any signs of seizures.

**Recording:** All recordings were performed in the morning (animal light phase). On the day of the experiment, the animal was connected to the head-stage, and neuronal activity during the light flickering protocol was recorded using an Intan RHD USB interface board with a sampling rate of 30 kHz for 10 minutes.

*Data analysis:* was performed with Python (Version 3.7), MATLAB R2019b, and Matplotlib (Version 3.4.1) Local field potentials (LFPs) were extracted from the *in-vivo* recordings using a 3rd-order low-pass Butterworth filter with a cut-off frequency of 300Hz, and were further downsampled to 3 kHz. A multi-tapering approach, as implemented in the Chronux toolbox (Version 2.12), was used to calculate the power spectra. Periodograms were estimated using 5 Slepian sequences with time-bandwidth product of 3 with zero-padding on the fast Fourier transforms. Periodograms were normalized over the total power from 0.5 to 100 Hz.

## QUANTIFICATION AND STATISTICAL ANALYSIS

All statistics were performed using R (version 3.4.4), if not otherwise indicated. Models were generated by changing the default contrast for unordered variables to “contr.sum”. This allows type III ANOVA to be applied to the model to evaluate the overall contribution of unordered effects on the response variable. Reported *P* values of post-hoc tests performed via the “multcomp” package (Hothorn et al., 2008) were corrected for multiple testing according to the default single-step method. If not otherwise indicated, all possible pairwise comparisons were performed. Linear regression was performed using the lme4 package (version 1.1-17) (Bates et al., 2015). Analyzed results were exported from Imaris into an Excel file, which was loaded into R via the xlsx package (version 0.6.1) (Dragulescu, 2014). Plots were generated using ggplot2 (version 3.0.0) (Wickham, 2016). All error bars in the manuscript represent standard error of the mean (SEM), calculated with mean\_se() as part of the hmisc package in ggplot2. A short description of each test and the results can be found in the figure legends. Details about the statistical models as well as the results of the statistical analysis for each figure are found in the Table S1. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , <sup>ns</sup>  $P > 0.05$ .

**Combined controls:** To compare experimental groups subjected to different injection paradigms, we first compared controls that received 1×, 3×, or 6× saline injections and obtained a mean value from the means of these conditions (control), which served as the control for further statistical testing.

**Perineuronal net density per mm<sup>3</sup>:** Each data point represents the count of neurons surrounded by PNNs in one image per animal (biological replicates). We calculated cell densities as a percentage, with the respective control set to 100%. We used linear regression to predict cell density per mm<sup>3</sup> (as absolute values or as percentages compared to the respective control) by experimental condition in cases where more than two levels were involved. We used a two-sided *t*-test to investigate differences between experimental conditions in cases where only two levels were involved. In this case, *P* values were adjusted using the “p.adjust” function and the method set to “BH”.

**Sex differences:** To compare removal and recovery of perineuronal nets between the sexes, we tested for differences between males and females within each experimental group using linear regression, and selected post-hoc contrasts for the desired comparisons. Models are described in Table S1 (Models, A).

**Ocular dominance plasticity experiments statistics:** To determine sample size, we ran a pilot experiment with 5 animals injected with 3× KX and the same timeline as the final dataset. We considered the subject and the properties of each time point (contralateral/ipsilateral ratio) to make the simulation using R. We simulated that the control (saline) group did not change over time, i.e. the mean value remained the same, with the given SD by the real data at baseline. We simulated that the treated group could change similar to the real data but with higher number of mice. The pilot data showed values decreasing compared to the baseline, with negative coefficients calculated using a linear model. We then performed 1000 simulations for 5, 10, 20, 40, 50 mice per group, and we calculated the two-way ANOVA for repeated-measures statistics and *P* value. We checked whether any difference (*P* value < 0.05) was detected between groups and between group:time interactions in every simulation. The results showed that we needed between 12 and 20 animals to observe >80% power. The final data set included 18 ketamine-treated and 16 saline-treated animals. We did not include the animals from the pilot experiment as that experiment was not performed blind to treatment.

We used Prism 8 to run statistical analyses and generate graphs on the final data set. To analyze the contra/ipsi ratio (Figure 1G) we performed a two-way repeated measures ANOVA to examine changes over time and across treatment conditions. We then ran a Dunnett’s multiple comparisons test to compare changes of baseline values over time. To analyze the VEP magnitude (Figures 1H and 1I), we ran a one-way repeated measures ANOVA after determining that the data were normally distributed (using the Shapiro-Wilk test) on ipsilateral responses in the saline group. For the contralateral saline-treated group and both the contralateral and ipsilateral ketamine-treated groups, the normality test showed that the data was not normally distributed and we therefore ran a Friedman test. If a significant effect (*P* < 0.05) was found, we then performed a multiple comparisons test using Dunnett’s correction.



**MMP volume within cells:** The total percentage of MMP volume within cell volume per image was calculated. A two-sided *t*-test was performed, and *P* values were adjusted using the “p.adjust” function and the method set to “BH” (Table S1, Models, B).

**Distance between Pvalb<sup>+</sup> neurons and microglia.**—The exported Imaris file contained the minimal distance values from five analyzed images per animal. We used linear regression to predict the minimal distance per experiment condition. Models are described in Table S1 (Models, C). A random effect was included to account for individual animals and the fact that their technical replicates were not independent.

**PNN volume within microglial CD68:** Each data point represented several microglia within one image per animal (biological replicates). We used linear regression to predict the percentage of PNN volume within microglial CD68 volume under experimental conditions. For the CD68 volume within microglia, we used linear regression to predict the percentage of CD68 volume within the microglia volume under experimental conditions. Models are described in Table S1 (Models, D).

**PNN volume within microglia in ex-vivo imaging:** We used linear regression to predict the square root-transformed percentage of PNN volume within the microglia volume under experimental conditions (Table S1, Models, A). To account for repeated measurements, we included an identifier for individual animals as a random factor.

**Cell density per mm<sup>3</sup>:** Each data point represented neurons stained for parvalbumin or microglia stained for Iba1 within one image per animal (biological replicates). We used a two-sided *t*-test to investigate differences between experimental conditions in cases where only two levels were involved. *P* values were adjusted with the “p.adjust” function and the method set to “BH”.

To evaluate the efficiency of microglial depletion via PLX in treated (PLX\_kxa\_3x) as well as saline-injected (PLX\_ctrlsal\_3x) animals, we compared their microglial density with saline-injected animals that were not exposed to PLX (ctrlsal\_3x). For this, we calculated the percentage with the no-PLX group set to 100%. We used linear regression to predict the percentage compared to the control under experimental conditions. Models are described in Table S1 (Models, E).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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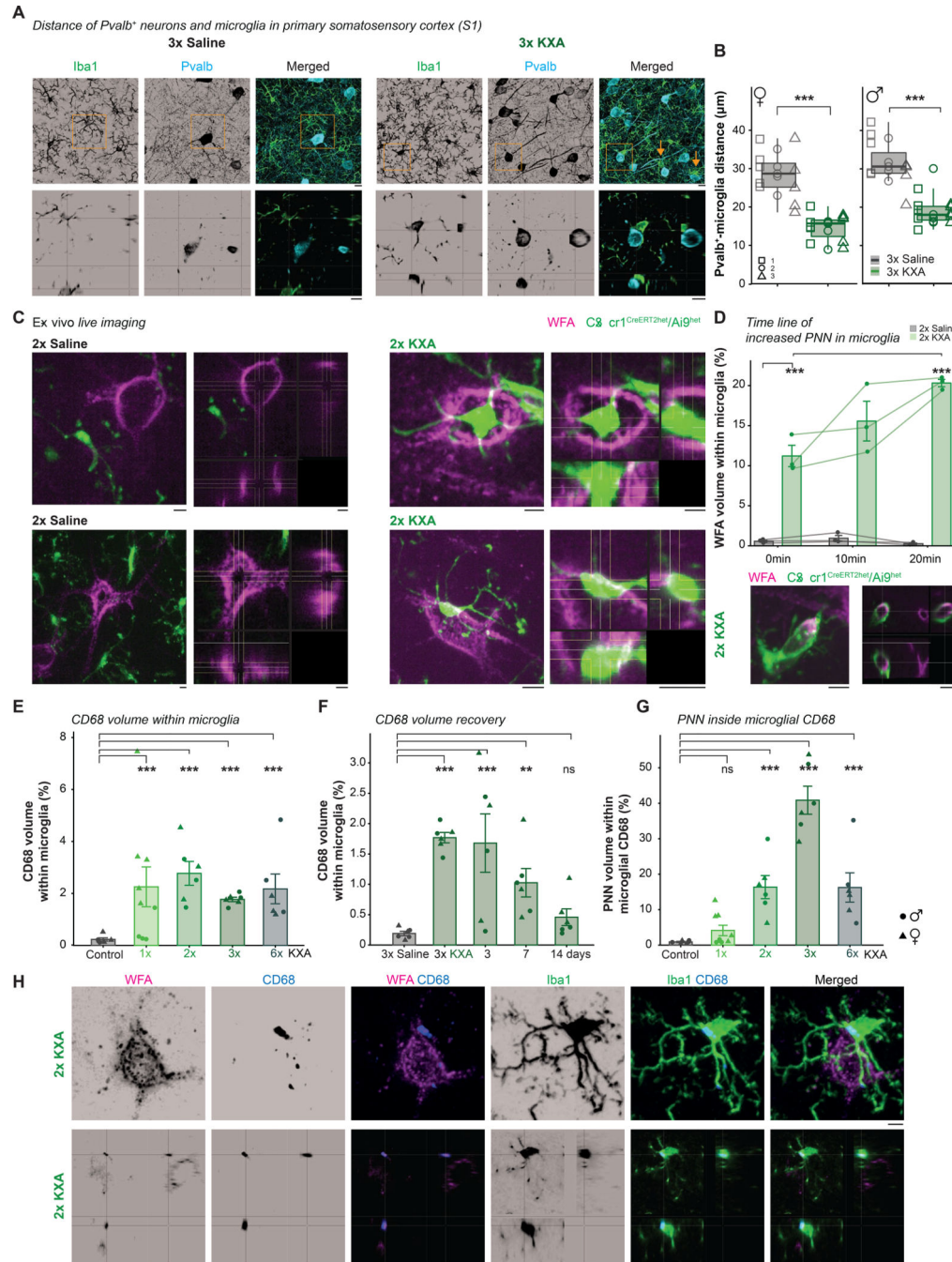
magnitudes  $\pm$  SEM with representative traces for (H) saline (contralateral, Friedman's test,  $P = 0.1550$ , ipsilateral, one-way ANOVA,  $P = 0.9102$ ), or (I) ketamine-treated mice (contralateral, Friedman's,  $P = 0.0097$  with selected post-hoc comparisons; ipsilateral, Friedman's  $P = 0.7546$ ). KXA, ketamine-xylazine-acepromazine. MD, monocular deprivation. S1(BF), somatosensory 1, barrel field. WFA, *Wisteria floribunda* agglutinin. Each dot represents one animal (3–5 animals per condition with exception of the control bar in Figure 1C, which shows the mean value of all controls. See also Figure S2B). SEM, standard error of the mean. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , <sup>ns</sup> $P > .05$ .

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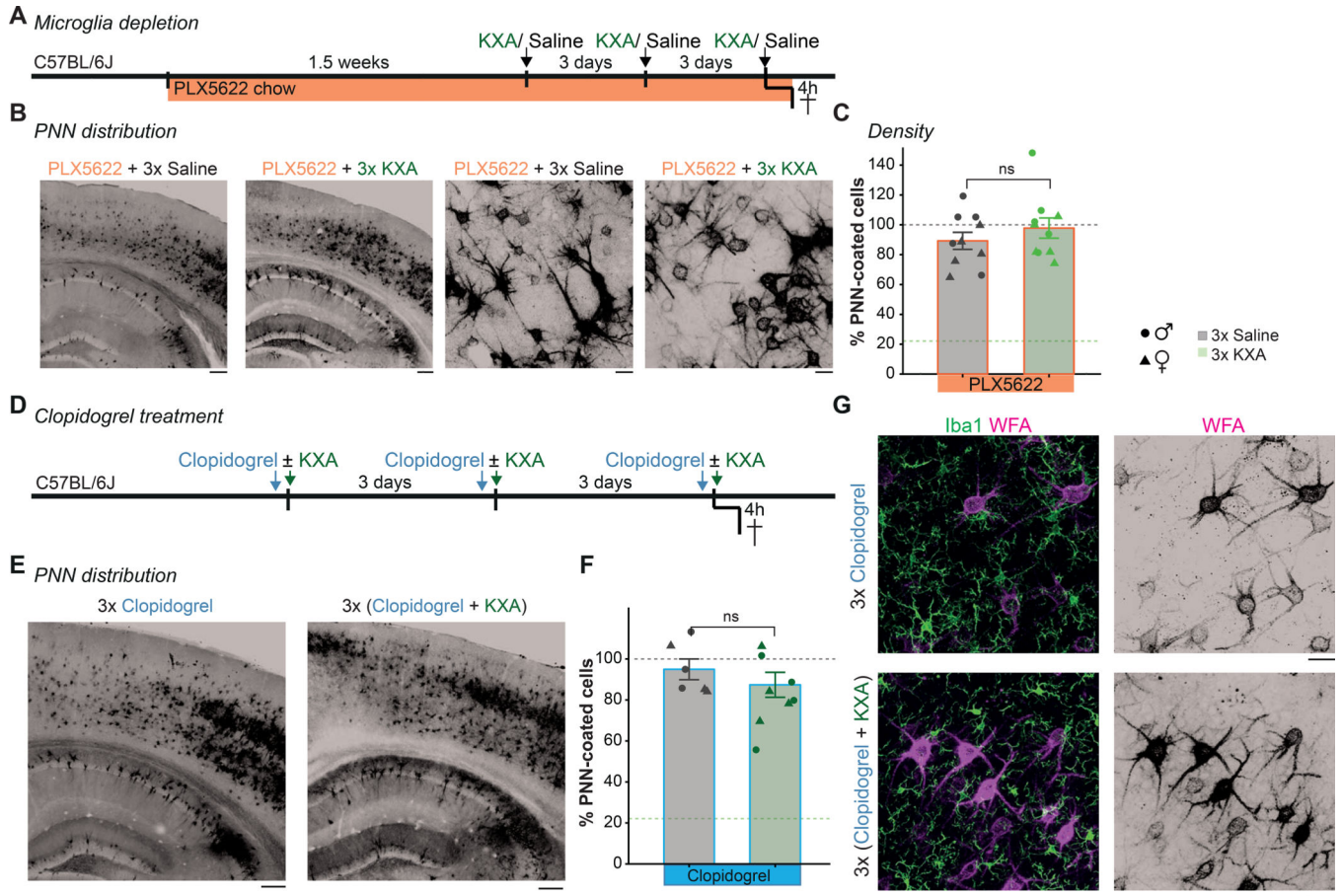
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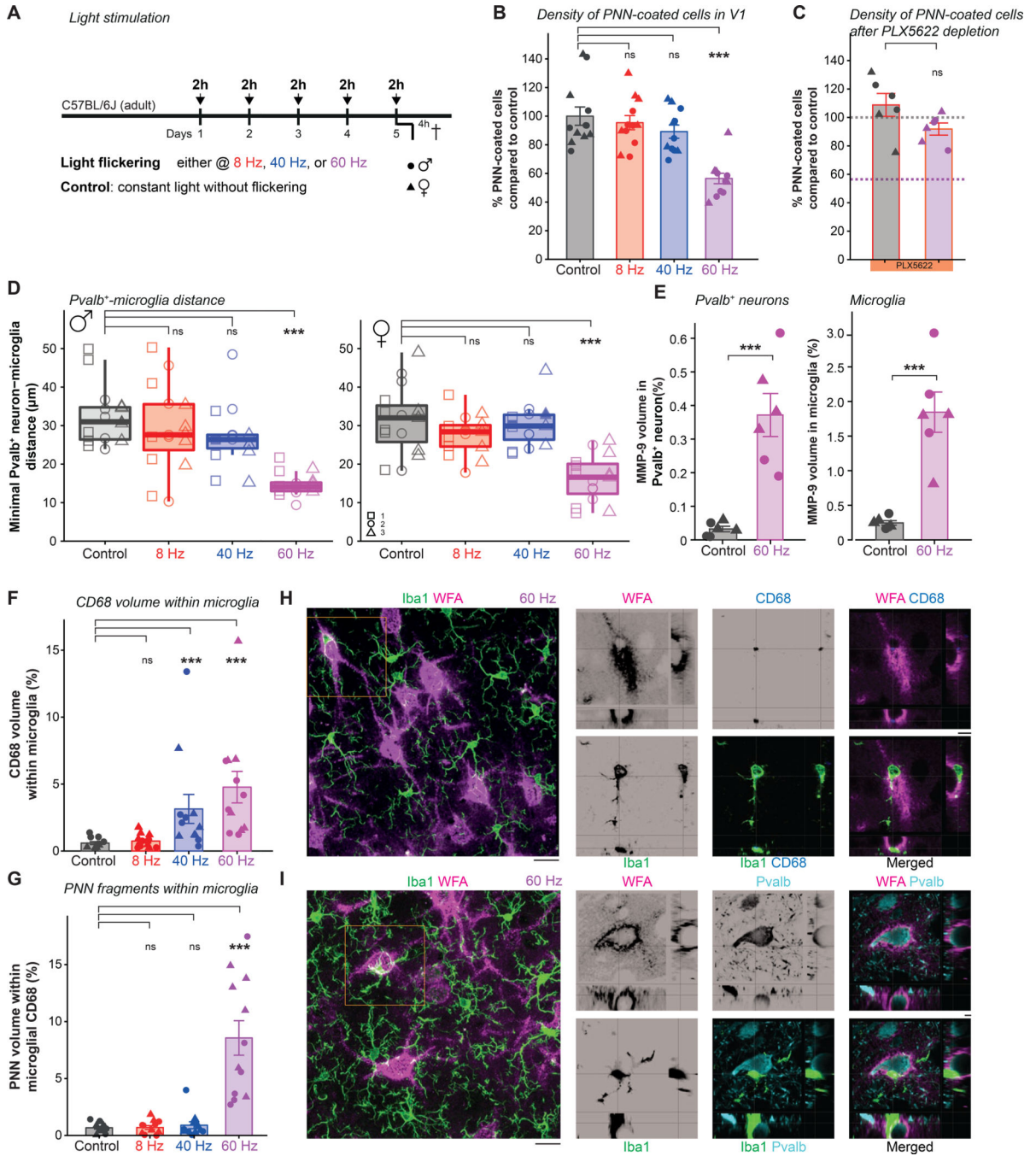
**Figure 2. Microglia interact with PNN-coated neurons upon repeated ketamine stimulation.** (A) Immunostaining for Iba1 (green) and parvalbumin (Pvalb, cyan) after 3× saline or KXA in primary somatosensory cortex S1. Zoom-in, single-plane orthogonal projection. Arrow, microglia in close distance of Pvalb<sup>+</sup> neurons. Scale bar: 10 μm. (B) Minimal Pvalb<sup>+</sup> neuron-microglia median distance in μm after treatment. Each condition 3 animals as indicated in legend, 5 microglia per animal. Linear regression model with animal ID as random effect:  $P_{\phi} < .0001$ ,  $P_{\sigma} < .0001$ . (C-D) *Ex-vivo* live imaging of brain slices of Cx3Cr1<sup>CreERT2</sup>*het*/A19<sup>het</sup> mice dissected 2-hours after 2× KXA. Snapshots of microglia



(green) and WFA (magenta) with orthogonal side projection. Scale bar: 10  $\mu\text{m}$ . (D) Time course of mean WFA volume within microglia percentage  $\pm$  SEM after treatment. Linear regression model:  $P < .0001$  with selected post-hoc comparisons. (E-F) Mean percentage CD68 volume within microglia  $\pm$  SEM after (E) saline or KXA or (F) 3 $\times$  KXA recovery. See also Figures S5A and S5B. Linear regression model: (C)  $P < .0001$ , (D)  $P < .0001$  with selected post-hoc comparisons. (G) Mean percentage of PNN volume within microglial CD68  $\pm$  SEM upon treatment. Linear regression model:  $P < .0001$  with selected post-hoc comparisons. Each dot represents one animal (3–5 animals per condition with exception of the control bar in (E) and (G), which shows the mean value of all controls. See also Figures S5A and S6B, respectively). (H) Immunostaining for PNN (WFA, magenta), endosomal-lysosomal marker CD68 (blue), and microglia (Iba1, green) after 2 $\times$  KXA. Below: single plane orthogonal projections. Scale bar: 5  $\mu\text{m}$ . \*\*\* $P < .001$ , \*\* $P < .01$ , <sup>ns</sup> $P > .05$ .



**Figure 3. Microglia depletion or clopidogrel exposure prior ketamine prevents PNN loss.** (A-C) Microglia depletion with Csf1r-inhibitor PLX5622 and 3× saline or KXA ketamine treatment. (A) Experimental timeline. (B) Immunostaining for WFA. Scale bar: 200 μm. Next, zoom-in into S1. Scale bar: 20 μm. (C) Mean density of PNN-coated cells ± SEM. Grey and green dashed lines: reference for mean value of 3× saline or KXA, respectively, from Figures 1C and S1D. Two sample t-test:  $P=0.3467$ . (D-G) Clopidogrel treatment, *i.p.* injected alone (3× clopidogrel) or 5 min before KXA application (3× (clopidogrel + KXA)). (D) Experimental timeline. (E) Immunostaining for WFA. Scale bar: 200 μm. (F) Mean density of PNN-coated cells ± SEM. Grey and green dashed lines: reference for mean value of 3× saline or 3× KXA, respectively, from Figures 1C and S1D. Two sample t-test:  $P=0.3808$ . Each dot represents an animal. (G) Higher magnification images for WFA (magenta or contrast-image) and Iba1 (green). Scale bar: 30 μm.  $^{ns}P>.05$ .



**Figure 4. 60-Hz light flickering recapitulates microglia response observed under repeated ketamine exposure.**

Light stimulation either with constant light (black) or light-flickering frequencies at 8 Hz (red), 40-Hz (blue), or 60-Hz (purple) in V1. (A) Experimental timeline. (B-C) Mean density of WFA-coated cells in percent ± SEM. (B) Linear regression model with selected post-hoc comparisons:  $P < .0001$ . (C) After microglia depletion. Two sample t-test:  $P = 0.0926$ . Grey and purple dashed lines: reference for mean value of constant light or 60 Hz from Figure 4B, respectively. (D) Median minimal parvalbumin (Pvalb)<sup>+</sup> neuron-microglia distance. Each condition 3 animals as indicated in legend, 5 microglia per animal. Linear regression model:

$P_{\sigma} < .0001$ ,  $P_{\varrho} < .0001$  with selected post-hoc comparisons. (E) Mean percent change of MMP-9 volume  $\pm$  SEM within each cell type. Two sample t-test: Pvalb<sup>+</sup>-neurons,  $P < .0001$ . Microglia,  $P < .0001$ . (F) Mean percentage of CD68 volume  $\pm$  SEM within microglia. Linear regression model:  $P < .0001$  with selected post-hoc comparisons. (G) Mean percentage  $\pm$  SEM of WFA volume within microglial CD68. Linear regression model:  $P < .0001$  with selected post-hoc comparisons. (H-I) Immunostained PNN (WFA, magenta), microglia (Iba1, green), endosomal-lysosomal marker CD68 (blue, H) and Pvalb (cyan, I) after 60-Hz light flickering stimulation. Scale bar: 20  $\mu$ m. Frame indicates zoom-in and single plane orthogonal projections. Scale bar: 7  $\mu$ m (H) and 4  $\mu$ m (I). \*\*\* $P < .001$ , <sup>ns</sup> $P > .05$ . With exception of (D), each dot represents an animal.