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Neurofibromatosis type 1-dependent alterations in mouse microglia function are not cell-intrinsic

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Abstract

We previously discovered a sex-by-genotype defect in microglia function using a heterozygous germline knockout mouse model of Neurofibromatosis type 1 (Nf1± mice), in which only microglia from male Nf1± mice exhibited defects in purinergic signaling. Herein, we leveraged an unbiased proteomic approach to demonstrate that male, but not female, heterozygous Nf1± microglia exhibit differences in protein expression, which largely reflect pathways involved in cytoskeletal organization. In keeping with these predicted defects in cytoskeletal function, only male Nf1± microglia had reduced process arborization and surveillance capacity. To determine whether these microglial defects were cell autonomous or reflected adaptive responses to Nf1 heterozygosity in other cells in the brain, we generated conditional microglia Nf1-mutant knockout mice by intercrossing Nf1flox/flox with Cx3cr1-CreER mice (Nf1flox/wt; Cx3cr1-CreER mice, Nf1MG± mice). Surprisingly, neither male nor female Nf1MG± mouse microglia had impaired process arborization or surveillance capacity. In contrast, when Nf1 heterozygosity was generated in neurons, astrocytes and oligodendrocytes by intercrossing Nf1flox/flox with hGFAP-Cre mice (Nf1flox/wt; hGFAP-Cre mice, Nf1GFAP± mice), the microglia defects found in Nf1± mice were recapitulated. Collectively, these data reveal that Nf1 sexually dimorphic microglia abnormalities are likely not cell-intrinsic properties, but rather reflect a response to Nf1 heterozygosity in other brain cells.

Keywords Neurofibromin, NF1, Purinergic signaling, Sex differences, Microglia, Cortex, Genetically engineered mice
Introduction

Microglia are highly adaptive cells, comprising 5% of the cells in the central nervous system, which serve numerous functions critical to brain homeostasis and response to disease [43, 68]. Changes in microglia function can originate from cell-intrinsic alterations in their innate properties or reactions to environmental stimuli (cell-extrinsic). For example, APOE4 genotype confers transcriptomic and functional alterations in primary mouse microglia in vitro [41], while microglia-specific ApoE loss reduces A-beta plaque size in a mouse model of Alzheimer’s disease [28]. Conversely, neurons and macroglia (oligodendrocytes and astrocytes) can communicate with microglia to influence their migration and phagocytosis through the elaboration of paracrine factors (e.g., chemokines; [25, 52] and/or expression of cell surface proteins (e.g., CD47; [38]). This adaptability is underscored by multiple reports demonstrating that microglia can undergo changes in gene expression and function when they are analyzed in vitro relative to their in vivo state [6, 43, 49, 62]. In this fashion, understanding microglia function requires a consideration of both cell-autonomous and non-cell-autonomous properties.

We have previously demonstrated that microglia from mice heterozygous for a germline inactivating mutation in the Neurofibromatosis type 1 (NF1) gene exhibit sexually dimorphic impairments in purinergic function [17, 18]. Using in situ analysis of microglia function, we showed that microglia from male, but not female, NF1 ± mice exhibit impaired phagocytosis, ATP-evoked membrane currents, and lesion-induced process accumulation relative to their wild-type counterparts. While these results could be interpreted as revealing cell-intrinsic effects of NF1 mutation on microglia function, it is equally possible that the observed abnormalities result from indirect effects on microglia, operating through the impact of NF1 mutation on other cell types (astrocytes, neurons, oligodendrocytes). This idea derives from recent studies in which we found that neurons with a heterozygous NF1 mutation elaborate paracrine factors that act on T cells [21] and oligodendrocyte precursor cells [50] to modify their function.

To distinguish cell-autonomous from non-cell-autonomous microglia defects, we leveraged two different Cre driver lines to heterozygously delete the NF1 gene in either microglia or neural progenitor cells and their progeny (astrocytes, neurons, oligodendrocytes) in vivo. Surprisingly, we found that heterozygous NF1 loss in microglia had no effect on microglia function, whereas heterozygous NF1 loss in neural progenitor cells (and their progeny) recapitulated the sexually dimorphic microglial defects observed in NF1 ± mice.

Results

Proteomic analysis reveals differences in microglial protein expression between male WT and NF1 ± mice

To define NF1-dependent alterations in murine microglia, we performed an unbiased proteomic analysis. Microglia were isolated from whole brains of male and female WT and NF1 ± mice (12-16 weeks of age) by MACS using CD11b antibodies and subjected to proteomics analysis as previously described Mertins et al. [46] using TMT-pro isobaric labeling. Hierarchical clustering of normalized intensities of all significantly differentially expressed proteins (adjusted p value: adj. p < 0.05) led to a clear separation into male and female, as well as WT and NF1 ± subclusters (Fig. 1A), indicating that differences in microglia protein expression occurred between, rather than within, groups. Clustering of differentially expressed genes revealed three major clusters, where cluster 2 and 3 were dominated by morphology- and membrane-related Gene Ontology (GO) networks and cluster 1 by transcriptional and mRNA-modifying terms (Additional file 1). Interestingly, among the data set there was a large number of differentially expressed proteins between male WT and NF1 ± microglia (2474), but none between female WT and NF1 ± microglia (Fig. 1B). As expected, and in accordance with our previous studies [20, 65], there were sex-dependent differences in the proteomes from WT microglia (3165 significantly regulated proteins), which were eliminated in the context of NF1 mutation (only five significantly regulated proteins observed). Consistent with a heterozygous NF1 mutation, there was a ~50% reduction in neurofibromin expression in.

(See figure on next page.)

Fig. 1 Proteomic analysis reveals sex-specific differences in WT and NF1 ± microglia. A: Heatmap of significantly regulated proteins (adj. p < 0.05) in male and female WT and NF1 ± microglia. Cluster analysis revealed high in-group similarities, indicating that each sample did highly correlate to samples from the same group and less to those from other groups. Furthermore, differentially expressed genes clustered into 3 different major clusters which are annotated and analyzed in more detail in Additional File 1: Fig. S1.B. Number of significantly regulated proteins between the indicated pairwise comparisons. Note that there were many expression changes between male WT and NF1 ±, as well as between male and female WT, microglia. Only a few significant differences were found between male NF1 ± vs. female NF1 ± microglia and female WT vs. female NF1 ± microglia. C: Comparison of NF1 ± protein levels in the analyzed samples. As expected, NF1 expression was reduced by ~50% in male and female NF1 ± compared to WT samples. D: GO term analysis of proteins upregulated in male NF1 ± compared to male WT microglia. Analysis was performed using Metascape [74]. Note that many terms refer to microglia morphology and surveillance. E: Volcano Plot comparing proteomic data from male WT and NF1 ± microglia. Position of NF1 is highlighted by an orange circle; those of the purinergic receptors P2y12, P2rx4 and P2rx7 by black squares.
Nf1± relative to WT microglia (male: p = 0.0006; female: p = 0.0003; Fig. 1C).

Focusing more on male WT and Nf1± microglia, we performed a GO analysis of the 789 proteins with increased expression in male Nf1± versus WT microglia using Metascape [74]. Many of the GO terms were related to cytoskeletal organization (Fig. 1D), suggesting Nf1-dependent alterations in morphology or motility.
Conversely, analysis of the downregulated proteins revealed GO terms that were associated with regulation of transcription and mRNA transport (Additional file 1: Fig. S2. There were three purinergic receptors found in our proteomic data set; P2ry12, P2rx4 and P2rx7, and in accordance to data from our previous study, these proteins were not differentially expressed between WT and Nf1 ± groups (Fig. 1E).

**Male Nf1 ± microglia exhibit reduced surveillance and ramification**

Based on the proteomic pathway findings, we next analyzed microglial properties related to cytoskeletal organization. First, we examined surveillance in acute cortical slices from 12–16 week old male and female mice, as previously reported Madry et al. [42]. The microglial cells in the slices were identified by transgenic EGFP labeling in WT and Nf1 ± mice intercrossed with MacGreen (Csfr1-EGFP) mice. To avoid any bias toward microglia activation at the slice surface [24, 27], all experiments were performed on microglial cells located 50–100 μm below the surface of the slice within 4 h of acute slice preparation. Consistent with the proteomic results, the determined surveillance parameters of male Nf1 ± microglia were reduced relative to their WT counterparts. The cumulative time courses of the surveyed area in maximum intensity projections (Fig. 2A and B) indicate that the initial rate of surveillance within 5 min was reduced in male Nf1 ± microglia (74 ± 6 μm²/min; n = 106 cells/4 mice; p < 0.0001) relative to male WT microglia (129 ± 9 μm²/min; n = 53 cells/2 mice), and that the cumulative area surveyed after 40 min was also smaller in male Nf1 ± (1488 ± 75 μm²; p < 0.0001) relative to male WT (2384 ± 117 μm²) microglia. Consequently, the surveillance index, which is a measure of the process retractions and extensions per unit time, was reduced in male Nf1 ± microglia (86.4 ± 5.1 μm²/min) compared...
to male WT microglia (153.5 ± 8.7 μm²/min; p < 0.0001; Fig. 2C). Second, we determined the ramification index, which is a normalized parameter expressing the ratio of the cell perimeter to the perimeter of a perfect circle with the same area as the cell and, thus, depends only on cell shape, rather than its overall size [36]. As shown in Fig. 2C, male Nf1± microglia had a ramification index (4.8 ± 0.1; n = 106 cells/4 mice) that was smaller than that observed for male WT microglia (6.2 ± 0.2; n = 53 cells/2 mice; p < 0.0001). The number of surveyed pixels and the surveillance index (Fig. 2B and C) might depend on the number and length of microglia processes or their speed of movement [36, 42]. We therefore normalized the surveillance indices to the mean area of each cell and found that there were no differences between male WT (0.25 ± 0.02 min⁻¹) and male Nf1± (0.23 ± 0.01 min⁻¹, p = 0.3007) microglia (Fig. 2D). Based on these results, we conclude that the decreased surveillance of male Nf1± microglia largely arises from their decreased ramification, rather than altered cell motility. Importantly, in female mouse brains, there were no differences in the surveillance of WT and Nf1± microglia processes (Fig. 2A and B), neither in the initial rates of surveillance (0–5 min; female WT: 134 ± 10 μm²/min; n = 78 cells/3 mice; female Nf1±: 108 ± 9 μm²/min; n = 95/3 mice; p = 0.0629) nor in the cumulative areas surveyed after 40 min (female WT: 2375 ± 90 μm²; female Nf1±: 2242 ± 86 μm²; p = 0.2891). In addition, the surveillance indexes were similar in female WT (162.1 ± 6.5) and Nf1± (146.7 ± 6.3; p = 0.0907) microglia. Taken together, these data demonstrate a sexually dimorphic reduction of ramification, resulting in reduced surveillance in Nf1± microglia.

To explore microglia cytoskeleton-dependent processes in more detail, we analyzed microglial morphology in fixed male and female WT and Nf1± mouse cortical brain slices (Fig. 3). Confocal images were taken with a 40X oil immersion objective on a Leica SPE upright microscope at a resolution of 0.156 × 0.156 × 1 μm/voxel and an excitation wavelength of 639 nm. Three-dimensional rendering was applied using Imaris software (Fig. 3A) to perform 3D Scholl analysis and quantify the average soma volumes, total process lengths and number of branch points per cell. As observed with microglia surveillance and ramification, only microglia from male Nf1± mice were less ramified relative to their WT counterparts, as indicated by the quantification of intersected processes within Scholl spheres at increasing distances from the soma (n = 50 cells from 3 mice per group; Fig. 3B). This result reflects reduced number of branch points per microglia in male Nf1± (39 ± 1) compared to WT mice (61 ± 2; p < 0.0001; Fig. 3C), whereas female WT (48 ± 2) and female Nf1± (51 ± 1; p = 0.3627) microglia had similar numbers. In addition, the cumulative process length per microglia was longer in male WT microglia (782 ± 20 μm) relative to male Nf1± microglia (526 ± 14 μm; p < 0.0001; Fig. 3D), but no differences were observed between female WT (667 ± 17 μm) and female Nf1± (670 ± 14 μm; p = 0.9988) microglia. In contrast to microglia process ramification, heterozygous Nf1 loss had no impact on microglial soma volumes (Fig. 3E), which were similar in male and female WT microglia (209 ± 5 μm³ and 197 ± 7 μm³, respectively) and in Nf1± male and female microglia (196 ± 5 μm³ and 198 ± 5 μm³, respectively). Collectively, these data provide additional support for a sex-by-genotype effect of Nf1 heterozygosity on microglia process ramification.

**Microglia morphology and function is not altered by microglia-intrinsic heterozygous Nf1 loss**

To determine whether the observed Nf1± sexually dimorphic microglia alteration in ramification was a direct consequence of Nf1 mutation on microglia biology (cell-intrinsic effect), we generated mice in which Nf1 was heterozygously deleted in microglia (Fig. 4A). CX3CR1-CreER [71] were intercrossed with Nf1floxflox [1] and R26R-EYFP mice [63] to generate litters heterozygous for CreER expression from the CX3CR1 locus, a single conditional (flox) Nf1 allele, and a conditional (LoxP-stop-LoxP; LSL) eYFP transgene in the Rosa26 locus (Nf1floxflox; Cx3cr1-CreER; LSL-eYFP mice; termed Nf1floxflox/Cx3cr1-CreER mice). Tamoxifen (100 mg/kg body weight) was intraperitoneally administered between P30 and P40 for five consecutive days to induce Cre recombinase activity in CX3CR1⁺ cells. WT (Cx3cr1-Cre) mice had the same genotype (Nf1+ /+ ; Cx3cr1-CreER; LSL-eGFP), except they lacked a conditional Nf1 allele. As a control for the successful activation of CreER in WT and Nf1floxflox/Cx3cr1-CreER mice by tamoxifen treatment, cortical brain slices were immunostained with Iba1 and YFP antibodies. We observed a clear induction of microglial YFP expression in mice treated with tamoxifen at both neonatal and adult stages (Fig. 4B). There was also weak expression of YFP in non-microglial cells, mainly in neuronal cells independent of CreER activation, as YFP was also seen in CX3CR1wt/wt mice, which do not express CreER recombinase (Additional file 1: FigS3). These unexpected findings likely reflect non-specific, off-target, expression of YFP from the Rosa26 locus, as has been previously reported for other reporter strains [73].

To determine the effect of heterozygous Nf1 loss in microglia, cortical brain slices from male and female WT and Nf1floxflox/Cx3cr1-CreER mice were analyzed (Fig. 4C). Confocal images were taken with a 40X oil immersion objective on a Leica SPE upright microscope at a...
resolution of 0.179 x 0.179 x 0.9 µm/voxel and an excitation wavelength of 635 nm. Three-dimensional rendering was applied using Imaris software to generate 3D quantification of microglia morphology. Interestingly, unlike microglia from Nf1 ± mice, in which Nf1 is heterozygously deleted in all cells, neither male nor female Nf1flox/wt Cx3cr1-CreER microglia exhibited changes in process ramification relative to their WT counterparts (Fig. 4C). Additionally, there were similar numbers of branch points per microglia in male Nf1 flox/wt Cx3cr1-CreER (38 ± 1, n = 50) relative to male WT (40 ± 2; n = 50; p = 0.3670; Fig. 4D) microglia, which were also similar in female WT (40 ± 2; n = 50) and female Nf1 flox/wt Cx3cr1-CreER (38 ± 1; n = 50; ANOVA p = 0.8230) microglia. Likewise, quantification of intersected processes within Scholl spheres at increasing distances from the soma revealed a similar distribution in microglia from Nf1 flox/wt Cx3cr1-CreER and WT mice of either sex (Fig. 4C).

There was no difference in the cumulative process length per microglial cell (male WT: 588 ± 17 μm; male Nf1 flox/wt Cx3cr1-CreER: 567 ± 16 μm; female WT: 650 ± 23 μm; female Nf1 flox/wt Cx3cr1-CreER: 603 ± 17 μm; n = 50 cells/3 mice per group; ANOVA p = 0.0711; Fig. 4E). There was also no effect of microglia-restricted heterozygous Nf1 loss on microglia soma volumes (Fig. 4F), which were similar in male and female WT (194 ± 6 μm³ and 186 ± 5 μm³, respectively) and Nf1 flox/wt Cx3cr1-CreER (183 ± 5 μm³ and 187 ± 7 μm³, respectively; ANOVA p = 0.0502) mice. Collectively, these data demonstrate that microglia-specific heterozygous Nf1 loss does not affect microglial morphology.

Second, to determine whether Nf1 ± microglial functional defects were cell-autonomous, we measured...
microglial responses in acute brain slices of adult (12 -16 weeks) male and female WT and Nf1flox/wt Cx3cr1-CreER mice following a laser lesion by two-photon imaging (Additional File 1: FigS4). The movement of microglial processes was quantified by determining the fluorescence distribution within the area of concentric circles (diameter: 20 µm and 90 µm) around the lesion site. In contrast to our previous findings using germline male WT flox/wt ER male Nf1 female WT male WT female WT male Nf1 female WT male WT female Nf1 female Nf1 Cx3cr1-CreER female Nf1 Cx3cr1-CreER female Nf1 Cx3cr1-CreER male WT Cx3cr1-CreER female WT Cx3cr1-CreER male WT Cx3cr1-CreER female WT Cx3cr1-CreER male WT Cx3cr1-CreER female WT Cx3cr1-CreER

**Fig. 4** Microglia morphology is not altered by intrinsic Nf1 reduction: A: CX3CR1-CreER mice were intercrossed with Nf1flox/flox and R26R-EYFP to obtain litters that carry a heterozygous knock-in of CreER at the CX3CR1 locus, a heterozygous knock-in of a flox cassette in the Nf1 locus, and a homozygous, floxed eYFP transgene in the Rosa26 locus (Nf1flox/wt Cx3cr1-CreER). Tamoxifen was administered between P30 and P40 on five consecutive days to activate Cre recombinase in microglia. B: Confocal microscopic images indicating the successful induction of microglial YFP reporter expression by tamoxifen treatment in the cortex of two male WT mice. All scale bars are 20 µm. C: Left, 3-dimensional renderings of example male and female WT and Nf1flox/wt Cx3cr1-CreER microglia in the somatosensory and parts of the motor cortex (layer 2–6). Scale bars denote 20 µm. Right, Sholl analysis of male (top) and female (bottom) WT and Nf1flox/wt Cx3cr1-CreER microglia. The number of intersected processes was plotted against their distance from the soma. There was no difference in the distribution of process branches around the soma between the four investigated groups. D-F: Summary of the number of intersections per cell (D), the total process length (E) and the soma volumes (F) for male and female WT and Nf1flox/wt Cx3cr1-CreER microglia. Number of quantified cells (mice): 50 (3) for each group.
**Nf1** ± mice [17, 18], YFP-based fluorescence in mice was much weaker; however, detection of process movements was still possible (Additional File 1: Fig. S4A). No translocation of microglial cell bodies was seen during the observation period. In addition, similar to morphology (Fig. 4), there were no defects in directed process motility of microglia from Nf1floxs/wtCx3cr1-CreERmice. The process response of male WT microglia 30 min following laser lesion was 24.6±5.8 (n=8), which was not different from that observed in male Nf1floxs/wtCx3cr1-CreERmice (25.4±4.0; n=8; p=0.9097). There were also no differences in process movements of female WT (27.4±5.3; n=8) relative to female Nf1floxs/wtCx3cr1-CreERmicroglia (23.7±5.2; n=12; p=0.6275).

Process movement in response to injury [17, 18, 20, 27, 42] is a P2RY12-mediated function in microglia [13, 48]. Microglial membrane current responses following the application of low (10 µM) ATP concentrations largely depend on P2RY12 activation [17, 18], which activates K+ currents through THIK-1 [3, 4, 20, 42, 67]. For these reasons, we analyzed purinergic responses of male WT microglia 30 min following tamoxifen administration at P30-40 using standard whole-cell patch clamp techniques in the voltage-clamp configuration. Microglial cells were identified in situ by eYFP fluorescence. WT and Nf1floxs/wtCx3cr1-CreER-microbial cells responded to 10 µM ATP with the induction of an outwardly rectifying current that reversed close to the equilibrium potential for potassium (Additional File 1: Fig. S4D-F). We next assessed the specific conductance between +20 mV and +60 mV to compare these currents in male and female microglia. The conductance of microglial ATP-evoked potassium currents was 13.5±4.6 pS/pF and 13.6±2.3 pS/pF for male and female WT microglia, respectively (n=3 and n=11 cells, respectively; p=0.9823, Additional File 1: Fig. S3H). There were no differences in microglial purinergic responses in male Nf1floxs/wtCx3cr1-CreER (15.0±3.4 pS/pF; n=14; p=0.8550 vs. male WT) or female Nf1floxs/wtCx3cr1-CreER (14.6±3.1 pS/pF; n=14; p=0.8060 vs. female WT) mice, indicating that the microglia-specific heterozygous Nf1 loss has no impact on P2RY-dependent membrane responses. Additionally, we compared the membrane characteristics of male and female WT and Nf1floxs/wtCx3cr1-CreER microglia, and repetitively patch clamped cortical microglia at potentials between −170 and +60 mV, starting from a holding potential of −70 mV. As shown in Additional File 1: Fig. SS, current density–voltage relations were characterized by a high input resistance and a small inwardly rectifying conductance between −40 and −170 mV, consistent with previous studies [33]. The reversal potentials, indicative of the resting membrane potential, were also similar among groups. There were no differences in the apparent membrane capacitance between the two sexes and genotypes. Taken together, these findings establish that the male microglia defects observed in Nf1 ± mice were unlikely to be cell-autonomous.

**Microglia defects result from heterozygous Nf1 loss in other brain cells**

Based on the above findings, we considered the possibility that the sex by genotype effects reflected microglia responses to heterozygous Nf1 mutation in other brain cells (e.g. neurons, astrocytes and oligodendrocytes). To address this question, we generated mice in which Nf1 is heterozygously deleted in non-microglial cells (neural progenitor cells by embryonic day 16.5, hGFAP-Cre mice; by intercrossing Nf1floxs/flox and hGFAP-Cre mice [1]).

To investigate microglia morphology, cells were analyzed as before, to quantify average soma volume, total process length and the number of branch points per cell (Fig. 5). Interestingly, microglia from male, but not female, Nf1floxs/wt; hGFAP-Cre mice had decreased ramification. The quantification of intersected processes within Scholl spheres at increasing distances from the soma (n=50 cells from 3 mice per group; Fig. 5A) revealed fewer intersections within a distance of 15–40 μm from the soma in male Nf1floxs/wt; hGFAP-Cre, compared to WT microglia. In addition, the number of branch points per microglial cell was reduced in male Nf1floxs/wt; hGFAP-Cre (32±1) compared to WT microglia (42±1; p<0.0001; Fig. 5B), whereas female WT (35±1) and female Nf1floxs/wt (35±1; p=0.9999) microglia were similar. Similar to Nf1 ± mice (see Fig. 3), there was a sexually dimorphic decrease in the cumulative process lengths in male Nf1floxs/wt; hGFAP-Cre microglia (500±14 μm) relative to male WT microglia (652±16 μm; p<0.0001; Fig. 5C), and no differences were observed between female Nf1floxs/wt; hGFAP-Cre (544±16 μm) and female WT (530±14 μm; p=0.9018) microglia. In contrast to microglia process ramification, heterozygous Nf1 loss in non-microglial cells had no impact on soma volume (Fig. 5D), which were similar in all groups (male WT: 185±5 μm3, male Nf1floxs/wt; hGFAP-Cre: 178±5 μm3, female WT: 189±5 μm3, female Nf1floxs/wt; hGFAP-Cre: 178±6 μm3, ANOVA p=0.1818). Collectively, these findings indicate that the sex-by-genotype effects of Nf1 reduction in microglia reflect the contributions of other cell types and are not cell-intrinsic to microglia.

**Discussion**

In the present study, we report proteomic changes and defects in cortical microglia process ramification in male, but not female, Nf1 ± mice. Using two different Cre driver
lines, we demonstrate that the sexually dimorphic microglia defects observed in germline Nf1± mice do not originate from heterozygous Nf1 loss in microglia, but rather reflect responses to signals from sex- and Nf1-dependent alterations in other cell types in the brain. Leveraging mice in which heterozygous Nf1 loss occurs in neural progenitor cells and their progeny (neurons, astrocytes and oligodendrocytes), but not in microglia, we show that heterozygous Nf1 loss in the cellular environment mimics the sexually dimorphic microglia defects observed in germline Nf1± mice. Taken together, these observations suggest that environmental factors from other brain cells induce microglia responses important for the formation and progression of NF1-related brain dysfunction.

Microglia are essential for the development and progression of numerous brain diseases [68], including neurodegenerative [72] and psychiatric disorders [44, 55], as well as brain tumors [23]; however, there is a great degree of heterogeneity between microglia associated with each brain disorder. In the early stages of Alzheimer’s disease, microglia facilitate the destruction of neuronal networks due to their ability to prune synapses [29, 29, 30, 30, 34], whereas the loss of phagocytic and surveillance activity contributes to enhanced Aβ plaque burden at later stages [35, 67]. Similarly, schizophrenia is initiated and closely linked to inflammatory mechanisms [10], leading to a pro-inflammatory microglial phenotype [44, 45]. In high-grade (malignant) gliomas, microglia are transcriptionally reprogrammed by tumor-derived factors and act as tumor-supporting cellular elements [22, 23, 68], whereas in low-grade gliomas, microglia are essential for tumor growth in response to cytokines released by T lymphocytes [7, 14, 21, 51].

The findings in the current study exclude a cell-autonomous effect of heterozygous Nf1 loss on microglia. As such, Nf1 differs from other genes that directly affect microglia function through intrinsic mechanisms, such as Cx3cr1 [52, 53], P2ry12 [27, 61], Gabbr1 [19], Chrm3 [11], Apoe [28], Tgfbr2 [75], Bmal1 [66], Ii6 [57], I110 [31, 70] or Bdnf [47]. It should, however,
be noted that all of these genes—in addition to their microglia-intrinsic effects—can also alter the behavior and function of non-microglial cells in a cell-autonomous fashion. Similarly, defects in neuronal Bdnf or Bmal1 expression [26] [60] or macroglia cells [16] can influence microglia function. In contrast, microglia can also respond to genetic mutation in other cell types: whereas microglia in a mouse model of Rett’s syndrome (Mecp2-/-) excessively engulf, and thereby eliminate, presynaptic inputs at end stages of disease, conditional loss of Mecp2 in microglia had little effect on synapse loss [59]. In addition, conditional loss of the calcitonin receptor (CalcR) in proopiomelanocortin (POMC) neurons of the hypothalamus leads to increased body weight gain, increased adiposity, and glucose intolerance [8] as a result of microglial IL-6 release [32, 37]. However, CalcR loss in selective in microglia had no effect on body weight or glucose tolerance (Coester et al. 2022).

In the setting of Nf1 heterozygosity, it is most likely that non-microglial cells (astrocytes, neurons or oligodendrocytes) are the initiating elements for the observed microglia morphological and functional changes. We acknowledge that it is conceivable that Nf1 mutational effects on microglia might require heterozygous Nf1 loss during embryonic development, prior to the Cre-mediated excision time point chosen for our experiments, which would require additional experimentation. Nonetheless, the simplest explanation for our findings suggest that normal microglia have the capacity to respond to paracrine or cell surface-bound cues provided by male Nf1 ± neurons or macroglia. In keeping with this mechanism, we found that Nf1 ± neurons secrete midkine, which can induce the expression of Ccl4 in both wild type and Nf1 ± T cells (Guo et al. 2019). Further investigation will be required to determine whether both male and female wild type microglia can alter their biological properties in response to Nf1 ± neurons or macroglia, or whether this sex by gene effect operates at the level of the neuron (or microglia). To this end, we have previously demonstrated sexually dimorphic differences between male and female Nf1 ± neurons [15], which reflect sex by genotype effects on cyclic AMP generation and dopamine homeostasis. Additional studies will be necessary to determine whether differences in neuronal dopamine (or other paracrine factors) underlie the sexually dimorphic differences observed in microglia [2, 19, 39, 40, 64, 69]. Understanding how this intercellular cross-talk is established and maintained will provide new insights into our understanding of sex differences and the interplay between risk factors and cellular function in the brain.

Materials and methods
Ethics statement
All procedures involving the handling of living mice were performed in strict accordance with the German Animal Protection Law, and were approved by the Regional Office for Health and Social Services in Berlin (Landesamt für Gesundheit und Soziales, Berlin, Germany, Permit Number G0164/19, X9005/18, A0376/17). Adult mice were euthanized by cervical dislocation or by transcardial perfusion of PBS or PFA after intraperitoneal injection of pentobarbital (Narcoren, Merial GmbH, Hallbergmoos, Germany). All efforts were made to minimize suffering. Similarly, all mice used at Washington University were performed under an approved Animal Studies protocol.

Mice
Wild type and Nf1 ± [5] mice used in this study were either bred onto a wild type or MacGreen (Csf1r-eGFP; [58] C57/BL6 background. Conditional microglia-specific Nf1 ± mice: CX3CR1-CreER [71] were intercrossed with Nf1flox/flox [1] and R26R-EYFP mice [63] to generate litters heterozygous for CreER expression from the CX3CR1 locus, a conditional (flox) Nf1 allele and a conditional (LoxP-stop-LoxP; LSL) eYFP transgene in the Rosa26 locus (Nf1flox/wt; Cx3cr1-CreER; LSL-eYFP mice; termed Nf1flox/wtCX3CR1-CreER mice). Tamoxifen (100 mg/kg body weight) was administered intraperitoneally between days P30 and P40 on 5 consecutive days to induce Cre recombinase activity in CX3CR1+ cells. Wild type (Nf1wt/wt; Cx3cr1-CreER; LSL-eYFP mice; termed WT) mice had the same genotype with the exception that they had no Nf1flox allele. Conditional non-microglia-specific Nf1 ± mice: hGFAP-CreER [5] were intercrossed with Nf1flox/flox [1] to generate litters heterozygous for CreER expression from the GFAP locus and a conditional (flox) Nf1 allele (Nf1flox/wt; hGFAP-Cre; termed Nf1flox/wt; hGFAP-Cre mice). Wild type (Nf1wt/wt; hGFAP-Cre; termed WT) mice had the same genotype with the exception that they had no Nf1flox allele. All mice were maintained under a 12 h/12 h dark–light cycle with food and water supply ad libitum, in accordance with German laws and IACUC recommendations (U.S.A.) for animal protection. All data in the current study are from 12–16 week old mice. Sexes and genotypes are indicated.

Immunohistochemistry and confocal microscopy
Mice were anesthetized with pentobarbital (Narcoren, Merial Hallbergmoos, Germany) and transcardially perfused with phosphate buffered salt solution (PBS) followed by 4% paraformaldehyde in PBS, de-capitated and sectioned in the sagittal plane at 40 μm thickness using
a sliding microtome (Leica SM2000 R, Leica Biosystems GmbH, Nussloch, Germany). Free-floating 40 µm sections were incubated in 5% donkey serum (EMD Millipore Corp., Burlington, Massachusetts, USA) and 0.1% Triton-X (Carl Roth® Karlsruhe, Germany) in Tris-buffered saline solution (TBStplus) together with the primary antibodies over-night at 4 °C. The following primary antibodies were used: goat monoclonal Iba-1 antibody targeting microglia (1:300; Abcam, Cambridge, UK); chicken polyclonal GFP antibody targeting eYFP (1:250; Abcam, Cambridge, UK); mouse anti-NeuN targeting neurons (1:100; Synaptic Systems). After washing, secondary antibodies were prepared in TBStplus. Iba-1 was visualized with donkey anti-goat IgG conjugated with Cy5 or Alexa488 fluorophores (both 1:200; Dianova, Hamburg, Germany); eYFP was visualized with donkey anti-chicken IgY conjugated with Alexa488 or Cy5 (both 1:200; Dianova), NeuN with donkey anti-mouse IgG (H+L) conjugated with Cy3 (1:200; Dianova). Sections were incubated with secondary antibodies at room temperature for 2 h and then mounted on glass slides with Aqua Polymount mounting medium (Polysciences Europe GmbH, Hirschberg an der Bergstraße, Germany). Cell nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI, 1:500; Dianova) before mounting. Images were acquired with either a Zeiss LSM700 (inverse) or a Leica SPE (upright; Leica Biosystems GmbH, Nussloch, Germany) using 40X oil immersion objectives. Z-stacks were taken at 1 µm z-step size, 35 steps to cover the whole thickness of the slice.

Morphological analysis
Morphological analysis of microglia was performed on 3-dimensional fluorescence images using Imaris × 64 version 9.6–9.9 (Bitplane, Zurich, Switzerland) algorithms. Microglial cells in which the nucleus was at least 15 µm away from the image border were selected for analysis. The modules "Filament tracer" and "Surface" were used for microglia reconstruction. A total of 50 cells from 3 different mice were analyzed for each group. The background was minimized with an appropriate filter width (20–40 µm) and the region of the analyzed cell was selected manually. The parameters Filament Length, Filament No, Dendrite Branch Pts, Filament No, Sholl Intersections, and Soma Volume were obtained from the specific values calculated by Imaris. Although tracing was performed automatically by the algorithm, we individually verified that processes originated from one defined cell. False connections were removed manually which were commonly less than 1%. The number of Sholl intersections was defined as the number of process intersecting concentric spheres, defining the spatial distribution of segments as a function of distance from the soma (Sholl analysis). All spheres have their center at the soma (beginning point) with a 5 µm step resolution for the spheres.

Acute brain slice preparation
Acute cortical brain slices were prepared as previously described [4]. In brief, mice were euthanized by cervical dislocation, and their brains removed and cooled in ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): 230 Sucrose, 2.5 KCl, 10 MgSO 4 , 0.5 CaCl 2 , 1.25 NaH 2 PO 4 , 26 NaHCO 3 , and 10 D-glucose, pH 7.4; gassed with 95% O 2 / 5% CO 2 . Brains were then mounted on a vibratome (HM650V, Thermo Scientific, Massachusetts, USA), and 250 µm thick coronal brain slices were generated and kept at room temperature for experiments for up to 5 h in gassed ACSF containing (in mM): 134 NaCl, 2.5 KCl, 1.3 MgCl 2 , 2 CaCl 2 , 1.26 K 2 HPO 4 , 26 NaHCO 3 , and 10 D-glucose (pH 7.4). Acute brain slices were used for patch clamp recordings and in situ 2-photon live-cell imaging.

Two-photon imaging and laser lesioning
Live imaging of microglial processes was performed on 250 µm coronal brain slices from Nf1+/− or Nf1flox/− mice and their WT littermates using a custom-built two-photon laser-scanning microscope (Till Photonics, Gräfelfing, Germany). EGFP or eYFP was excited by a Chameleon Ultra II laser (Coherent, Dieburg, Germany) at a wavelength of 940 nm. A 40X water-immersion objective (NA 0.8, Olympus, Hamburg, Germany) was used, with scanned 60 µm thick z-stacks and a step size of 3 µm covering a field of 320 × 320 µm. Laser lesions were set to 40 µm under the slice surface in the cortex by focusing the laser beam, set to a wavelength of 810 nm and to maximum power in the selected imaging volume, and scanned until autofluorescence of the injured tissue was visible. This procedure resulted in lesions of ~20 µm in diameter in the middle of the observed region. For the recording of microglia surveillance, no laser lesion was performed. IGOR Pro 6.37 (Lake Oswego, USA) was used for data analysis as in Davalos et al. [13] and Madry et al. [42]. The sequences of 3D image stacks were converted into sequences of 2D images by a maximum intensity projection algorithm. Grayscale images were first converted into binary form using a threshold. For quantification of laser lesion-induced movements, microglial response to focal lesion was defined as EGFP + pixel count in a proximal circular region 45 µm around the lesion site over time (Rx(t)). Distal fluorescence of the first time point was determined within a diameter of 45 µm to 90 µm around the lesion site for normalization (Ry(0)). Microglial responses were represented as R(t)= (Rx(t)-Rx(0))/Ry(0). For the quantification of baseline surveillance, cells
of interest were individually selected by manually drawing a region of interest (ROI) around an area including all their process extensions throughout the 20 min movie and erasing data around that ROI. Starting with the second frame, we subtracted from each binarized frame the preceding frame and counted the number of pixels < 0 (retracting = PR) and > 0 (extending = PE). The surveillance index for each frame is then given by the sum of PR ad PE. The surveillance index of a given cell was then calculated by averaging the indices of the first 20 images in the movie. For ramification index (RI), we used the equation RI = (peri/area)/(2sqrt(pi/area)), where peri and area are respectively the perimeter and area of a given cell in pixels. For the quantification of these two parameters, the ImageAnalyzeParticles operation in IGOR Pro 6.37 was applied on binarized images in which all analyzed microglia were manually examined and, if necessary, somata and processes connected.

Electrophysiological recordings
A conventional patch-clamp amplifier was used (EPC9, HEKA Elektronik, Lambrecht, Germany). Acute 250 µm coronal brain slices were prepared from Nf1flox/wt and WT mice, and microglial cells were identified by their transgenic EYFP fluorescence on an epifluorescent microscope. Patch pipettes were pulled from borosilicate microscope. Patch pipettes were pulled from borosilicate glasses and had resistances of 4—6 MOhm. The following intracellular solution was used (in mM): KCl, 130; MgCl2, 2; CaCl2, 0.5; Na-ATP, 2; EGTA, 5; HEPES, 10 and sulforhodamine 101, 0.01 (Sigma Aldrich,) and had an osmolarity of 280—290 mOsm/L adjusted to a pH of 7.3 with KOH. The extracellular solution contained (in mM): NaCl, 134; KCl, 2.5; MgCl2, 1.3; CaCl2, 2; K2HPO4, 1.25; NaHCO3, 26; D-glucose, 10; pH 7.4; 310—320 mOsm/L and was gassed with carbogen (95% O2/ 5% CO2). Experiments with series resistances less than ~ 65 MOhm were used for data analysis. All experiments were performed in the voltage-clamp configuration. To obtain current–voltage curves during continuous recordings, the membrane was clamped every 5 s from a holding potential of -70 or -20 mV (before and during the ATP response, respectively) to a series of de- and hyperpolarizing voltages ranging from -140 mV to 60 mV with 20 mV increment, 100 ms in duration. Membrane currents were averaged for quantification between 30 and 45 ms after clamping the membrane to a given value from the resting potential. Membrane capacitance was quantified based on an exponential fit of the current decay in response to a -10 mV test pulse. The same pulse was used to quantify series resistance from the peak amplitude of the membrane capacitance currents. Comparisons of membrane currents between different groups were always normalized to the membrane capacitance.

Microglia isolation by MACS
12–16 week male and female mice were transcardially perfused with ice-cold Phosphate Buffered Saline (PBS) to harvest the brain. Brains were subsequently homogenized at 0–4 °C in dissection buffer (HBSS, 45% glucose, 1 M HEPES) and cell pellets were resuspended in 25 ml of 22% Percoll (GE Healthcare, Little Chalfont, UK). 5 ml PBS were added as a layer on top. Centrifugation was performed for 20 min at 950 g with medium acceleration and no brakes to remove myelin and debris. Pellets were resuspended in ice-cold MACS buffer and incubated with anti-mouse CD11b antibodies coupled to magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 15 min at 4 °C. Cells were resuspended in MACS buffer and passed through medium-sized MACS columns (Miltenyi Biotech) attached to a magnet. The flow-through was discarded and the cells were flushed out of the columns in MACS buffer, collected by centrifugation, and stored at -80 °C for cAMP ELISA.

Mass spectrometry and proteomic analysis
Global proteome analysis was conducted using isobaric TMTpro 16-plex labeling (Thermo Fisher Scientific) essentially as described in Mertins et al. [46]. Samples were lysed in urea buffer and digested with trypsin (Promega). Of each sample, 12 µg peptide was assigned to channels 1 through 13 as well as channel 15 in a randomized fashion. Channel 16 was used as a booster channel and loaded with 70 µg of a mix of the remaining peptide material. Channel 14 was kept empty. The combined TMT cassette was deeply fractionated using high-pH HPLC separation into 30 fractions using a 1290 Infinity II LC System (Agilent Technologies). An estimate of 1 µg of each fraction was injected into LC–MS analysis on an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) in data-dependent mode using a 110 min gradient on an EASY-nLC 1200 system (Thermo Fisher Scientific) with an in-house packed column (C18-AQ 1.9 µm beads, Dr. Maisch Reprosil-Pur 120). For database search, MaxQuant version 1.6.10.43 [12] was used whilst enabling TMTpro 16-plex reporter ion quantitation with a PIF setting of 0.5. Downstream analysis was done in R. For quantitation, a complete data matrix was required for the experimental conditions (14 out of 16 channels). Reporter ion intensities were normalized and scaled using median-MAD normalization. For significance calling two-sample moderated t-tests were applied (limma R package; [56]). P-values were adjusted using the Benjamini–Hochberg method.

Statistics
All data are expressed as mean ± SEM. A combination of one-way ANOVA tests with Bonferroni post hoc
tests were employed using Prism 7 (GraphPad Software, San Diego, CA, USA) to compare data between the four experimental groups. Significance is given as *** p < 0.001, ** p < 0.01, * p < 0.05, n.s. p > 0.05.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s40478-023-01525-w.

**Additional file 1:** Supplementary Figures.

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**Author contributions**

M.S. performed the electrophysiology, confocal microscopy and data analysis; FL performed the 2-photon surveillance and laser lesion experiments; L.G. performed the morphology analysis; O.P. and P.M. performed the mass spectrometry; E.C.C. performed brain dissections and fixation; M.S. wrote the first draft of the manuscript and prepared the figures; M.S., H.K., and D.H.G. designed the experiments; H.K. and D.H.G. oversaw the studies, revised the manuscript and provided funding for this project.

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**Availability of data and materials**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomexchange.proteomeexchange.org) via the PRIDE partner repository [54] with the dataset identifier PXD0035881.

**Declarations**

**Competing interests**

The authors declare no relevant conflicts of interest.

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**References**


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36. Kyriazoglou V et al (2020) P2Y13 receptors regulate microglial morpho-

logy, surveillance, and resting levels of interleukin 1beta release. Glia 68(2):328–344
38. Lehman EK et al (2018) CD47 Protects Synapses from Excess Microglia-

Mediated Pruning during Development. Neuron 100(1):120-134.e12
50. Pan Y et al (2021) NF1 mutation drives neuronal activity-dependent initia-

58. Sasmono RT et al (2005) A macrophage colony-stimulating factor recep-

tor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. Blood 101(3):1155–1163

ronal transcripts in microglia relevant to central nervous system disease. Glia 63(4):531–548
71. Yona S et al (2013) Fate mapping reveals origins and dynamics of mono-

cytes and tissue macrophages under homeostasis. Immunity 38(1):79–91

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