NPC1 deficiency impairs cerebellar postnatal development of microglia and climbing fiber refinement in a mouse model of Niemann-Pick Type C disease.

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Summary Statement

Genetic deficiency of Npc1 impairs postnatal development of microglia and climbing fiber synaptic pruning in the mouse cerebellum.

Key Words

Cerebellum, CLEC7A, NPC, VGLUT2, Microglia, Pruning
Abstract

Little is known about the effects of NPC1 deficiency in brain development and if they contribute to neurodegeneration in Niemann-Pick Type C disease. Since cerebellar Purkinje cells die early and to a higher extent in NPC, here we analyzed the effect of NPC1 deficiency in microglia and climbing fiber synaptic refinement during cerebellar postnatal development using the Npc1<sup>nmf164</sup> mouse. Our analysis revealed that NPC1 deficiency leads to early phenotypic changes in microglia that are not associated with an innate immune response. However, the lack of NPC1 in Npc1<sup>nmf164</sup> mice significantly affected the early development of microglia by delaying the radial migration, increasing the proliferation and impairing the differentiation of microglia precursor cells during postnatal development. Additionally, increased phagocytic activity of differentiating microglia was found at the end of the second postnatal week in Npc1<sup>nmf164</sup> mice. Moreover, significant Climbing-fiber (CF) synaptic refinement deficits along with an increased engulfment of CF synaptic elements by microglia were found in Npc1<sup>nmf164</sup> mice, suggesting that profound developmental defects in microglia and synaptic connectivity precede and predispose Purkinje cells to early neurodegeneration in NPC.
Introduction

Niemann-Pick Type C disease (NPC) is a recessive genetic lysosomal storage disease caused by mutations in the NPC1 or NPC2 proteins, important transporters of cholesterol from endosomes and lysosomes (Patterson, 1993). Accumulation of cholesterol inside these intracellular organelles leads to progressive neurodegeneration, dementia, and death in children. Developmental regression, ataxia, and cognitive impairment are found among the symptomatology of NPC. Although the average age of diagnosis is 10yrs, 50% of the NPC patients are diagnosed before age 7 and die before 12.5yrs of age. However, the average age of death is 16yrs, indicating that NPC onset and severity are variable but progressive (Garver et al., 2007). In fact, the nature and severity of neurological symptoms in NPC are directly correlated to the onset of the disease. Infantile manifestations of NPC include delay in motor milestones, gait problems, clumsiness and speech delay, while symptomatic manifestations of juvenile and adult-onset of NPC include learning deficits, ataxia, dystonia and psychiatric symptoms (Mengel et al., 2013). Questions remain as to how the deficiency of the NPC1 protein perturbs developmental processes in the brain that could contribute to the early development of dementia and neurodegeneration.

Studies in mice and cats have revealed that Purkinje cells (PCs) are particularly hypersensitive to the loss of the NPC1 protein; in NPC these cells degenerate earliest and to a greater severity than other neurons in the brain (Vanier and Millat, 2003). This early degeneration of cerebellar PCs contributes to the development of early neurological symptoms such as clumsiness, gait defects and ataxia in both the human disease and animal models. Identifying the timeline and nature of pathological changes at the cellular and molecular level in the cerebellum
caused by mutations in Npc1 is critical to understand the mechanisms underlying the early dysfunction and degeneration of PCs. Interestingly, recent studies have shown that genetic inactivation of reactive microglia in Npc1<sup>−/−</sup> mice reduces neurological impairment and increases their life span (Cougnoux et al., 2018). We recently found that engulfment and phagocytosis of dendrites by activated microglia occur early and precede PC loss in NPC (Kavetsky et al., 2019), demonstrating that activated microglia contribute to PC degeneration in NPC.

Although NPC is a childhood disorder, little is known about the effects of NPC1 deficiency on brain development. Moreover, how developmental deficits precede and contribute to neurodegeneration in NPC is not completely understood. Developmental delay in motor skill acquisition and significant reductions in synaptic and myelin proteins were reported in the postnatal Npc1<sup>nmf164</sup> mouse (Caporali et al., 2016). In contrast to mice with complete deletion of the Npc1 gene, the Npc1<sup>nmf164</sup> mutant mice present a late onset and slower disease progression where severe motor deficits and neurodegeneration are evident at a young adult stage (Maue et al., 2012). Since no degeneration of PC and no severe motor deficits are found at early developmental stages, the Npc1<sup>nmf164</sup> mouse is an ideal model to study potential “silent” cerebellar developmental defects and behavioral changes caused by NPC1 deficiency that precede degeneration of PC. Contrary to most common late-onset dementias that are age-associated, the early-childhood onset of neurological manifestations in NPC and other lysosomal storage disorders (LSD), indicates the potential disruption of neurodevelopmental processes. The impacts of disrupted cholesterol trafficking by NPC1 deficiency in neural cells development is unknown, leading to a poor understanding of the origin and preclinical mechanisms that lead to childhood dementia. Understanding the mechanisms by which NPC1
deficiency affects neural cells during development not only will expand our current knowledge of brain-behavior developmental processes, but also will provide potential therapeutic avenues to identify and delay the progression of NPC and other childhood dementias (Ford et al., 1951; Shapiro E.G., 1994).

Previous work in our laboratory showed significant changes in microglia number, morphology and phagosome content in the cerebellum of Npc1<sup>nmf164</sup> mice at post-weaning age and before PC degeneration (Kavetsky et al., 2019). Interestingly, the migration, proliferation and differentiation of cerebellar microglia occur postnatally along with the development and differentiation of cerebellar neurons. In the brain, microglia play important roles during normal development, including the clearance of apoptotic cells and the elimination of redundant synapses. Importantly, when compared to microglia from other regions of the brain, normal cerebellar microglia strongly display cellular and gene expression patterns mostly associated with cell clearance (Ayata et al., 2018), suggesting that cerebellar microglia are more phagocytic. To determine the impact of NPC1 deficiency in cerebellar microglia during postnatal development, different stages of microglia development such as migration, proliferation and differentiation were examined at the corresponding postnatal age. The findings of this study suggest that NPC1 deficiency not only affects the different phases of microglia development, but also increases phagocytosis activity in these cells that promotes and amplifies profound synaptic defects during the postnatal development of the cerebellum.
Results

Early changes in microglia are not the result of an innate immune response in the cerebellum of post-weaning \textit{Npc1}\textsuperscript{nmf164} mice.

Since changes in \textit{Npc1}\textsuperscript{nmf164} microglia are evident at P30 (Kavetsky et al., 2019, Fig. 1A), we proceeded to test if genes associated with an innate immune response were upregulated in the cerebellum of P30 \textit{Npc1}\textsuperscript{nmf164} mice. A PCR array plate containing primers for ~92 genes associated with the mouse innate-immune response was used. RNA from the cerebellum of P90 \textit{Npc1}\textsuperscript{nmf164} mice was used as a positive control since at this age a severe loss of PCs is detected and the changes in microglia morphology and proliferation are more remarkable than at P30 (Fig. 1A) (Kavetsky et al., 2019). As expected, several genes including cytokines (\textit{Lif}, \textit{Tnf}, \textit{Csf3}, \textit{Il1a}, \textit{Ccl2}, and \textit{Ccl3}), endothelial-inflammatory genes (\textit{Sele} and \textit{Vcam1}), T-cell associated genes (\textit{Gzmb}, \textit{Cd3e}, \textit{Cd28}, \textit{Stat4}, \textit{PRF1}, and \textit{Tnfrsf18}) and other proinflammatory molecules (\textit{C3} and \textit{Ptgs2}) were significantly increased (>5 fold) in the cerebellum of P90 \textit{Npc1}\textsuperscript{nmf164} mice when compared to WT mice (Fig. 1B). However, no significant expression changes were found in the cerebellum of P30 \textit{Npc1}\textsuperscript{nmf164} mice when compared to WT mice (Fig. 1B), suggesting that microglia changes at that early age are not the consequence of an immune or inflammatory response in \textit{Npc1}\textsuperscript{nmf164} mice. To determine if microglia changes at P30 were associated with effects of NPC1 deficiency on microglia development, a comprehensive analysis of cerebellar and microglia postnatal development was performed.
Early radial migration of microglial precursors into the developing cerebellar cortex is reduced in Npc1\textsuperscript{nmf164} mice.

In the mouse, most of the cerebellar development occurs postnatally. Therefore, at early postnatal stages such as P4, only round and ameboid IBA1\textsuperscript{+} microglial precursors were observed in the developing cerebellar medulla (CbM) while ramifying microglia were already found at this stage in the cerebral cortex (Fig. 2A). As described by others (Ashwell, 1990; Cuadros et al., 1997; Mosser et al., 2017), in the P4 WT cerebellum microglial precursors were concentrated in the developing CbM and migrating tangentially towards the primitive cerebellar folia following tomato lectin\textsuperscript{+} blood vessels (Fig. 2B). Also, at this early postnatal stage, abundant microglial precursors were observed at the pial surface (PS) of the meninges (Fig. 2B). The beginning of the radial migration of microglial precursors into the different regions of the cerebellar cortex that include the inner granule layer (IGL), Purkinje cell layer (PCL) and external granule layer (EGL), is expected to occur at this early stage. Quantitative analysis of the density of IBA1\textsuperscript{+} microglial precursors in the CbM revealed no differences between P4 WT and Npc1\textsuperscript{nmf164} mice (Fig. 2B and C). However, a significant reduction in the number of IBA1\textsuperscript{+} microglial precursors reaching the IGL and circulating the PS was found in Npc1\textsuperscript{nmf164} mice when compared to WT mice (Fig. 2B and D). At this early postnatal stage, very few IBA1\textsuperscript{+} microglial precursors have reached the PCL, and the EGL is completely devoid of them as reported by others (Cuadros et al., 1997; Nakayama et al., 2018). No changes in vascularization (tomato lectin\textsuperscript{+} blood vessels) were observed between the P4 WT and Npc1\textsuperscript{nmf164} mice (Fig. 2B, Fig. S1).

Proliferative activity in CbM microglial precursors was detected in both P4 WT and Npc1\textsuperscript{nmf164} mice, but a significantly higher number and percentage of these CbM
KI67+/IBA1+ microglial precursors were found in $Npc1^{nmf164}$ mice (Fig. 2E-G). Meanwhile, proliferative activity in microglial precursors at the IGL, PCL, and PS was very low in P4 WT and $Npc1^{nmf164}$ mice, but a further significant reduction in proliferative microglial precursors was found in $Npc1^{nmf164}$ mice (Fig. 2H and I), mostly because of the delayed migration of these cells in the $Npc1^{nmf164}$ mice. Overall, these results suggest that NPC1 deficiency affects the ability of microglial precursors to migrate radially while increasing their proliferation during early postnatal cerebellar development.

**Increased density of precursor and maturing microglia in the developing cerebellum of P10 and P14 $Npc1^{nmf164}$ mice**

Studies have shown that the proliferative activity of microglial precursors in the developing cerebellar CbM and folias white matter region (WMR) start at birth and peak at P7 in normal mice (Ashwell, 1990; Li et al., 2019). In fact, an abundant subpopulation of microglia in the WMR region was recently identified as proliferative-region-associated microglia (PAM) (Li et al., 2019). When we examined the cerebellum of P10 WT and $Npc1^{nmf164}$ mice, we found that the volume occupied by IBA1+ microglia in the WMR (axonal tracts) was significantly higher in $Npc1^{nmf164}$ mice than in WT mice (Fig. 3A-B and D). However, the percentage of proliferative microglia in this region, as assessed by KI67 immunostaining, was similar between WT and $Npc1^{nmf164}$ mice (Fig. 3B and E). Similarly, the density of IBA1+ microglia in the PCL and molecular layer (ML) was significantly increased in $Npc1^{nmf164}$ mice without significant changes in KI67 immunoreactivity when compared to WT mice (Fig. 3C and F-G). In addition, microglia in the cerebellar cortex of WT and $Npc1^{nmf164}$ mice were evidently maturing and ramifying (Fig. 3C), while the microglia
in the WMR were ameboid shape and less ramified, an indication of a more immature cell (Fig. 3B). Since no differences in Ki67 immunoreactivity at the cerebellar WMR were observed between P10 WT and \textit{Npc1}\textsuperscript{nmf164} mice, our results suggest that an increased number of microglia was produced in the cerebellar WMR of \textit{Npc1}\textsuperscript{nmf164} mice prior to the P10 stage (P4-P7) as reported by others (Li et al., 2019). To test this possibility, P10 WT and \textit{Npc1}\textsuperscript{nmf164} cerebellar slices were immunostained with CLEC7A (Fig. 4), a specific marker for PAM cells, which proliferation rate in WT mice is significantly reduced after peaking at P7 (Li et al., 2019). Interestingly, a significant higher number of CLEC7A\textsuperscript{+} clusters were observed in \textit{Npc1}\textsuperscript{nmf164} developing WMRs (Fig. 4A and C). Also, the area of these clusters with CLEC7A\textsuperscript{+} (Fig. 4D) and IBA1\textsuperscript{+} cells (Fig. 4E), as well as the fraction of the IBA1\textsuperscript{+} area that was CLEC7A\textsuperscript{+} (Fig. 4F), were significantly larger in \textit{Npc1}\textsuperscript{nmf164} mice when compared to WT. The majority of P10 CLEC7A\textsuperscript{+} microglial precursors lacked processes and were primarily ameboid shape in both WT and \textit{Npc1}\textsuperscript{nmf164} mice (Fig. 4G-I). Although, CLEC7A expression is absent in differentiated microglia (Li et al., 2019), we found a higher tendency of CLEC7A\textsuperscript{+}/IBA1\textsuperscript{+} microglial precursors to have more terminal points in \textit{Npc1}\textsuperscript{nmf164} mice (Fig. 4H-I), and some CLEC7A\textsuperscript{+} differentiating microglia were observed in \textit{Npc1}\textsuperscript{nmf164} mice (Fig. 4J). Finally, because CLEC7A\textsuperscript{+}/IBA1\textsuperscript{+} microglia actively phagocytose oligodendrocyte progenitor cells (OPCs) at P7 (Li et al., 2019), and decreased levels of myelin basic protein (MBP) have been found in P15 \textit{Npc1}\textsuperscript{nmf164} mice (Caporali et al., 2016), analysis of MBP immunostained cerebella was performed specifically in the regions where CLEC7A\textsuperscript{+}/IBA1\textsuperscript{+} cells were located (Fig. 4K, blood vessels staining is artifactual). We found that MBP intensity inside the CLEC7A\textsuperscript{+} clusters tended to be decreased in
Npc1\textsuperscript{164} mice, but due to variability in WT mice the result was not statistically significant (Fig. 4L).

At P14, the density of IBA1\textsuperscript{+} microglia was also significantly higher in the PCL/ML of Npc1\textsuperscript{164} mice when compared to WT mice (Fig. 5A-C). However, the levels of IBA1\textsuperscript{+}/Kl67\textsuperscript{+} cells in P14 WT and Npc1\textsuperscript{164} mice were very low in the cerebellar cortex layers (Fig. 5B and D). Overall, our results suggest that the increased proliferation of WMR microglial precursors in Npc1\textsuperscript{164} mice leads to an increased density of microglia in the cerebellar cortex region since no increased microglia proliferative activity is detected in the cerebellar cortex at P10 and P14 (Fig. 5E).

**NPC1 deficiency affects microglia differentiation and ramification**

In the early days of cerebellar postnatal development, microglial precursors in the WMR were distinctly recognized by their round or ameboid shape (Fig. 2A-B). It was also evident that as these cells migrate to the cerebellar cortex, where PC dendrites and synaptic connections are developing, they began to differentiate, ramify and extend their processes through the ML where neuronal synaptic connections are found (Fig. 3C). To determine if NPC1 deficiency alters microglia differentiation, a quantitative analysis of IBA1\textsuperscript{+} cells morphology was performed in P10 and P14 WT and Npc1\textsuperscript{164} mice, using the “Filament Tracer” tool (Imaris\textsuperscript{TM}). Differences in microglia volume were not detected between WT and Npc1\textsuperscript{164} mice at P10 (Fig. 6A-B), however, at this stage, the microglia total length and the number of terminal points were significantly reduced in Npc1\textsuperscript{164} mice (Fig. 6C-D). At P14, the microglia volume, total length, and terminal points were significantly lower in Npc1\textsuperscript{164} mice when compared to WT mice (Fig. 6A and E-G). These results show that microglia in Npc1\textsuperscript{164} mice are less ramified and have shorter processes,
suggesting that NPC1 deficiency impairs microglia differentiation and ramification during postnatal development.

**Phagocytic activity is increased in developing Npc1<sup>nmf164</sup> microglia**

Microglia play an important role in the clearance of apoptotic cells during development (Mosser et al., 2017). While analyzing microglia morphology in P14 postnatal mice, we noticed an abundant number of maturing microglia in the ML containing phagocytic cups, especially in Npc1<sup>nmf164</sup> mice. Phagocytic cups are cup-shaped endocytic vacuolar structures in ramified microglia that are formed by the ingestion of particles or cells during phagocytosis (Swanson, 2008). The number of microglia with phagocytic cups was significantly higher in Npc1<sup>nmf164</sup> mice when compared to WT mice (Fig. 7A-C). We also found that microglia with at least two phagocytic cups and phagocytic cups per image area, were more abundant in the ML of Npc1<sup>nmf164</sup> mice (Fig. 7B, D-E). Interestingly, the number of phagocytic cups containing pyknotic bodies in the ML was higher in Npc1<sup>nmf164</sup> mice than in WT mice, suggesting that NPC1 deficiency increases microglial phagocytic activity in the developing cerebellum. Immunostaining of microglia with the CD68 antibody, a phagosome marker, showed that P14 WT and Npc1<sup>nmf164</sup> microglia were actively phagocytosing at this developmental stage, since CD68<sup>+</sup> phagosomes were abundant in microglia from both mouse strains. Markedly, WT IBA1<sup>+</sup> microglia at P14 had many small CD68<sup>+</sup> phagosomes distributed through the cell body and processes (Fig. 7G), while in the Npc1<sup>nmf164</sup> microglia the majority of the CD68<sup>+</sup> phagosomes were accumulated in the cell body (Fig. 7G). Quantitative analysis showed that the mean volume of CD68<sup>+</sup> phagosomes per microglia at P14 was larger in Npc1<sup>nmf164</sup> mice than in WT mice (Fig. 7G-H), however, no differences were found in the total
volume of CD68 between WT and \( Npc1^{\text{nmf164}} \) microglia at this stage (Fig. 7I). Since CLEC7A expression is reactivated specifically in actively phagocytic disease-associated microglia (DAM) (Krasemann et al., 2017), to test if P14 phagocytic cells were similar to DAM, P14 and P60 (NPC neurodegeneration stage) WT and \( Npc1^{\text{nmf164}} \) cerebella were immunostained with CLEC7A. We found that CLEC7A+ microglia only reappear in the ML of \( Npc1^{\text{nmf164}} \) mice during neurodegeneration at P60 (Fig. S2), and not at P14. Our results suggest that postnatal changes in NPC microglia are developmental alterations caused by NPC1 deficiency and not as an immunological response. These results indicate that NPC1 deficiency alters the phagocytic activity and the distribution of phagosomes in the developing cerebellar microglia.

**Microglia function in Climbing fiber synapse elimination is altered in \( Npc1^{\text{nmf164}} \) mice.**

During the first three weeks after birth, PCs develop their dendritic tree and establish synaptic connections with two major excitatory inputs in the molecular layer (ML): a single CF from the Inferior Olive nuclei and parallel fibers (PFs) from cerebellar granule cells. In the first week, multiple immature synapses with PCs are formed, however mature and functional synapses are established after the completion of three different periods of synaptic refinement: the early (P8-P11) and late (P12-P17) phases of CF synapses elimination, and the PF synapse elimination phase (P15-P30) (Hashimoto and Kano, 2013; Watanabe and Kano, 2011). During the late phase of CF refinement, the excess of presynaptic terminals is pruned while the terminals of the winning CF are translocating from the PC soma to the proximal regions of PC dendrites (Hashimoto and Kano, 2013; Watanabe and Kano, 2011).
The role of microglia during this phase of CF synaptic refinement is not completely understood, however recent studies have shown that CF synapse elimination is impaired in mouse cerebella depleted of microglial cells (Kana et al., 2019; Nakayama et al., 2018) suggesting a key role of microglia in CF synapse elimination. When we examined P14 cerebella from WT and *Npc1<sup>nmf164</sup>* mice, we found that the volume of CF VGLUT2<sup>+</sup> presynaptic inputs in the proximal region of CALB<sup>+</sup> PC dendrites was significantly reduced in *Npc1<sup>nmf164</sup>* mice when compared to WT mice (Fig. 8A-B), as previously reported by others (Caporali et al., 2016). However, we also noticed differences in the distribution of VGLUT2<sup>+</sup> inputs between WT and *Npc1<sup>nmf164</sup>* mice (Fig. 8A-B). In fact, a higher percentage of CALB<sup>+</sup> PC somas in *Npc1<sup>nmf164</sup>* mice contained VGLUT2<sup>+</sup> inputs, and in higher numbers (VGLUT2 puncta/soma) than in WT mice (Fig. 8C-E). Interestingly, significantly more VGLUT2<sup>+</sup> inputs in the proximal region of CALB<sup>+</sup> PC dendrites of P14 WT mice were contacted by IBA1<sup>+</sup> microglia than in the *Npc1<sup>nmf164</sup>* mice (Fig. 8F-H). However, a significantly larger percentage of CALB<sup>+</sup> PC somas in *Npc1<sup>nmf164</sup>* mice were contacted by IBA1<sup>+</sup> microglia, suggesting a possible link between the excess of CF synaptic inputs in PC somas and the increased interaction of microglia with this region of the PC.

Since previous studies in other regions of the brain have shown that microglia actively engulf and phagocytose presynaptic terminals during developmental pruning (Gunner et al., 2019; Schafer et al., 2012; Tremblay et al., 2010), and P14 cerebellar microglia were evidently phagocytic as they contained high levels of CD68<sup>+</sup> phagosomes (Fig. 7G), we analyzed the interaction of individual microglial cells with VGLUT2<sup>+</sup> presynaptic inputs. To assess whether IBA1<sup>+</sup> microglia are contacting or engulfing VGLUT2<sup>+</sup> inputs in WT and *Npc1<sup>nmf164</sup>* mice, confocal microscopy and 3D surface rendering analysis (Imaris™) were used in P14 cerebella. At this stage of
postnatal development (P14, late phase of CF synapse elimination), WT microglia were actively contacting and engulfing VGLUT2\(^+\) inputs in the ML (Fig. 9A).

Quantitative analysis of the total volume of VGLUT2 puncta per microglia at the PCL and ML indicated that \(Npc1^{nmf164}\) microglia contacted or engulfed significantly more VGLUT2\(^+\) inputs than WT microglia at P14 (Fig. 9A-B). By examining the Z-stack sequence images of the microglial cell shown in figure 8A, the interactions of the IBA1\(^+\) cell processes with VGLUT2\(^+\) inputs innervating CALB\(^+\) PC dendrites can be observed in Fig. 9C. Some VGLUT2\(^+\) inputs were completely engulfed (arrows) by the IBA1\(^+\) cell processes, while others were only contacted (Fig. 9C), demonstrating that CF presynaptic inputs are contacted or engulfed by microglia during the late phase of CF refinement. In contrast, Z-stack imaging sequence of the \(Npc1^{nmf164}\) microglial cell presented in figure 8A clearly shows the interaction of the IBA1\(^+\) cell with CALB\(^+\) PC soma while contacting or engulfing VGLUT2\(^+\) inputs that were found abundantly in this region of the PC in P14 \(Npc1^{nmf164}\) mice (Fig. 9C). These results suggest that NPC1 deficiency not only impairs CF synapse formation, but that it also alters the elimination and translocation of CF synapses in addition to the normal interaction and synaptic pruning function of microglia during the postnatal developmental refinement of CF synapses. Overall, our results show severe impairments in cerebellar microglia and synaptic development that precede and may contribute to early behavioral deficits and neurodegeneration in NPC.

Discussion

In this study, we demonstrate that deficiency of NPC1 affects the postnatal development and function of cerebellar microglia, contributing to profound defects in developmental synaptic pruning and connectivity in the cerebellum. Specifically, we
found that lack of NPC1 in mice reduced radial migration, increased proliferation and impaired differentiation of microglial precursors during the first two postnatal weeks. Increased engulfment of pyknotic bodies and CF presynaptic elements was characteristic of \textit{Npc}^{\text{nmf164}} differentiating microglia at two weeks of age. These developmental deficiencies precede the pathological changes in microglia, behavioral deficits and the degeneration of PCs found in young adult mice (Kavetsky et al., 2019), suggesting that developmental defects in the cerebellum significantly contribute to psychiatric and neurological symptoms in NPC.

Neuroinflammation is a remarkable pathological hallmark in neurodegenerative diseases that contributes to the progression of neuronal dysfunction and degeneration (Perry et al., 2010; Soto and Howell, 2014). It is thought that neuroinflammation is initiated by microglia activation in response to CNS insult or injury. Microglia activation and neuroinflammation occur in NPC1 patients and mouse models of the disease (Cologna et al., 2014; Cougnoux et al., 2018; Erickson and Bernard, 2002; Kavetsky et al., 2019). Importantly, genetic inactivation of microglia in \textit{Npc1}^{\text{−/−}} mice significantly reduces NPC symptoms while increasing lifespan (Cougnoux et al., 2018), demonstrating a major role of microglia in NPC progression. Recent work in our laboratory demonstrated that changes in morphology and accumulation of phagosomes in microglia are already observed at early stages of the disease when no loss of PC is detected in \textit{Npc}^{\text{nmf164}} mice (Kavetsky et al., 2019). Since these early changes were observed in \textit{Npc}^{\text{nmf164}} mice at the end of postnatal development, we investigated whether these changes were an early inflammatory response of microglia or simply a consequence of impaired postnatal development. Gene expression analysis of innate immune response genes in P30 and P90 \textit{Npc}^{\text{nmf164}} cerebella revealed that the upregulation of these genes
was only detected at the P90 stage, when a severe degeneration of PCs is found, suggesting that the early changes observed in microglia may be the result of disrupted postnatal development. Moreover, at this early age, Npc1<sup>nmf164</sup> mice present behavioral deficits, such as repetitive behavior, that seem to be associated with the early pathological changes found in the cerebellum (Kavetsky et al., 2019). Similarly, in the human NPC disease, where the classic presentation of the disease is often found between middle to late childhood, early neurological symptoms associated with cerebellar dysfunction, such as clumsiness, gait disturbances, and eventually ataxia, are observed before the manifestation of other neurological symptoms (Patterson, 1993). These findings suggest that deficiency of NPC1 causes developmental disturbances in the cerebellum that precede neurodegeneration.

Microglial precursors actively proliferate, migrate and undergo morphological changes during normal brain development (Mosser et al., 2017). A substantial portion of the anatomical and functional development of the mouse cerebellum occurs during postnatal development (Butts et al., 2014). Therefore, in early postnatal days, round and ameboid shape microglial precursors are accumulated in the developing CbM, where they actively proliferate and migrate radially to the developing cerebellar cortex (Ashwell, 1990; Mosser et al., 2017). Since microglia are the cells in the brain with the highest expression of Npc1 (Bennett et al., 2016), we hypothesized that NPC1 deficiency severely affects the postnatal development of cerebellar microglia in Npc1<sup>nmf164</sup> mice. Indeed, our data demonstrated that early radial migration of microglial precursors was reduced or delayed in Npc1<sup>nmf164</sup> mice since fewer microglial precursors were found at the IGL in P4 mice. NPC1 deficiency has been previously implicated in the reduced <i>in vitro</i> migration and invasion of CHO and fibroblast cells from NPC patients implicating dysfunctional
recruitment and function of integrins in focal adhesion during cell migration (Hoque et al., 2015). It is possible that the intrinsic ability of microglial precursors to migrate is affected by the lack of NPC1 and the lysosomal accumulation of cholesterol.

Another important finding in this study was the increased density of microglia in the developing WMR and cerebellar cortex regions of Npc1<sup>nmf164</sup> mice. In the normal brain, microglia are highly proliferative during the first two postnatal weeks, particularly in the developing CbM and WMR (Li et al., 2019; Nikodemova et al., 2015). A recent study demonstrated that the density of a subset of microglial precursors named PAM peaks at P7 exclusively in the cerebellar WMR (Li et al., 2019). In our study, we found higher proliferative activity in microglial precursors at P4 in the CbM, followed by a significantly increased number of microglia in the cerebellar WMR and in the PCL/ML of P10 Npc1<sup>nmf164</sup> mice. Furthermore, the number of CLEC7A<sup>+</sup> PAM in the WMR was also increased in Npc1<sup>nmf164</sup> mice suggesting that NPC1 deficiency amplifies the proliferative activity of microglial precursors during highly proliferative stages. An increased number of microglia was still found at P14 and in post-weaning Npc1<sup>nmf164</sup> mice (Kavetsky et al., 2019), indicating that the active proliferation of microglial precursors in the WMR leads to a higher number of these cells in the cerebellar cortex. A few CLEC7A<sup>+</sup> differentiating microglia were observed in Npc1<sup>nmf164</sup> mice at P10, but these cells were no longer seen at P14 indicating a possible failure of these cells to rapidly downregulate Clec7a expression during their differentiation at P10. Interestingly, CLEC7A is not only a specific marker for PAM, but also for DAM, which are only found in neurodegenerative conditions (Keren-Shaul et al., 2017; Krasemann et al., 2017), including NPC (Cougnoux et al., 2018). We also confirm the reappearance of CLEC7A<sup>+</sup> microglia during NPC neurodegeneration in P60 Npc1<sup>nmf164</sup> cerebella, but
at this stage these CLEC7A+ DAM are mainly found at the ML where PC dendrites are degenerating. It is speculated that PAM-derived microglia is transcriptionally predisposed to phagocytosis (Li et al., 2019), therefore the NPC cerebellum possesses a higher density of these type of microglia that is more phagocytic. In fact, decreased levels of MBP in Npc1<sup>nmf164</sup> mice (Caporali et al., 2016) correlate to the increased density of PAM, which actively phagocyte OPCs (Li et al., 2019). Future studies targeting PAM in NPC are needed to understand the role of this subset of cells in the early onset and neurodegeneration of this disease.

Developmental genes like Clec7a are reactivated during neurodegenerative conditions by the TREM2/APOE pathway (Krasemann et al., 2017), which does not activate these genes in PAM during development (Li et al., 2019). Recent in vitro studies have shown that lysosomal accumulation of cholesterol in NPC1 deficient cells causes constitutive activation of the mTOR pathway, which is involved in cell growth and proliferation (Castellano et al., 2017; Lim et al., 2019). Intriguingly, overactivation of the mTOR pathway in mouse mature microglia in vivo leads to less ramified microglia, increased proliferation and robust phagocytic activity in the absence of an inflammatory response in a mouse model of epilepsy (Zhao et al., 2018). It is highly probable that NPC1 deficiency causes the pathological changes in developmental microglia through the overactivation of the mTOR pathway, since increased proliferation, impaired differentiation and increased phagocytic activity were hallmarks of postnatal Npc1<sup>nmf164</sup> microglia. Further studies are warranted to determine the role of the mTOR signaling pathway in NPC microglia pathology.

Microglial cells play an important role in the clearance of apoptotic cells during neuronal developmental death (Ashwell, 1990; Mosser et al., 2017). However, it has been also demonstrated, that microglia can induce apoptosis in the neurons they
phagocytose (Mosser et al., 2017). In this study, an abundant number of maturing microglia in the ML containing phagocytic cups in both WT and Npc1<sup>nmf164</sup> mice were found at the end of the second postnatal week. It was also evident that the number of phagocytic cups and phagocytic cups containing pyknotic bodies was significantly higher in Npc1<sup>nmf164</sup> mice than in WT mice. A high content of phagosomes in P14 microglia at the ML confirmed that at this stage of postnatal development microglial cells were engaged in phagocytic activity. Previous work in the developing rat cerebellum found that the density of phagocytic cups peak around P17 (Perez-Pouchoulen et al., 2015), supporting our findings that microglia is highly phagocytic by the end of the second postnatal week. It is presumed that pyknotic bodies observed at the ML are apoptotic granule precursor cells that were migrating from the ECL into the IGL during postnatal development (Wood et al., 1993). Interestingly, a reduced number of cerebellar granule cells and reductions in cerebellar lobule size at the end of postnatal development have been found in Npc1<sup>−/−</sup> mice (Nusca et al., 2014). It is possible that the increased number of phagocytic cups and the engulfed pyknotic bodies in Npc1<sup>nmf164</sup> mice are caused by the increased number of noninflammatory microglia in the developing mutant cerebellum, which could also increase the developmental apoptotic death of cells at the ML. Indeed, an increased number of apoptotic cells was found in mice with elevated microglial phagocytic activity due to the constitutive activation of the mTOR pathway in noninflammatory microglia (Zhao et al., 2018), suggesting that phagocytic microglia can induce and increase developmental neuronal apoptosis.

In the cerebellum, developmental CF synapse elimination and refinement occur during the first three postnatal weeks (Kano and Watanabe, 2019). Initially, multiple CFs innervate the soma of PCs, however, the selective synaptic
strengthening of a single CF (P3-P7) begins the early phase of synapse elimination of the redundant CFs synapses (P8-P11). Translocation and expansion of the strongest CF to the proximal region of PC dendrites occur along with the second phase of CF synapse elimination (P12-P17), which is also dependent on excitatory synapse formation between PFs and PC dendrites (Kano and Watanabe, 2019). Here we found that VGLUT2+ synaptic inputs from CFs were significantly reduced in the ML of Npc1<sup>nmf164</sup> mice at P14, suggesting that NPC1 deficiency affects CF synapse formation. Our results also indicate that translocation of CF synaptic inputs from the PC soma to the proximal region of PCs dendrites was impaired since a higher number of PC somas contained VGLUT2+ and a greater number of VGLUT2+ puncta per PC soma were found in Npc1<sup>nmf164</sup> mice. A previous study found that not only the glutamatergic CF synaptic inputs were reduced in Npc1<sup>nmf164</sup> mice, but also the GABAergic (basket/stellate cells) inputs, indicating that deficiency of the NPC1 protein broadly impairs synaptic connectivity in the cerebellum (Caporali et al., 2016). These synaptic defects were also associated with developmental deficits in motor skill acquisition in the Npc1<sup>nmf164</sup> mouse.

Importantly, previous studies have demonstrated that microglia have a role in developmental activity-dependent synaptic pruning in the brain (Gunner et al., 2019; Schafer et al., 2012; Tremblay et al., 2010). In fact, microglia engulf and remove intact presynaptic elements during the process of developmental synaptic pruning (Gunner et al., 2019; Schafer et al., 2012; Tremblay et al., 2010). The role of microglia in developmental CF synapse refinement is not completely understood. However, recent studies have shown that genetic or pharmacological depletion of microglia in the cerebellum impairs the early and late stages of CF synapse elimination during postnatal development leading to behavioral and motor deficits.
Furthermore, it is thought that microglia facilitate developmental CF synapse elimination by promoting GABAergic inhibition of PCs (Nakayama et al., 2018). Here, we aimed to determine if cerebellar microglia engulf CF presynaptic inputs at P14 (late-phase of CF synapse elimination) and if NPC1 deficiency alters this microglial pruning function. In fact, we found that at P14, microglia were contacting and engulfing VGLUT2\textsuperscript{+} inputs in the ML of WT mice. These results are in accordance with the abundant density of CD68\textsuperscript{+} phagosomes observed in P14 microglia, indicating that microglia are highly phagocytic in the cerebellum at this postnatal age. Interestingly, at this age, the increased density of somatic VGLUT2\textsuperscript{+} in $Npc1^{nmf164}$ PCs coincided with a higher percentage of PC somas contacted by microglia. Furthermore, P14 microglia contacted and engulfed more VGLUT2\textsuperscript{+} inputs in $Npc1^{nmf164}$ mice than in WT mice. It is possible that the reduced elimination and translocation of VGLUT2\textsuperscript{+} inputs in $Npc1^{nmf164}$ PC somas could be the consequence of decreased GABAergic stimulation to PCs (Caporali et al., 2016), which is also modulated by microglia (Nakayama et al., 2018). Also, it is thought that microglia preferentially engulf and remove presynaptic inputs with decreased activity (Gunner et al., 2019; Schafer et al., 2012; Tremblay et al., 2010), which could explain why a higher number of VGLUT2\textsuperscript{+} inputs are contacted or engulfed by microglia in $Npc1^{nmf164}$ mice. Disrupted presynaptic terminals in NPC can predispose neurons to early neurodegeneration, as demonstrated in a mouse model of the lysosomal storage disease mucopolysaccharidosis type IIIA, where restoration of presynaptic function delayed neurodegeneration (Sambri et al., 2017). Current work in our laboratory is investigating if this phagocytic activity of NPC microglia affects other synaptic refinement and remodeling programs in PCs. Overall, our data demonstrate that deficiency of NPC1 affects microglia and synapse development.
during the postnatal development of the cerebellum, leading to behavioral deficits and predisposing PCs to neurodegeneration.

**Materials and Methods**

**Animals**

All experiments involving mice were conducted in accordance to policies and procedures described in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Animal Care and Use Committees at the Rowan University School of Osteopathic Medicine. The C57BL/6J-*Npc1*<sup>nmf164</sup>/J mouse strain (Jax stock number 004817) was provided by Dr. Robert Burgess at The Jackson Laboratory. *Npc1*<sup>nmf164</sup> heterozygous mice were bred and housed in a 12/12-hour light/dark cycle to generate both WT and *Npc1*<sup>nmf164</sup> homozygous mutant mice. Both males and females were used in this study, at a ratio of 2:2 when 4 mice were used.

**Mouse Perfusion and Tissue Preparation**

Mice were euthanized with CO<sub>2</sub> and transcardially perfused with 1X PBS followed by 4% paraformaldehyde. After perfusion, mice were decapitated and their brains were carefully dissected and fixed by immersion in 4% paraformaldehyde overnight. After fixation, brains were rinsed in 1X PBS, immersed in 30% sucrose/PBS solution overnight at 4°C, frozen in OCT, and cryosectioned at 25μm or 50μm (floating sections).
**Immunohistochemistry**

For immunostaining, brain sections in slides (25 μm) or as floating sections (50 μm) were rinsed once in 1X PBT (PBS + 1% Triton 100X) and incubated in primary antibodies diluted with 1X PBT + 20% normal donkey serum for overnight at 4°C. After incubation with primary antibodies, sections were rinsed three times with 1X PBT for 10 min and incubated for two hours in the corresponding secondary antibodies (1:800, Jackson-ImmunoResearch or Invitrogen). Tissue was then washed three times with 1X PBT for 10–15 min, incubated with DAPI and mounted in Poly aquamount (Polysciences). The following primary antibodies were used: rabbit anti-IBA1 (1:200, Wako, # 019-19741), rat anti-KI67 (1:200, SolA15, ThermoFisher, #14-5698-82), rat anti-CLEC7A (1:50, 2A11, Bio-Rad, #MCA2289), mouse anti-CALB (calbindin, 1:200, Sigma-Aldrich, #C9848), rat anti-CD68 (1:200, Bio-Rad, #MCA1957), Lycopersicon esculentum(Tomato)-Lectin (1:200, Sigma-Aldrich, #L0401) and guinea-pig anti-VGLUT2 (1:800, Synaptic Systems, #135 404).

**Microscopy image analysis**

To keep consistency between samples, imaging and quantitative analyses to determine changes in the number of IBA1+ cells and IBA1+/KI-67+ cells were performed in the first four anterior cerebellar lobules (I-IV). For quantification of IBA1+ and IBA1+/KI67+ cells in the developing cerebellum, four images (1 per lobule) were taken from two cerebellar cryosections for each mouse (8 images per mouse) with an inverted Leica DMI8 fluorescent microscope. For the WMR, one image per section (two sections) were taken using the Kyence microscope. The imaged regions were randomly selected and investigators were blinded to the genotype. Once the images were taken, a box of 250 X 350 pixels (cerebellar cortex) or 350 X 450 pixels...
(CbM) was used to crop the images (1-2 boxes per image), so that the area used for the cell counting was consistent between images/animals, and included IGL, PCL/ML, EGL and PS in P4 mice, PCL/ML, EGL and PS in P10 mice, and PCL/ML, EGL and PS in P14 mice. The cropped images were manually counted using the cell counter plugin from the ImageJ (1.47 d) software. For quantification of Lectin+ IGL capillaries total length, a region of 400 X 300 pixels was cropped and the Simple Neurite Tracer from ImageJ was used to trace the capillaries and obtain the length measure of every capillary in the image. For quantification of CLEC7A/IBA1 area and MBP intensity, two images per section were taken using the Kyence microscope. The measure of CLEC7A/IBA1 and MBP immunostained area was selected by threshold and measured by the Analyze plugin of ImageJ. Investigators were blind to the genotype of the tissue while counting the cells or immunostained areas.

For 3D image reconstructions and analyses, three sagittal 50μm cerebellar sections were immunostained by free floating immunohistochemistry. All the images analyzed by the Bitplane Imaris™ software, were acquired using a Nikon A1R Confocal System equipped with Live Cell 6 Laser Line and Resonant Dual Scanner. Confocal image stacks were acquired using a 40X objective lens with a 1μm interval through a 50μm z-depth of the tissue. Three confocal images per mouse were taken from the first three lobes (1 per lobe), in the CbM (P4), in the WMR (P10) and in the cerebellar cortex (P10 and P14). To quantify microglial precursors in the WMR of P10 mice, a box of 500 X 500μm was used and Imaris™ surface rendering tool was used to calculate the volume of IBA1+ cells and colocalization of IBA1+ and KI67+ cells inside the box. The quantification of microglia with phagocytic cups and the number of phagocytic cups were quantified manually in 40X confocal images of the
ML in P14 cerebella using the cell counter plugin from ImageJ. Two to three images per mouse (n=4) were used for the quantifications in confocal images.

Quantitative analysis of 3D microglia morphology was performed using the Surface rendering tool for cell volume and the Filament Tracer for processes volume and ramification, both tools are part of the Bitplane Imaris™ software. Confocal z-stack images of ~50μm were taken and twenty IBA1+ (5-6 per mouse, n=4 mice) were segregated using 3D surface rendering to be used for the Filament Tracer tool that determines processes length, volume and ramification. The 3D surface rendering was also used to segregate IBA1+ microglia and quantified CD68+ phagosomes inside microglia, or VGLUT2+ synaptic terminals contacted or engulfed by microglia, by using the “Mask all” tool which creates a new channel of the immunostained areas that are inside the created surface (in this case IBA1 surface) and clearing all the fluorescence that is not found overlapping/contacting the rendering surface. The sum of the CD68 or VGLUT2 volume contacted or inside the IBA1 surface was calculated and provided by the software and used for the data analysis presented here. The quantifications of VGLUT2 volume in the ML of P14 mice was performed by cropping the ML region (300μm height X 400μm wide) in 40X confocal images and creating a 3D surface rendering that was used to obtain the sum of the volume of all the VGLUT2+ inputs inside the cropped image. To quantify the volume of VGLUT2+ inputs contacted or engulfed by microglia in the ML, the “Mask all” tool, which creates a new channel of the IBA1 immunostained area that are in contact or inside the created surface (in this case the VGLUT2 surface) was used, then a new surface was created for the IBA1/VGLUT2 overlapping inputs and the calculated volume sum values were collected. The quantification of the percentage of CALB+ PCs with VGLUT2+ inputs and the number of VGLUT2+ inputs
per cell were quantified manually in 1μm Z-sections from 40X confocal images using the cell counter plugin from the ImageJ (1.47 d) software. Two to three images per mouse (n=4) were used for these quantifications.

Quantitative Real-Time Polymerase chain reaction (PCR) array

To measure gene expression changes in mouse innate immune response genes in the cerebellum of WT and \(Npc1^{nmf164}\) mice Real-Time PCR array TaqMan™ Array Mouse Immune Response. Cerebella from P30 WT (n=4), P30 \(Npc1^{nmf164}\) (n=4) and P90 \(Npc1^{nmf164}\) mice (n=3) were collected after mice were perfused with 1X PBS and treated overnight in RNAlater for long-term storage. For RNA extraction, 30mg of cerebellum from each mouse was used and total cellular RNA was extracted and purified from each individual tissue according to the TRizol™ Plus RNA Purification Kit (ThermoFisher) manufacturer protocol; RNA concentration and purity were determined using the Qubit® 2.0 Fluorometer using the RNA quantification kit (Invitrogen). RNA (1 μg) was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). Real-time quantitative PCR was performed using the 96-Well TaqMan™ Array Mouse Immune Response (ThermoFisher) according to the manufacturer protocol and one PCR array plate per mouse was used. Briefly, cDNA samples were diluted appropriately, 540 μl cDNA template was added to 540 μl of 2X real-time quantitative reaction mixture (TaqMan™ Fast Advanced Master Mix, ThermoFisher), and 10 μl of reaction liquid plus cDNA were added to each well of the PCR array, containing gene specific primers. Conditions for the real-time quantitative PCR reaction were as follows: UNG incubation 50°C for 2 min, enzyme activation 95°C for 20 seconds, 40 amplification cycles of denaturing at 95°C for 3 s, annealing/extension at 60°C for 30 s, followed
by acquisition of fluorescence signal. Data analysis is based on the ΔΔCt method with normalization of raw signal data to housekeeping genes incorporated on the TaqMan™ Array Mouse qPCR plate.

Statistical Analysis
Data were analyzed using GraphPad Prism software. Significance was calculated using unpaired t tests for comparisons between two groups. p-values are provided as stated by GraphPad Prism software and significance was determined with p-values less than 0.05.

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Competing Interests
No competing interests declared.

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References

Ashwell, K. (1990). Microglia and cell death in the developing mouse cerebellum. *Brain Res Dev Brain Res* **55**, 219-230.

Ayata, P., Badimon, A., Strasburger, H. J., Duff, M. K., Montgomery, S. E., Loh, Y. E., Ebert, A., Pimenova, A. A., Ramirez, B. R., Chan, A. T., et al. (2018). Epigenetic regulation of brain region-specific microglia clearance activity. *Nat Neurosci* **21**, 1049-1060.

Bennett, M. L., Bennett, F. C., Liddelow, S. A., Ajami, B., Zamanian, J. L., Fernhoff, N. B., Mulinyawe, S. B., Bohlen, C. J., Adil, A., Tucker, A., et al. (2016). New tools for studying microglia in the mouse and human CNS. *Proc Natl Acad Sci U S A* **113**, E1738-1746.

Butts, T., Green, M. J. and Wingate, R. J. (2014). Development of the cerebellum: simple steps to make a 'little brain'. *Development* **141**, 4031-4041.

Caporali, P., Bruno, F., Palladino, G., Dragotto, J., Petrosini, L., Mangia, F., Erickson, R. P., Canterini, S. and Fiorenza, M. T. (2016). Developmental delay in motor skill acquisition in Niemann-Pick C1 mice reveals abnormal cerebellar morphogenesis. *Acta Neuropathol Commun* **4**, 94.

Castellano, B. M., Thelen, A. M., Moldavski, O., Feltes, M., van der Welle, R. E., Mydock-McGrane, L., Jiang, X., van Eijkeren, R. J., Davis, O. B., Louie, S. M., et al. (2017). Lysosomal cholesterol activates mTORC1 via an SLC38A9-Niemann-Pick C1 signaling complex. *Science* **355**, 1306-1311.

Cologna, S. M., Cluzeau, C. V., Yanjanin, N. M., Blank, P. S., Dail, M. K., Siebel, S., Toth, C. L., Wassif, C. A., Lieberman, A. P. and Porter, F. D. (2014). Human and mouse neuroinflammation markers in Niemann-Pick disease, type C1. *J Inherit Metab Dis* **37**, 83-92.

Cougnoux, A., Drummond, R. A., Collar, A. L., Iben, J. R., Salman, A., Westgarth, H., Wassif, C. A., Cawley, N. X., Farhat, N. Y., Ozato, K., et al. (2018). Microglia activation in Niemann-Pick disease, type C1 is amendable to therapeutic intervention. *Hum Mol Genet* **27**, 2076-2089.

Cuadros, M. A., Rodriguez-Ruiz, J., Calvente, R., Almendros, A., Marin-Teva, J. L. and Navascues, J. (1997). Microglia development in the quail cerebellum. *J Comp Neurol* **389**, 390-401.

Erickson, R. P. and Bernard, O. (2002). Studies on neuronal death in the mouse model of Niemann-Pick C disease. *J Neurosci Res* **68**, 738-744.

Ford, F. R., Livingston, S. and Pryles, C. (1951). Familial degeneration of the cerebral gray matter in childhood with convulsions, myoclonus, spasticity, cerebellar ataxia, choreoathetosis, dementia, and death in status epilepticus; differentiation of infantile and juvenile types. *J Pediatr* **39**, 33-43.

Garver, W. S., Francis, G. A., Jelinek, D., Shepherd, G., Flynn, J., Castro, G., Walsh Vockley, C., Coppock, D. L., Pettit, K. M., Heidenreich, R. A., et al. (2007). The National Niemann-Pick C1 disease database: report of clinical features and health problems. *Am J Med Genet A* **143A**, 1204-1211.

Gunner, G., Cheadle, L., Johnson, K. M., Ayata, P., Badimon, A., Mondo, E., Nagy, M. A., Liu, L., Bemiller, S. M., Kim, K. W., et al. (2019). Sensory lesioning induces microglial synapse elimination via ADAM10 and fractalkine signaling. *Nat Neurosci* **22**, 1075-1088.

Hashimoto, K. and Kano, M. (2013). Synapse elimination in the developing cerebellum. *Cell Mol Life Sci* **70**, 4667-4680.
Hoque, M., Rentero, C., Conway, J. R., Murray, R. Z., Timpson, P., Enrich, C. and Grewal, T. (2015). The cross-talk of LDL-cholesterol with cell motility: insights from the Niemann Pick Type C1 mutation and altered integrin trafficking. Cell Adh Migr 9, 384-391.

Kana, V., Desland, F. A., Casanova-Acebes, M., Ayata, P., Badimon, A., Nabel, E., Yamamura, K., Sneboer, M., Tan, I. L., Flanigan, M. E., et al. (2019). CSF-1 controls cerebellar microglia and is required for motor function and social interaction. J Exp Med 216, 2265-2281.

Kano, M. and Watanabe, T. (2019). Developmental synapse remodeling in the cerebellum and visual thalamus. F1000Res 8.

Kavetsky, L., Green, K. K., Boyle, B. R., Yousufzai, F. A. K., Padron, Z. M., Melli, S. E., Kuhnel, V. L., Jackson, H. M., Blanco, R. E., Howell, G. R., et al. (2019). Increased interactions and engulfment of dendrites by microglia precede Purkinje cell degeneration in a mouse model of Niemann Pick Type-C. Sci Rep 9, 14722.

Keren-Shaul, H., Spinrad, A., Weiner, A., Matcovitch-Natan, O., Dvir-Szternfeld, R., Ulland, T. K., David, E., Baruch, K., Lara-Astaiso, D., Toth, B., et al. (2017). A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. Cell 169, 1276-1290 e1217.

Krasemann, S., Madore, C., Cialic, R., Baufeld, C., Calcagno, N., El Fatimy, R., Beckers, L., O'Loughlin, E., Xu, Y., Fanek, Z., et al. (2017). The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases. Immunity 47, 566-581 e569.

Li, Q., Cheng, Z., Zhou, L., Darmanis, S., Neff, N. F., Okamoto, J., Gulati, G., Bennett, M. L., Sun, L. O., Clarke, L. E., et al. (2019). Developmental Heterogeneity of Microglia and Brain Myeloid Cells Revealed by Deep Single-Cell RNA Sequencing. Neuron 101, 207-223 e210.

Lim, C. Y., Davis, O. B., Shin, H. R., Zhang, J., Berdan, C. A., Jiang, X., Counihan, J. L., Ory, D. S., Nomura, D. K. and Zoncu, R. (2019). ER-lysosome contacts enable cholesterol sensing by mTORC1 and drive aberrant growth signalling in Niemann-Pick type C. Nat Cell Biol 21, 1206-1218.

Maue, R. A., Burgess, R. W., Wang, B., Woolley, C. M., Seburn, K. L., Vanier, M. T., Rogers, M. A., Chang, C. C., Chang, T. Y., Harris, B. T., et al. (2012). A novel mouse model of Niemann-Pick type C disease carrying a D1005G-Npc1 mutation comparable to commonly observed human mutations. Hum Mol Genet 21, 730-750.

Mengel, E., Klunemann, H. H., Lourenco, C. M., Hendriksz, C. J., Sedel, F., Walterfang, M. and Kolb, S. A. (2013). Niemann-Pick disease type C symptomatology: an expert-based clinical description. Orphanet J Rare Dis 8, 166.

Mosser, C. A., Baptista, S., Arnoux, I. and Audinat, E. (2017). Microglia in CNS development: Shaping the brain for the future. Prog Neurobiol 149-150, 1-20.

Nakayama, H., Abe, M., Morimoto, C., Iida, T., Okabe, S., Sakimura, K. and Hashimoto, K. (2018). Microglia permit climbing fiber elimination by promoting GABAergic inhibition in the developing cerebellum. Nat Commun 9, 2830.

Nikodemova, M., Kimyon, R. S., De, I., Small, A. L., Collier, L. S. and Watters, J. J. (2015). Microglial numbers attain adult levels after undergoing a rapid decrease in cell number in the third postnatal week. J Neuroimmunol 278, 280-288.
Nusca, S., Canterini, S., Palladino, G., Bruno, F., Mangia, F., Erickson, R. P. and Fiorenza, M. T. (2014). A marked paucity of granule cells in the developing cerebellum of the Npc1(-/-) mouse is corrected by a single injection of hydroxypropyl-beta-cyclodextrin. *Neurobiol Dis* 70, 117-126.

Patterson, M. (1993). Niemann-Pick Disease Type C. In *GeneReviews(R)* (ed. R. A. Pagon, M. P. Adam, H. H. Ardinger, S. E. Wallace, A. Amemiya, L. J. H. Bean, T. D. Bird, N. Ledbetter, H. C. Mefford, R. J. H. Smith, et al.). Seattle (WA).

Perez-Pouchoulen, M., VanRyzin, J. W. and McCarthy, M. M. (2015). Morphological and Phagocytic Profile of Microglia in the Developing Rat Cerebellum. *eNeuro* 2.

Perry, V. H., Nicoll, J. A. and Holmes, C. (2010). Microglia in neurodegenerative disease. *Nat Rev Neurol* 6, 193-201.

Sambri, I., D'Alessio, R., Ezhova, Y., Giuliano, T., Sorrentino, N. C., Cacace, V., De Risi, M., Cataldi, M., Annunziato, L., De Leonibus, E., et al. (2017). Lysosomal dysfunction disrupts presynaptic maintenance and restoration of presynaptic function prevents neurodegeneration in lysosomal storage diseases. *EMBO Mol Med* 9, 112-132.

Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., Ransohoff, R. M., Greenberg, M. E., Barres, B. A. and Stevens, B. (2012). Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron* 74, 691-705.

Shapiro E.G., K. K. A. (1994). Dementia in Childhood: Issues in Neuropsychological Assessment with Application to the Natural History and Treatment of Degenerative Storage Diseases. In *Advances in Child Neuropsychology* (ed. H. S. R. Tramontana M.G.), pp. 119-171. New York: Springer.

Soto, I. and Howell, G. R. (2014). The complex role of neuroinflammation in glaucoma. *Cold Spring Harb Perspect Med* 4.

Swanson, J. A. (2008). Shaping cups into phagosomes and macropinosomes. *Nat Rev Mol Cell Biol* 9, 639-649.

Tremblay, M. E., Lowery, R. L. and Majewska, A. K. (2010). Microglial interactions with synapses are modulated by visual experience. *PLoS Biol* 8, e1000527.

Vanier, M. T. and Millat, G. (2003). Niemann-Pick disease type C. *Clin Genet* 64, 269-281.

Watanabe, M. and Kano, M. (2011). Climbing fiber synapse elimination in cerebellar Purkinje cells. *Eur J Neurosci* 34, 1697-1710.

Wood, K. A., Dipasquale, B. and Youle, R. J. (1993). In situ labeling of granule cells for apoptosis-associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum. *Neuron* 11, 621-632.

Zhao, X., Liao, Y., Morgan, S., Mathur, R., Feustel, P., Mazurkiewicz, J., Qian, J., Chang, J., Mathern, G. W., Adamo, M. A., et al. (2018). Noninflammatory Changes of Microglia Are Sufficient to Cause Epilepsy. *Cell Rep* 22, 2080-2093.
Figure 1. Changes in innate immune responses are not evident in the cerebellum at post-weaning age in Npc1<sup>nmf164</sup> mice. A) Images from P30 and P90 mice showing IBA1<sup>+ </sup>microglia cells containing CD68<sup>+ </sup>phagosomes at the ML. B) Expression changes of genes associated to the innate immune response comparing Npc1<sup>nmf164</sup> mice at P30 (red, n=3) and P90 (blue, n=3) versus WT mice (n=4). Scale bars: (A) 30μm.
Figure 2. Decreased radial migration of microglial precursors in P4 Npc1<sup>nmf164</sup> mice.

A) IBA1<sup>+</sup> differentiating microglia in the cortex versus IBA1<sup>+</sup> round/ameboid microglial precursors in the cerebellum at P4 in WT mice. B) IBA1<sup>+</sup> microglial precursors concentrated in the developing CbM of the cerebellum (dashed-semicircle). IBA1<sup>+</sup> microglial precursors are observed migrating radially toward the cerebellar cortex layers (IGL, PCL and EGL) following Tomato Lectin<sup>+</sup> capillaries. C) Quantification of
IBA1+ cells in the developing CbM at P4. D) Quantification of the number of IBA1+ cells in the IGL and PS at P4. E) IBA1+ and KI67+ cells in the developing CbM at P4. F) The number of IBA1+/KI67+ cells is significantly higher in the CbM of *Npc1*<sup>nmf164</sup> mice. G) The percentage of KI67+ microglia is significantly higher in the CbM of *Npc1*<sup>nmf164</sup> mice. H) IBA1+ and KI67+ cells in the developing cerebellar cortex at P4. I) The number of IBA1+/KI67+ cells is significantly lower in the IGL of *Npc1*<sup>nmf164</sup> mice. Data are presented as mean ± SEM n= 3-4 mice. ***P < 0.001, and ****P < 0.0001. Scale bars: (A, E and H) 50µm, (B) 100µm upper row, 50µm lower row. In graphs: u.a.= unit area.
Figure 3. Increased number of microglial precursors and differentiating microglia in Npc1^{nmf164} mice at P10. A) Images of P10 whole cerebellum sections immunostained with IBA1 showing the distribution and abundance of IBA1+ cells in WT and Npc1^{nmf164} mice. Squares are indicating some regions in the WM. B) High magnified images in the WMR (axonal tract, AT) immunostained with IBA1 and KI67 showing higher number of IBA1+ cells in the Npc1^{nmf164} mouse. C) High magnified images in a region of the cerebellar cortex immunostained with IBA1 and KI67 showing higher number of IBA1+ cells in the Npc1^{nmf164} mouse. D) Quantification of the volume of IBA1+ microglia clusters in the cerebellar WMR. E) No differences were found in the percentage of WM IBA1+/KI67+ cells between WT and Npc1^{nmf164} mice. F) Quantification of the number of IBA1+ cells in the PCL/ML and PS. G) Quantification of the number of IBA1+/KI67+ cells in the PCL/ML and PS. Data are presented as mean ± SEM (D & E) n= 2 images per mouse n= 4 mice, (F & G) n= 4 mice. *P < 0.05. Scale bars: (A) 250μm (B) 30μm, (C) 30μm. In graphs: u.a.= unit area.
Figure 4. Proliferative-Region-Associated Microglia (PAM) are increased in the cerebellar WMR of P10 \textit{Npc1}^{nmt164} mice. A) Images of P10 whole cerebellum sections immunostained with CLEC7A showing the distribution and abundance of CLEC7A$^+$ cells in WT and \textit{Npc1}^{nmt164} mice. B) High magnified images from squares at (A) showing CLEC7A$^+$ cells colocalizing with IBA1 in the cerebellar WMR of WT and \textit{Npc1}^{nmt164} mice. C) Quantification of the number of CLEC7A$^+$ clusters (CLS, arrowheads) per cerebellum (CB) in WT and \textit{Npc1}^{nmt164} mice. D) Quantification of the percentage area immunostained by CLEC7A in WT and \textit{Npc1}^{nmt164} mice. E) Quantification of the percentage area immunostained by IBA1 in WT and \textit{Npc1}^{nmt164} mice. F) Fraction area of IBA1 immunostained by CLEC7A$^+$ in WT and \textit{Npc1}^{nmt164} mice. G) Images of the morphology of P10 IBA1$^+$ microglia that are CLEC7A$^+$ in WT and \textit{Npc1}^{nmt164} mice. H-I) Quantitative analysis of the total length (H) and number of terminal points (I) of P10 CLEC7A$^+$ microglia. J) Evidence of CLEC7A$^+$ differentiating microglia in P10 \textit{Npc1}^{nmt164} mice. K) Immunostaining of WM track (dashed red line)
with MBP showing myelinated axons intermingling between CLEC7A+/IBA1+ microglia in WT and Npc1<sup>nmf164</sup> mice. White asterisk (*) is showing WM region with high levels of MBP. L) Differences in MBP integrated density were not statistically significant between P10 WT and Npc1<sup>nmf164</sup> mice. Data are presented as mean ± SEM (C-F & L) n= 2 images per mouse n= 4 mice, (H & I) 19-20 cells per mouse, n= 4 mice. **P < 0.01. Scale bars: (A) 250μm (B) 50μm, (G) 10μm, (J & K) 30μm. In graphs: u.a.= unit area, a.u.=arbitrary units.
Figure 5. Increased number of differentiating microglia in Npc1<sup>nmf164</sup> mice at P14. A) Images of P14 whole cerebellum sections immunostained with IBA1 showing the distribution and abundance of IBA1<sup>+</sup> cells in WT and Npc1<sup>nmf164</sup> mice. B) High magnified images of the cerebellar cortex (PCL, ML, EGL and PS) immunostained with IBA1 and KI67 showing higher number of IBA1<sup>+</sup> cells in the Npc1<sup>nmf164</sup> mouse. C) Quantification of the number of IBA1<sup>+</sup> cells in the PCL/ML and PS at P14. D) Quantification of the number of IBA1<sup>+</sup>/KI67<sup>+</sup> cells in the PCL/ML and PS at P14. E)
NPC1 deficiency affects migration and proliferation events of microglial precursors (MPC), PAM and differentiating microglia (MGC) in the postnatal cerebellum. Data are presented as mean ± SEM (C) n= 4 (WT) and 6 (Npc1<sup>nmf164</sup>) mice, (D) n= 4 mice. ****P < 0.0001. Scale bars: (A) 250µm (B) 30µm. In graphs: u.a.= unit area.
Figure 6. Microglia differentiation is impaired in *Npc1*\textsuperscript{nmf164} mice. A) Representative images of IBA1 microglia from P10 and P14 WT and *Npc1*\textsuperscript{nmf164} mice show less ramified microglia with shorter processes in *Npc1*\textsuperscript{nmf164} mice. B) Quantitative analysis of cell volume at P10. C-D) Total length (C) and number of terminal points (D) of microglia processes at P10. E-G) Total length (E) and number of terminal points (G) of microglia processes at P14. Data are presented as mean ± SEM, (B-D) n= 25 cells (WT) from 4 mice, n= 19 cells (*Npc1*\textsuperscript{nmf164}) from 4 mice. (E-G) n= 20 cells (WT) from 4 mice, n= 23 cells (*Npc1*\textsuperscript{nmf164}) from 4 mice. **P < 0.01, ***P < 0.001. Scale bars: (A) 10µm.
Figure 7. Increased number of phagocytic cups in \textit{Npc1}^{nmf164} mice at P14. A) Images of P14 cerebellar sections at the ML showing the IBA1\(^+\) cells in WT and \textit{Npc1}^{nmf164} mice containing phagocytic cups. B) High magnified images from insets in (A) showing \textit{Npc1}^{nmf164} microglia with two phagocytic cups containing pyknotic bodies (arrows). C) Quantification of the number of IBA1\(^+\) cells with phagocytic cups in the ML of P14 mice. D) Quantification of the number of IBA1\(^+\) cells with 1, 2 or 3 phagocytic cups in WT and \textit{Npc1}^{nmf164} mice. E) The total of phagocytic cups per unit area in the ML. F) The number of phagocytic cups containing pyknotic bodies. G) Images showing P14 microglia immunostained with IBA1 and CD68 in WT and \textit{Npc1}^{nmf164} mice. H) Quantification of the mean volume of CD68\(^+\) phagosomes. I) Total (summed) volume of CD68\(^+\) phagosomes between WT and \textit{Npc1}^{nmf164} mice.
Data are presented as mean ± SEM, (C-F) WT n=13 images from 4 mice, \(Npc1^{nmf164}\) n=11 images from 4 mice. *P < 0.05, ***P < 0.001, ****P < 0.0001. Scale bars: (A) 40\(\mu\)m, (B and G) 20\(\mu\)m. In graphs: u.a.= unit area.
Figure 8. NPC1 deficiency disrupts CF synapse elimination and translocation in
*Npc1<sup>nmf164</sup>* mice. A) (A’) Representative confocal images from P14 WT and
*Npc1<sup>nmf164</sup>* mice showing CALB<sup>+</sup> PCs innervated by VGLUT2<sup>+</sup> inputs from CF at the
ML. (A’’) 3D surface rendering images where only VGLUT2<sup>+</sup> inputs in the ML from
WT and *Npc1<sup>nmf164</sup>* mice were selected (VGLUT2<sup>+</sup> inputs in PCL were not included).
(A’’’) Overlap of VGLUT2<sup>+</sup> inputs segregated in (A’’) with CALB<sup>+</sup> PCs to show how
only dendritic VGLUT2 was segregated for quantitative analysis. Images of the P14
WT and P14 *Npc1<sup>nmf164</sup>* are from littermate mice. B) Quantitative analysis of
dendritic VGLUT2<sup>+</sup> inputs at the ML. C) Representative Z-section of confocal images
showing VGLUT2⁺ inputs in the PCL region innervating CALB⁺ PC somas. D) Percentage of PC somas containing VGLUT2⁺ inputs. E) Average of VGLUT2 puncta per PC soma. F) Confocal images from P14 WT and Npc1<sup>nmf164</sup> mice showing the distribution and interaction of IBA1⁺ microglia with CALB⁺ PCs innervated by VGLUT2⁺ inputs from CF. G) Images showing segregated VGLUT2⁺ inputs from the ML contacted or engulfed by microglia (MG-CT= microglia contacts). H) Quantitative analysis of the volume of VGLUT2⁺ inputs in the ML (innervating dendrites only) contacted by microglia. I) High magnified images from P14 WT and Npc1<sup>nmf164</sup> mice showing the increased interaction of IBA1⁺ microglia with CALB⁺ PC somas innervated by CF VGLUT2⁺ inputs in Npc1<sup>nmf164</sup> mice. J) Quantitative analysis of the percentage of CALB⁺ PC somas contacted by microglia shows a significant increase in Npc1<sup>nmf164</sup> mice. Data are presented as mean ± SEM, WT n= 8 images from 4 mice, Npc1<sup>nmf164</sup> n= 9 images from 4 mice. *P < 0.05, ***P < 0.001, ****P < 0.0001. Scale bars: (A, F and G) 30μm, (C and I) 15μm. In graphs: u.a. = unit area.
Figure 9. Increased engulfment of CF presynaptic inputs by microglia in P14 Npc1<sup>nmf164</sup> mice. A) Confocal images (A'), and 3D surface renderings (A'') showing segregated IBA1<sup>+</sup> microglial cells from P14 WT and Npc1<sup>nmf164</sup> mice contacting or engulfing VGLUT2<sup>+</sup> inputs. (A''') 3D surface renderings showing only the contacted or engulfed VGLUT2<sup>+</sup> inputs. B) Quantitative analysis of the total volume of contacted or engulfed VGLUT2<sup>+</sup> inputs in P14 IBA1<sup>+</sup> microglia. C) High magnified serial sections from the confocal Z stack in (A) showing VGLUT2<sup>+</sup> inputs innervating CALB<sup>+</sup> dendrites (WT) or PC somas (Npc1<sup>nmf164</sup>) contacted or engulfed by IBA1<sup>+</sup> microglia at P14. Data are presented as mean ± SEM, WT n= 21 cells from 4 mice, Npc1<sup>nmf164</sup> n= 22 cells from 4 mice. *P < 0.05. Scale bars: (A) 10µm, (C) 10µm.
**Supplementary Information**

**Fig. S1.** Capillary density in the cerebellar WMR is similar between WT and \(Npc1^{nmf164}\) mice at P4. A) Sample images showing Lectin\(^+\) capillaries in the WMR of P4 WT and \(Npc1^{nmf164}\) mice. B) Quantitative analysis of the total length of Lectin\(^+\) capillaries in the WMR shows no differences between P4 WT and \(Npc1^{nmf164}\) mice. Data are presented as mean ± SEM (B) n= 3 mice. Scale bars: (A) 20\(\mu m\).
**Fig. S2.** Reappearance of CLEC7A in microglia is observed at P60 when PC degeneration and inflammatory activation of microglia are occurring. At P14, CLEC7A$^+$ microglia were barely detected in the cerebellar white matter (WM) region (or any other region) in WT and *Npc1*$_{nmf164}$ mice. At P60, the appearance of CLEC7A$^+$ microglia is evident primarily at the ML where PC dendrite degeneration is occurring. White circle is indicating an artifact. Scale bars: (A) 50µm