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Faculty of Medicine and Life Sciences
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Master of Biomedical Sciences

Master's thesis

Disrupted-in-Schizophrenia 1 controls microglial cytoskeletal remodeling via regulation of actin dynamics and adhesion

Adelaïde Margoosian Gharghani

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :
Prof. dr. Bert BRONE

MENTOR :
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www.uhasselt.be
Universiteit Hasselt
Campus Hasselt:
Martelarenlaan 42 | 3500 Hasselt
Campus Diepenbeek:
Agoralaan Gebouw D | 3590 Diepenbeek

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Disrupted-in-Schizophrenia 1 controls microglial cytoskeletal remodeling via regulation of actin dynamics and adhesion

Adelaide Margoosian Gharghani[1], Melanie Mertens1[1], Jerome Hendriks[2] and Bert Brône [1]

[1]Neurology research group, Biomedical Research Institute, Universiteit Hasselt, Campus Diepenbeek, Agoralaan Gebouw C - B-3590 Diepenbeek

[2]Physiology research group, Biomedical Research Institute, Universiteit Hasselt, Campus Diepenbeek,
Agoralaan Gebouw C - B-3590 Diepenbeek

DISC1's regulates microglial actin dynamics

To whom correspondence should be addressed: Bert Brône, **Tel: +32(0)11 26 92 37;** Email: bert.brone@uhasselt.be

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ABSTRACT

Microglia are the primary immune cells of the central nervous system, playing a vital role in brain development by shaping neuronal networks through synaptic pruning and phagocytosis. Their functions depend on dynamic cytoskeletal remodeling that enables migration, particularly through the extension and retraction of actin-based protrusions such as filopodia and lamellipodia, as well as the formation of focal adhesions (FAs) for membrane anchoring. Alterations in their functions might contribute to neurodevelopmental disorders (NDDs). Disrupted-in-Schizophrenia 1 (*Disc1*), a genetic risk factor for NDDs, regulates neuronal migration and cytoskeletal organization during development, but its function in microglia remains poorly understood. Previously, we observed that *Disc1* locus impairment (LI) reduced embryonic saltatory migration in microglia *in vitro* and *in situ*. We therefore aimed to investigate the role of *Disc1* in the cytoskeletal regulation during this process. We hypothesized that DISC1 controls microglial migration via actin cytoskeleton control. Using a *Disc1* LI mouse model, we show that DISC1 dysfunction alters microglial protrusion dynamics, increasing filopodia motility while reducing lamellipodia movement, indicating impaired protrusive activity. Furthermore, FA density was decreased in *Disc1* LI microglia, suggesting deficits in adhesion formation. Analysis of actin polymerization showed a significant decrease in G-actin levels, resulting in an increased F/G-actin ratio that indicates impaired actin turnover. These findings correspond with impaired microglial migration previously observed. Our findings identify DISC1 as a critical regulator of the microglial actin cytoskeleton. These results suggest that DISC1 dysfunction may contribute to neurodevelopmental and psychiatric disorders by impairing normal microglial function.

INTRODUCTION

The development of the brain is an extraordinarily intricate process involving the generation and organization of diverse neuronal and non-neuronal cells into functional circuits (1). Within this elaborate system, cell migration is fundamental for shaping brain architecture and establishing proper neural connections (2). Disruptions in these processes are often linked to neurodevelopmental disorders (NDDs), such as schizophrenia and autism spectrum disorder (ASD), which are characterized by synaptic defects (3). Mutations affecting cytoskeletal proteins are a hallmark of these disorders, underscoring the critical role of the cell's cytoskeleton in brain development (3,4). This is particularly evident in neuronal synapses, where the dynamic regulation of the cytoskeleton is essential for synaptic function and plasticity (5). Moreover, microglia, the brain's resident immune cells, rely heavily on

their cytoskeleton for key functions such as migration, phagocytosis, and synaptic pruning (6). This dependence underscores the importance of cytoskeleton dynamics in both neurons and glial cells for proper brain development and functioning (7).

Microglia emerge early during embryonic development and play a crucial role in brain development (8). Microglia originate from yolk sac-derived erythro-myeloid progenitors (9). In mice, a subset of these progenitors differentiates into *Cx3cr1*$^{+}$ microglial precursors, which migrate into the brain between embryonic days 9.5 and 14.5, coinciding with blood-brain barrier formation (10). During the development of the CNS, microglia regulate synaptogenesis and synaptic pruning, mainly shaping neuronal plasticity (11,12). In adulthood, microglia primarily function as surveying cells, actively monitoring the CNS for pathogens, cellular debris, or protein aggregates by surveying the parenchyma using motile processes and ramified morphology to sense molecular cues (13,14). Although previously considered inactive under normal physiological conditions, microglia are now known to be highly dynamic, constantly surveilling the brain parenchyma even in their 'resting' state (15,16). Their ability to detect, evaluate, and respond to the environment is essential for synaptic refinement, forming neural circuits, and maintaining brain homeostasis from development through adulthood (13,17). These essential microglial functions depend heavily on cellular movement, making cytoskeletal remodeling crucial for proper microglial activity (18,19). Microglia rely on two distinct forms of movement: migration, which involves the active displacement of the cell body through interactions with the surrounding environment, and motility, which refers to the dynamic extension of cellular protrusions to scan the parenchyma. Both types of movement rely on the dynamic rearrangement of the microglial cytoskeleton (20,21). This structural network, crucial for cell shape and movement, primarily comprises filamentous (F-) actin, formed by the polymerization of actin subunits (19). This occurs in three phases: nucleation, where globular (G-) actin monomers form stable nuclei; the elongation phase, where actin monomers rapidly add to the growing ends of the actin filament; and the steady-state phase, where monomer addition and dissociation reach equilibrium, maintaining actin filament length (22,23). Microglia tightly regulate actin polymerization in a spatiotemporal manner, where actin filaments are constructed to form membrane protrusions with unique morphologies and functions, such as lamellipodia and filopodia (21,24). Lamellipodia are dynamic, wave-like plasma membrane extensions characterized by thin (0.1–0.3 μm), elongated (1–5 μm) projections at the cell's leading edge, containing densely packed branched actin filaments (22). They are crucial for various microglial functions, including chemotaxis, environmental surveillance and movement (25). Formation and dynamics of lamellipodia are tightly controlled by signaling pathways involving Rho family GTPases (26). Rac1 is a central regulator of lamellipodia, promoting actin branching and polymerization at the leading edge, acting through effectors such as the WAVE regulatory complex (WRC), which in turn activates the Arp2/3 complex, responsible for actin nucleation and branching (27). Filopodia are long, slender, actin-rich protrusions that extend from the cell body (28). In microglia, filopodia are critical for cell-cell interactions, sensing of chemical cues, and transient movement (24). To enable effective migration and interaction with the extracellular environment, microglia rely on the formation of focal adhesions (FAs), which are actin-linked multi-protein complexes that anchor the cell to the extracellular matrix (ECM) (29). These structures serve as critical junctions that physically connect the actin cytoskeleton to the ECM (30). Specifically, integrins located at these FAs are linked to the actin cytoskeleton through adaptor proteins such as vinculin, serving as anchor points where stress fibers, which are bundles of actin filaments, can attach to the ECM and provide mechanical support and promote cell movement (31,32).

Actin polymerization, along with the branching and network formation of actin filaments, is crucial for regulating actin-dependent structures (33). However, the precise mechanisms and key players regulating actin polymerization and, consequently, microglial migration during development remain unclear. Since microglial dysfunction is linked to various brain disorders, including developmental abnormalities and neurodegenerative diseases, understanding key

molecular regulators of their function is essential (34,35).

Disrupted-in-schizophrenia 1 (DISC1) is a multifunctional intracellular scaffolding protein, encoded by the *Disc1* gene. It plays a critical role in brain development by engaging in a wide-ranging network of interactions (36). The role of DISC1 has been primarily studied in neurons, where it has been shown to be involved in cytoskeletal functions and migration. DISC1 interacts with a diverse array of proteins involved in key processes such as neuronal migration (*e.g.*, APP, Dixdc1, LIS1, NDE1, NDEL1) (37,38). Moreover, DISC1 interacts with cytoskeletal proteins such as Girdin, facilitating the cross-linking of actin filaments (39). DISC1 dysfunction in migrating interneurons reduced F-actin levels at the tips of leading processes and decreased Girdin and the Girdin-activator pAkt. This disruption in actin dynamics within growth cone-like structures impaired proper neuronal migration during development (40). Additionally, alterations in DISC1 are linked to major mental disorders such as schizophrenia, bipolar disorder, and impairments in motor skills, learning, language, and communication (41).

Although *Disc1* mutations and their associated protein functions have been investigated in neurons, their potential role in microglia remains uncertain. Research from our team suggests that DISC1 is involved in the microglial cytoskeleton. Specifically, *Disc1* locus impaired (*Disc1* LI) microglia exhibited disrupted cytoskeletal organization, marked by reduced actin density and impaired branch extension, correlating with diminished saltatory microglial migration. Moreover, single-cell RNA sequencing of *Disc1* LI microglia revealed differential expression of genes involved in actin cytoskeleton regulation (42). However, the precise mechanisms through which DISC1 regulates microglial cytoskeletal dynamics and movement remain to be elucidated.

Based on our findings, we aim to investigate whether these migratory deficits are driven by dysregulated actin cytoskeleton-dependent processes due to the impairment of DISC1. Our study therefore expands the understanding of microglial actin cytoskeletal dynamics by examining the role of DISC1 using a *Disc1* LI mouse model. We focus on alterations in filopodia and lamellipodia dynamics, FA density, and the polymerization balance between G-actin and F-actin. We hypothesize that DISC1 dysfunction impairs actin polymerization and disrupts these actin-dependent structures, ultimately impairing microglial migration during brain development.

EXPERIMENTAL PROCEDURES

Mouse models **–** Mice were group-housed in a 12-hour light/dark cycle in temperature- and humidity-controlled rooms with *ad libitum* access to water and food. All animal experiments were complied with the institutional guidelines and approved by the Ethical Committee for Animal Experiments at Hasselt University. Homozygous *Disc1* wild-type ($Disc1^{WT/WT}$) and *Disc1* LI ($Disc1^{LI/LI}$) littermates were bred from heterozygous *Disc1* LI mice. The $Disc1^{LI/LI}$ model was created by Prof. Akira Sawa of Johns Hopkins University in Baltimore, USA. Here, a 40-kb targeted deletion was introduced, spanning exons 1, 1b, 2, and 3, including a miRNA in intron 1 and Tsnax/Trax-*Disc1* intergenic region. A spontaneous 25-base pair deletion in exon 6, previously identified in the 129SvEv mouse strain, was also incorporated. This genetic modification resulted in the absence of the full-length 100 kDa DISC1 isoform. A double mutant $Disc1^{WT/WT}$ $Cx3cr1^{eGFP/+}$ and $Disc1^{LI/LI}$ $Cx3cr1^{eGFP/+}$ mouse model was created in-house, expressing eGFP under the CX3 chemokine receptor 1 (Cx3cr1) promoter, marking the microglia fluorescently green. $Cx3cr1^{eGFP/eGFP}$ mice were sourced from the European Mouse Mutant Archive (EMMA) institute with Steffen Jung's approval (Weizmann Institute of Science). Experiments were conducted using age-matched mice of both sexes, using littermate controls.

Genotyping **–** To determine mouse genotype, tissue biopsies were obtained to perform genotyping using the KAPA Mouse Genotyping Kit (KAPA Biosystems). Genomic DNA was extracted using an extraction buffer containing Milli-Q water, 10x KAPA Express Extract Buffer and 1 U/µl KAPA Express Extract Enzyme. Samples were heated at 75 °C for 15 min. The DNA extract was added to a PCR mix with 2x KAPA2G Fast Genotyping Mix, Milli-Q water, and primers (DISC1; *forward primer:5'-GCTGTGACCTGATGGCACT-3', reverse primer: 5'-GCAAAGTCACCTCAATAACCA-3'*). Polymerase chain reaction (PCR) was

performed: Initial denaturation at 95 °C for 1 min, followed by 30 cycles of 95 °C for 10 s, 64 °C for 10 s (with -0.2 °C decrement per cycle), 72 °C for 10 s and a final extension at 72 °C for 1 min, followed a hold at 12 °C. Samples were loaded on a 2% agarose gel (Invitrogen, Thermo Fisher Scientific) and separated by electrophoresis at 145 V for 60 min. A 100 bp ladder was used to detect amplicons at 196 bp (*Disc1*$^{WT/WT}$) and 171 bp (*Disc1*$^{LI/LI}$).

Primary microglia cell isolation – Primary microglial cells were harvested from *Disc1*$^{WT/WT}$ Cx3cr1$^{eGFP/+}$ and *Disc1*$^{LI/LI}$ Cx3cr1$^{eGFP/+}$ and *Disc1*$^{WT/WT}$ and *Disc1*$^{LI/LI}$ pups from postnatal days 2 to 5 (P2-P5). Brains were dissected post-decapitation and placed in cold Hank's Balanced Salt Solution (HBSS, Gibco, 4 °C) after skull removal. The meninges were removed, and brains were transferred to ice-cold Dulbecco's Modified Eagle Medium (DMEM, Gibco, UK). The brains were mechanically dissociated by triturating the cortices in DMEM and centrifuging (300 g, 5 min, 4 °C) the homogenate, followed by enzymatic digestion with papain (17 U/mg, Sigma-Aldrich, Germany) and DNase I (10 mg/ml, Roche, Switzerland). This was incubated for 20 min at 37 °C. Digestion was halted with cold DMEM, followed by centrifugation (300 g, 5 min, 4 °C). The pellet was resuspended in 10.10.1 medium (DMEM with 10% fetal calf serum (FCS, Bio-West), 10% horse serum (HS, Sigma-Aldrich), and 1% penicillin/streptomycin (P/S, Invitrogen)). Cells were seeded on poly-D-lysine (PDL, 20 µg/ml, Gibco)-coated flasks and incubated at 37 °C, 5% CO_2. Medium was renewed on days 3, 7 and 11 with fresh 10.10.1 medium, supplemented with one-third L929-conditioned medium (LCM). On day 14, microglia were isolated via orbital shaking (230 rpm, 3h, 37 °C), filtered through a 70 µm cell strainer (Corning) and seeded onto pre-PDL coated glass coverslips in 24-well plates (50 x 10^3 cells/well) and in 35mm glass bottom dishes (300x10^3 cells/dish).

Microglia transduction with LifeAct lentivirus – Primary microglia were seeded on 35 mm glass bottom dishes (300x10^3 cells/dish). 10.10.1 medium was removed and fresh medium was added with polybrene (8 µg/ml, 28728-55-4, Santa Cruz Biotechnology, Inc., USA). Cells were transduced with a lifeAct-mScarlet Lentivirus (0.7%) for 7-8 hours at 37 °C, 5% CO_2 and washed afterwards. Five days after transduction cells were rinsed and imaged in Krebs (in mM: 150 NaCl, 6 KCl, 10 HEPES, 10 glucose, 1.5 $CaCl_2$, 1 $MgCl_2$, pH 7.4). Live-cell imaging was performed on the Zeiss Elyra PS.1 (Carl Zeiss AG, Germany) with the Plan-ApoChromat 63×/1.40 Oil DIC M27 objective for the Structured Illumination Microscopy (SIM) with an excitation at 647 nm. Images were taken at 6-second intervals over 5 min. Acquired images were analyzed using Particle Image Velocimetry (PIV) lab (43) designed for MATLAB (The MathWorks, Inc., MA, USA).

Filopodia dynamics analysis – Filopodia dynamics were analyzed using PIVlab. Before PIV analysis, SIM videos were preprocessed in FIJI with median filtering (3), contrast enhancement (0.3%) and noise reduction, and saved as BMP files. Images were loaded into PIVlab in time-resolved mode. Preprocessing included CLAHE (window size = 20), Wiener2 denoising (window size = 3). PIV analysis used FFT window deformation with a 32-pixel interrogation area, 16-pixel step size, and two passes. Calibration was based on pixel-to-micron conversion using FIJI measurements, with a time step of 6000 ms. Post processing steps included velocity- and image-based validation for noise removal. Velocity magnitude was plotted using fixed scale limits determined by control samples to allow consistent comparison across conditions. Final data (u and v components) were exported to calculate vector magnitudes ($\sqrt{(U^2 + V^2)}$) in µm/s. Median velocities per frame were calculated.

Lamellipodia displacement analysis – Lamellipodia displacement was analyzed following Beeken et al.'s protocol (44). SIM images were pre-processed in Fiji with background subtraction (rolling ball radius: 30), median filtering (radius: 1.0), and drift correction using the StackReg plugin (rigid body). Lamellipodia were highlighted by thresholding (Huang method, dark background) with manual adjustment. Frame-by-frame subtraction (*e.g.*, frame 2 - frame 1) was performed using the Image Calculator, generating 49 difference images per ROI. Newly formed actin signal, indicating protrusive activity, was measured in each image. Displacement was quantified as the cumulative area (µm²) of these newly formed protrusions across all frames, resulting in a

single value representing total lamellipodia displacement per ROI.

Immunocytochemistry **–** Cells were fixed with 2% Paraformaldehyde (PFA), 0.05% Glutaraldehyde and 0.2 M HEPES for 15 min at room temperature (RT) followed by fixation with 4% PFA, 0.2 M HEPES for an additional 15 min at RT. After fixation, cells were washed with washing buffer (0.2% Triton X-100 in 1X PBS), 3x5 min. Cells were incubated with anti-Actin Monoclonal Antibody to visualize total actin (ACTN05, mouse, 1:200, Thermo Fisher Scientific, USA) overnight at 4 °C. Secondary antibody Alexa Fluor 555 (Donkey anti-mouse, 1:500, Invitrogen, USA) was added together with Alexa Fluor 647-Phalloidin (1:400, Thermo Fisher Scientific, USA) to visualize F-actin and Deoxyribonuclease Alexa Fluor 488 for G-actin (DnaseI, 0.5 µM Thermo Fisher Scientific, USA) in 1X PBS with 2% BSA and incubated for 1 h at RT. Lastly, cells were incubated with 6-diamino-2-phenylindole (DAPI, 1:10 000, Thermo Fisher Scientific) for nuclear staining. For FA visualization, a mouse anti-vinculin monoclonal antibody (2 µg/ml, Thermo Fisher Scientific) with an Alexa Fluor 555 goat anti-mouse (1:500, Invitrogen) secondary antibody was used. Coverslips were mounted on glass cover slides with Fluoromount-G (Thermo Fisher Scientific, USA). Imaging of actin-stained samples was carried out on the Zeiss Elyra PS.1 (Carl Zeiss AG, Germany) with the Plan-ApoChromat 63×/1.40 Oil DIC M27 objective for SIM imaging with an excitation at 555 nm, 647 nm and 488 nm. Vinculin-stained samples were imaged with a Zeiss LSM880 confocal microscope equipped with an Airyscan detector and the Plan-ApoChromat 63×/1.40 Oil DIC M27 objective (NA 1.40, 0.19 mm working distance).

Actin polymerization analysis **–** To quantify fluorescence intensity of F and G-actin, cells were manually outlined in Fiji using the polygon tool to determine cell area. Corrected Total Cell Fluorescence (CTCF) was calculated using the formula: CTCF = Integrated Density – (Area of selected cell × Mean background fluorescence). Background fluorescence was measured from a cell-free region in the same image. F/G-actin ratio was calculated by dividing the CTCF values for each cell.

Focal adhesion analysis **–** Microglial FAs were quantified using the protocol of Horzum *et al.* (45). Fluorescence images were analyzed in Fiji. Image processing included background subtraction (sliding paraboloid, radius 50), CLAHE (block size 19, histogram bins 256, max slope 6), exponential filtering, Log3D filtering (sigma X = 5, sigma Y = 5), thresholding (default automatic), and particle analysis (size 50–∞, circularity 0.00–0.99). FA outlines were overlaid on original images to verify detection accuracy. FA density was calculated as the number of FAs normalized to cell area to account for differences in cell size. Average FA size was calculated as the total FA area divided by the number of FAs. The FA area fraction was determined by dividing the total FA area by the cell area. Cell area was measured by manually outlining the cell using the polygon selection tool in Fiji.

Statistics **–** Statistical analysis was performed using GraphPad Prism 9. Data distributions were assessed for normality using the Shapiro-Wilk tests, and appropriate parametric or non-parametric tests applied as necessary. Detailed statistical information, including details on sample size and statistical analyses, is provided in the figure legends. P-values < 0.05 were considered significant.

RESULTS

Disc1 locus impairment enhances filopodia dynamics in microglia **–** Previously, we observed that *Disc1* LI affects microglial migration during embryonic development, suggesting that DISC1 plays a key role in regulating this process. Since cell migration is strongly influenced by cytoskeletal dynamics, we sought to investigate whether DISC1 affects the formation and behavior of microglial protrusions, specifically filopodia (46). Filopodia are dynamic, actin-rich projections that extend from the leading edge of cells and rapidly retract and extend in a stochastic surveillance pattern, enabling microglia to perform fast, nanoscale environmental sensing within localized regions (24). To assess whether DISC1 influences these dynamic structures, we analyzed filopodial behavior in *Disc1*$^{WT/WT}$ and *Disc1*$^{LI/LI}$ microglia. To quantify filopodia motility, we performed live-cell SIM of primary cultured *Disc1*$^{WT/WT}$ and *Disc1*$^{LI/LI}$ microglia, transduced with LifeAct-mScarlet lentivirus. Single filopodia were cropped out and analyzed over time (3 min), using PIVlab in MATLAB to generate velocity vectors as a measure for filopodia dynamics.

SIM overlays illustrate filopodia movement over a 3-min interval (0 min in green and 3 min in red) **(Fig. 1a)**. Vector magnitudes in µm/min are represented in the heat map where *Disc1*$^{LI/LI}$ microglia show increased filopodia activity compared to *Disc1*$^{WT/WT}$ microglia, reflected by high-magnitude regions (yellow) representing faster movement in contrast to the low-magnitude regions (blue) **(Fig. 1a)**. The median vector velocity over a 3-min interval showed significant higher actin-rich filopodia movements in *Disc1*$^{LI/LI}$ cells compared to *Disc1*$^{WT/WT}$ cells ($P < 0.0001$) **(Fig. 1b)**. Next, we averaged the median *Disc1*$^{LI/LI}$ vector velocities across all time frames to obtain one value per cell. This data confirms that *Disc1*$^{LI/LI}$ microglia show significantly elevated median vector velocities ($P = 0.0242$) as well as a broader distribution compared to *Disc1*$^{WT/WT}$ microglia, suggesting dysregulated actin dynamics **(Fig. 1c)**. Moreover, previous data showed that *Disc1*$^{LI/LI}$ microglia exhibit a significantly higher number of filopodia (47). Taken together, these findings suggest that DISC1 is critical for regulating balanced filopodial behavior in microglia, with its impairment likely driving a shift toward excessive filopodia formation and activity.

***Disc1* locus *impairment disrupts lamellipodia formation and movement in microglia* –** While filopodia function mainly as sensory structures for environmental scanning, effective cell migration relies on the extension and movement of broad, actin-rich lamellipodia (25). Given the reduced migration observed in *Disc1*$^{LI/LI}$ microglia, we next examined whether DISC1 impairment alters lamellipodia dynamics. SIM images of LifeAct-mScarlet lentivirus transduced *Disc1*$^{WT/WT}$ and *Disc1*$^{LI/LI}$ microglia were obtained and lamellipodia-rich regions were selected **(Fig. 2a)**. We quantified lamellipodia displacement over a 5-min time period by measuring changes in lamellipodia

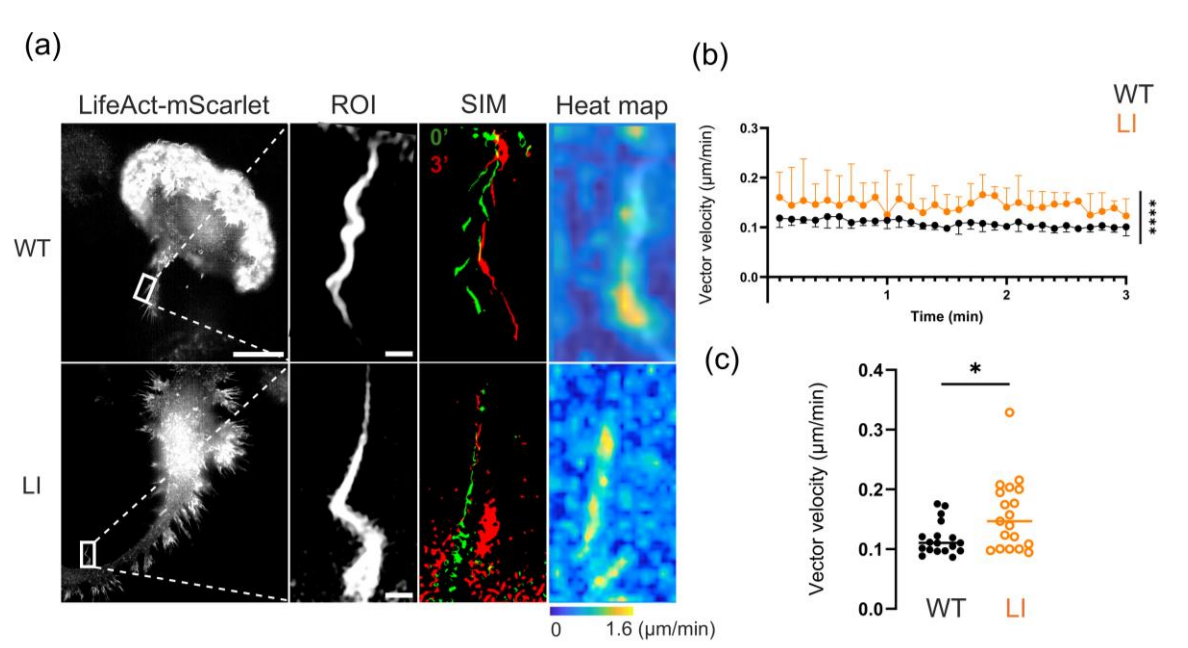

Figure 1. *Disc1* locus impairment enhances microglia filopodia movement. (a) Representative live-cell SIM images of primary cultured *Disc1*$^{WT/WT}$ (top, WT) and *Disc1*$^{LI/LI}$ (bottom, LI) microglia transduced with LifeAct-mScarlet lentivirus to visualize F-actin. Regions of interest (ROIs) highlight individual filopodia, with SIM overlays showing differences in filopodia movement between min 0 (green) and min 3 (red). Heat maps represent filopodia vector velocities derived from PIV analysis in MATLAB. Color scale represents vector magnitude in µm/min Scale bar = 10 µm (left panel), 1 µm (ROI). (b) Median vector velocity (µm/min) over a 3-min interval, sampled every 6 s (*i.*e., 30 time points) of *Disc1*$^{WT/WT}$ and *Disc1*$^{LI/LI}$ microglia. Data points represent median vector velocity per time point (20 filopodia per genotype derived from 5 microglia). (c) Scatter plot showing the average of the median vector velocity per ROI ($P = 0.0242$). Data points represent individually analyzed ROIs of different images (n= 20 regions per condition, from two independent experiments). Horizontal bars are reported as the median. Mann-Whitney U test. * $P < 0.05$, **** $P < 0.0001$.

protrusion area using a threshold-based image subtraction approach in Fiji. Visually, *Disc1*$^{WT/WT}$ cells exhibited a more extensive lamellipodia activity over time, with lamellipodia expanding noticeably in the whole-cell overlay as well as the ROI panels **(Fig. 2a)**. In contrast, *Disc1*$^{LI/LI}$ cells displayed reduced lamellipodia activity with diminished area changes in the corresponding ROI overlays. Quantification of lamellipodia displacement revealed a significant reduction in *Disc1*$^{LI/LI}$ cells compared to *Disc1*$^{WT/WT}$ microglia ($P < 0.0001$), indicating that DISC1 dysfunction impairs lamellipodial dynamics in microglia **(Fig. 2b).**

anchoring points that link the actin cytoskeleton to the ECM and regulate protrusion stability and migration efficiency, assessing differences in their presence between *Disc1*$^{WT/WT}$ and *Disc1*$^{LI/LI}$ microglia may provide important insight into the migratory behavior of *Disc1*$^{LI/LI}$ microglia (29) *Disc1*$^{WT/WT}$ and *Disc1*$^{LI/LI}$ microglia were stained for vinculin, a common FA marker and phalloidin-647 to visualize the F-actin cytoskeleton and assess cell structure. Immunohistochemistry and confocal microscopy were conducted by A. Janssens **(Fig. 3a)**. Three metrics were quantified to assess differences in FA characteristics between *Disc1*$^{WT/WT}$ and *Disc1*$^{LI/LI}$ microglia:

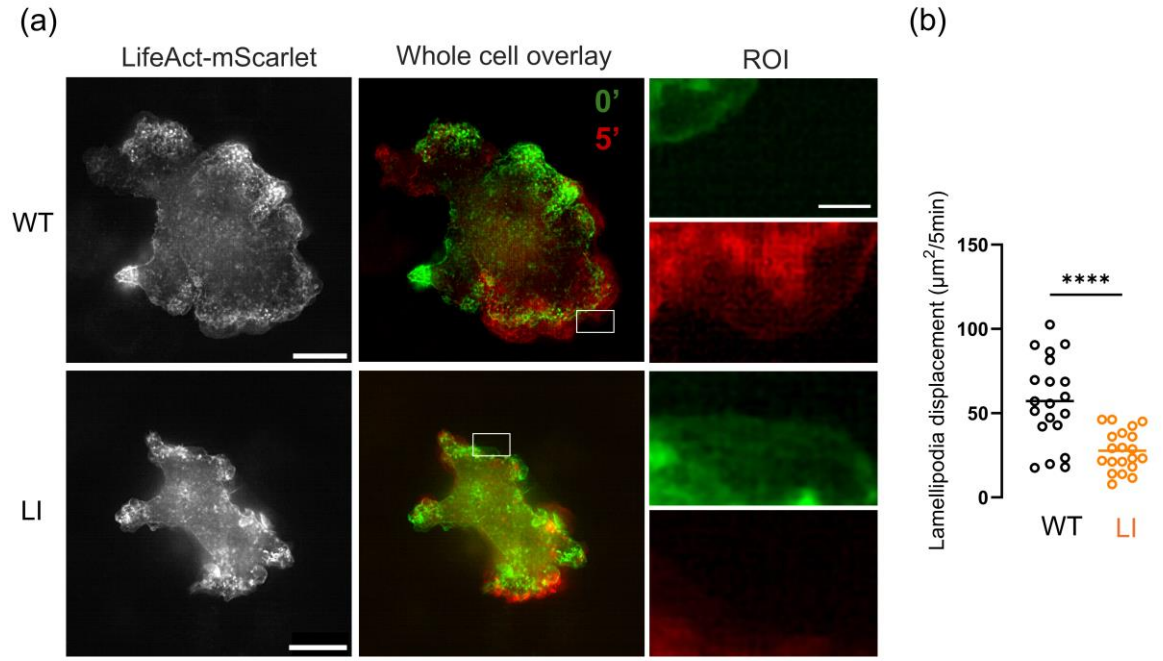

Figure 2. *Disc1* locus impairment alters microglial lamellipodia dynamics. (a) Representative SIM live-cell images of primary cultured *Disc1*$^{WT/WT}$ (top, WT) and *Disc1*$^{LI/LI}$ (bottom, LI) microglia transduced with LifeAct-mScarlet lentivirus to visualize actin dynamics. Scale bar = 5 µm. Regions of interest (ROIs) show zoomed-in lamellipodial areas where displacement is shown between min 0 (green) and min 5 (red). Scale bar = 2 µm. **(b)** Quantification of lamellipodia displacement (µm²/5 min) over time per ROI (4 ROIs per cell, 5 cells per genotype, n= 20 data points per genotype) ($P < 0.0001$). Horizontal bars are reported as the median. Unpaired T-test. Data represent cells from two independent experiments. **** $P < 0.0001$.

Disc1 locus impairment reduced focal adhesion density in microglia – Building on our previous findings that *Disc1*$^{LI/LI}$ microglia exhibit altered actin dynamics, characterized by increased filopodia activity and reduced lamellipodia displacement, we next investigated whether these cytoskeletal differences were accompanied by changes in FA formation. Since FAs serve as critical

FA density, average FA size, and FA area fraction. To account for differences in cell size, FA counts were normalized to cell area, which was determined by outlining the cells based on their cytoskeleton. FA density was calculated as the number of FAs per unit cell area, and

compared between *Disc1*$^{WT/WT}$ and *Disc1*$^{LI/LI}$ microglia. *Disc1*$^{LI/LI}$ microglia showed reduced FA density compared to WT cells ($P = 0.0200$), indicating fewer adhesions relative to cell size

significant difference was observed between the genotypes ($P = 0.3428$), suggesting that while *Disc1*$^{LI/LI}$ cells form fewer adhesions, the size of individual FAs remains comparable to

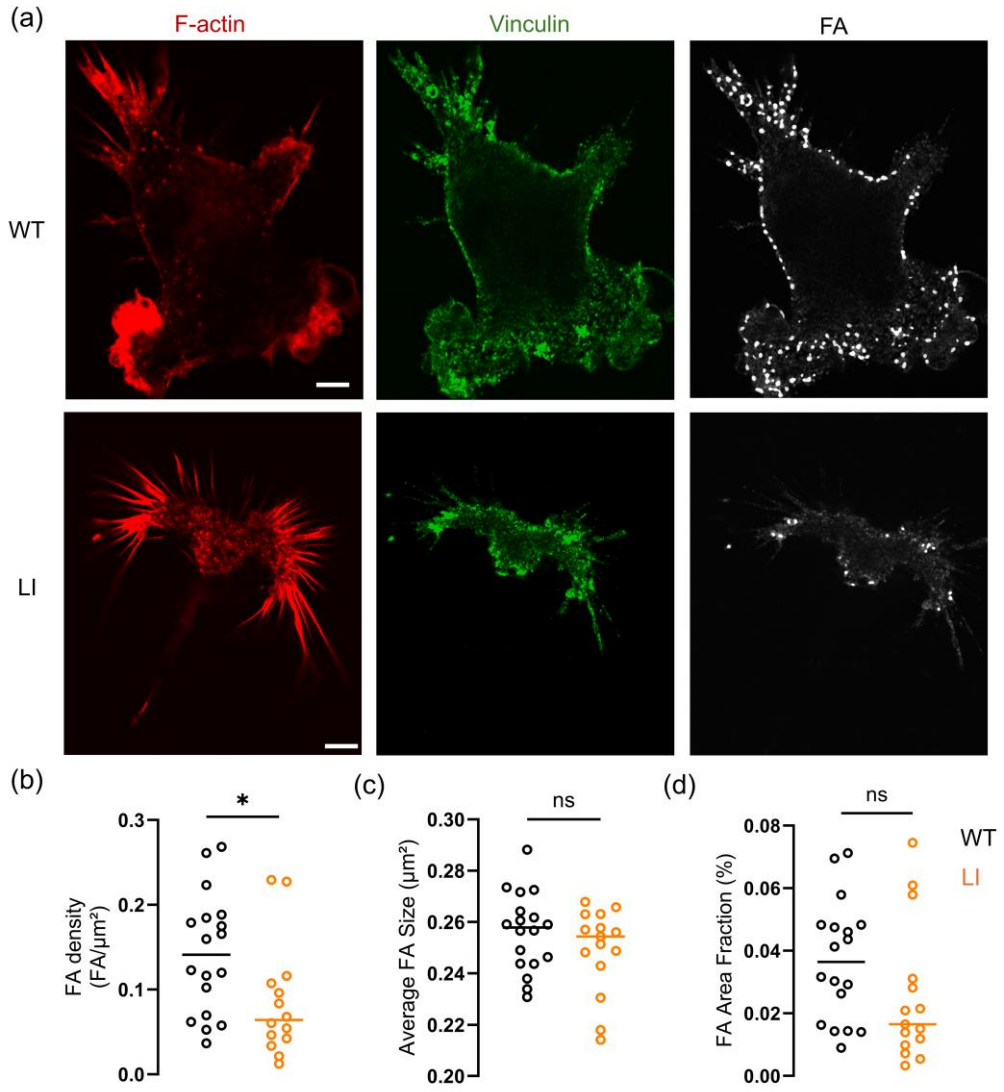

Figure 3. *Disc1* locus impairment disrupts FA formation in microglia. (a) Representative confocal images of microglia from *Disc1*$^{WT/WT}$ (top, WT) and *Disc1*$^{LI/LI}$ (bottom, LI) microglia stained for phalloidin-647 (F-actin, red), vinculin (green), and processed images to visualize Focal Adhesions (FA, greyscale). Scale bar = 5 µm. (b-d) Quantitative analysis of FA characteristics in *Disc1*$^{WT/WT}$ and *Disc1*$^{LI/LI}$ microglia. (b) FA density (FA count per µm² cell area) ($P = 0.0200$). (c) Average FA size (total FA area divided by FA count). (d) FA area fraction (total FA area per cell area in %) ($P = 0.0747$, ns = non-significant). Data points represent individual microglia (n= 18 *Disc1*$^{WT/WT}$ microglia and n=15 *Disc1*$^{LI/LI}$ microglia). Immunostaining and imaging performed by A. Janssens. Horizontal bars are reported as the median. Mann-Whitney U test. ** $P < 0.05$, ns = non-significant.*

(Fig. 3b). To determine whether this reduction in FA number was accompanied by a compensatory change in adhesion size, we calculated the average FA size by dividing the total FA area by the number of FAs. No

those in *Disc1*$^{WT/WT}$ cells **(Fig. 3c)**. Lastly, we obtained the FA area fraction, representing the proportion of the cell area occupied by adhesions. Although this value was lower in *Disc1*$^{LI/LI}$ cells, this difference did not reach

statistical significance (P = 0.0747) **(Fig. 3d)**. Importantly, since FA area fraction reflects a combination of FA density and their size, the observed reduction in density, together with the unchanged FA size, likely contribute to the overall trend toward reduced adhesive coverage

average FA size remains largely unchanged. The reduced FA density may underlie the impaired embryonic microglial migration in *Disc1*$^{LI/LI}$ microglia by limiting anchoring points, thereby restricting the traction forces essential for effective movement.

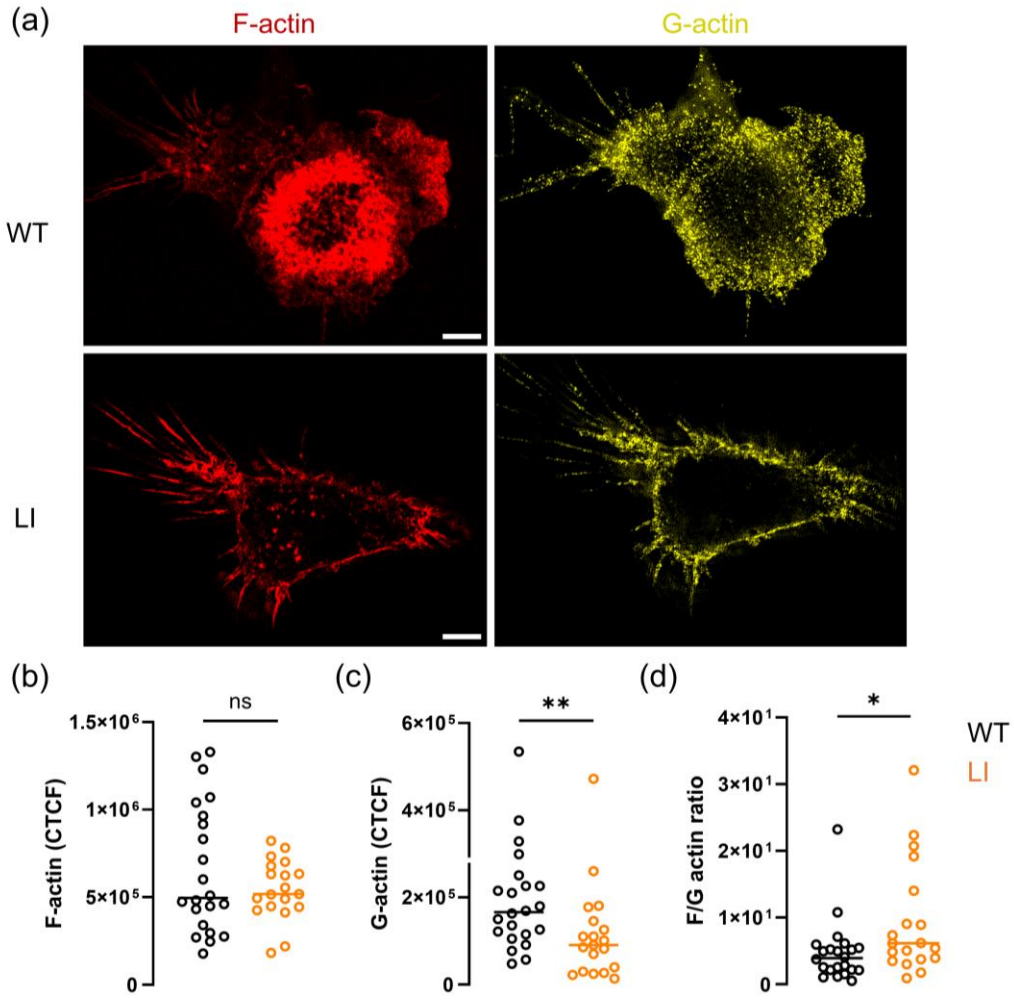

Figure 4. *Disc1* locus impairment shifts actin balance in microglia. (a) Representative confocal images of primary *Disc1*$^{WT/WT}$ and *Disc1*$^{LI/LI}$ microglia showing filamentous actin (F-actin, red), and monomeric actin (G-actin, yellow). Scale bar = 5 µm. (b-d) Quantification of F-actin (b); G-actin (c); and the F/G-actin ratio (d) using corrected total cell fluorescence (CTCF). (P = 0.6885 (b); P = 0.0059 (c); P = 0.0299 (d)). Data points represent individual microglia (n= 22 *Disc1*$^{WT/WT}$ microglia and n=19 *Disc1*$^{LI/LI}$ microglia). Horizontal bars are reported as the median. Mann-Whitney U test. * P < *0.05,* ** P < *0.01, ns = non-significant.*

in *Disc1*$^{LI/LI}$ microglia. Nonetheless, the trend aligns with the overall reduction in FA density and supports the possibility of a generalized defect in adhesion complex formation and their stabilization. These findings therefore suggest that DISC1 dysfunction is associated with a significant reduction in FA density, while

***Disc1* locus impairment disrupts actin polymerization capacity in microglia** - As we previously revealed that *Disc1*$^{LI/LI}$ microglia exhibit increased filopodia motility, reduced lamellipodia displacement, with the presence of fewer FAs compared to *Disc1*$^{WT/WT}$ cells, our analyses suggest that DISC1 dysfunction may

lead to abnormal actin-based protrusion dynamics and anchoring of the cell to the ECM. These findings implicate a fundamental disruption in cytoskeletal organization, particularly which relies tightly on regulated actin polymerization. This process involves the formation of F-actin from monomeric G-actin (23). We investigated whether DISC1 dysfunction impairs this actin polymerization capacity. To test this, we quantified the presence of G- and F-actin in *Disc1*$^{LI/LI}$ and *Disc1*$^{WT/WT}$ microglia, and determined the F/G-actin ratio as a quantitative measure of polymerization. We used Alexa Fluor 488–conjugated DNase I and Alexa Fluor 647–conjugated phalloidin to mark G- and F-actin respectively **(Fig. 4a)**. Quantitative analysis using corrected total cell fluorescence (CTCF) confirmed that F-actin levels did not differ significantly between genotypes **(Fig. 4b)** (P = 0.6885). However, G-actin levels were significantly decreased in *Disc1*$^{LI/LI}$ microglia **(Fig. 4c)** (P = 0.0059). As a result, the F/G-actin ratio was significantly elevated in *Disc1*$^{LI/LI}$ cells compared to *Disc1*$^{WT/WT}$ microglia **(Fig. 4d)** (P = 0.0299), indicating a shift toward monomeric actin and impaired actin polymerization in *Disc1*$^{LI/LI}$ microglia.

These results suggest that DISC1 dysfunction leads to a reduced G-actin pool without affecting total F-actin levels, indicating a disruption in the equilibrium between monomeric G-actin and F-actin.

DISCUSSION

Microglia play a crucial role in brain development by supporting the formation of complex neural networks (48). In the early stages of brain development, they are actively involved in synaptic pruning, promoting neuronal plasticity, and preserving tissue integrity (49,50). To fulfill these dynamic roles, microglia depend on their ability to migrate through the brain parenchyma (51). Disruption of microglial migration and synaptic pruning has been linked to NDDs, highlighting the critical importance of the cytoskeleton during brain development (52). One gene that has emerged as a key player in neurodevelopmental risk is *Disc1,* which encodes the multifunctional scaffolding protein DISC1 (40,53). While DISC1 is well studied in neurons, where it is known to regulate cytoskeletal organization, its role in microglia remains poorly understood (40). Interestingly, we have previously observed that DISC1 dysfunction impairs embryonic microglial saltatory migration, indicating a potential role in regulating the microglial cytoskeleton (42). Microglial movement depends largely on dynamic remodeling of the actin cytoskeleton, which supports the formation of lamellipodia, broad membrane ruffles that drive the cell forward, and filopodia, thin exploratory protrusions that sense the surrounding environment (54–57). In addition, tightly regulated interactions with the ECM through FAs anchor the microglia and generate the contractile forces needed for movement (58). As these actin-based structures are essential for migration, we aimed to investigate whether DISC1 influences microglial migration by regulating these protrusions and adhesion sites (54).

We here examined the role of DISC1 in microglial filopodia and lamellipodia dynamics. Live-cell SIM revealed a striking shift in actin-rich protrusion dynamics in *Disc1*$^{LI/LI}$ microglia. Specifically, PIV analysis of LifeAct-mScarlet transduced microglia revealed that *Disc1*$^{LI/LI}$ microglia consistently exhibit enhanced filopodia dynamics compared to *Disc1*$^{WT/WT}$ microglia. On the other hand, *Disc1*$^{LI/LI}$ microglia revealed reduced lamellipodia movement. These findings are consistent with earlier observations, which show that *Disc1*$^{LI/LI}$ microglia exhibit reduced lamellipodia and increased filopodia formation (47). Interestingly, lamellipodia formation depends on Arp2/3-mediated nucleation (59). The Arp2/3 complex initiates the formation of branched actin networks by binding to the side of preexisting actin filaments and generating new filaments at a characteristic 70° angle (60). This branching activity is essential for the formation and dynamic behavior of lamellipodia (61). Specifically, lamellipodia expansion is sustained by the coordinated activity of the Arp2/3 complex, the WAVE complex, and their upstream regulators (25). Small GTPases such as Rac accumulate at the tips of these protrusions, promoting the localization and activity of the WAVE complex, where it acts as a polymerase to promote branched actin filament elongation, securing the actin network to the plasma membrane, and functions as a nucleation-promoting factor for the Arp2/3 complex (62). This activity near the plasma membrane ensures persistent actin polymerization, driving the

extension of lamellipodia (25). Interestingly, our single-cell RNA sequencing has revealed downregulation of *Arp2/3* in *Disc1*$^{LI/LI}$ microglia (42). Therefore, DISC1 possibly modulates lamellipodia dynamics via upstream pathways involving Rac and Rho. Within neurons, DISC1 was found to regulate Rac1 signaling, particularly influencing glutamatergic synapse spine formation (63). Since Rac1 activation is essential for Arp2/3-mediated lamellipodia expansion, disruption of DISC1 may impair Rac1 signaling in microglia, which in turn compromises Arp2/3 function (64). This likely limits lamellipodia formation and movement and ultimately impairs microglial migration. In contrast, filopodia formation mainly relies on formin-driven linear actin polymerization (65). Formins facilitate the nucleation and processive elongation of linear, unbranched actin filaments by associating with filament barbed ends and protecting them from capping proteins (23). *Disc1*$^{LI/LI}$ microglia show upregulation of *Fmn1*, a formin family member (42). Taken together, the increased filopodial dynamics observed in *Disc1*$^{LI/LI}$ microglia may therefore reflect a compensatory or dysregulated upregulation of formin activity in response to impaired lamellipodial stability. While formins, such as mDia1/2, can nucleate and elongate unbranched actin filaments essential for filopodia, these protrusions are slender and transient, and do not generate the broad, traction-bearing forces necessary for sustained migration (66,67). This demonstrates that DISC1 has a specific role in the expansion and maintenance of these structures. Our findings suggest that DISC1 dysfunction disrupts actin cytoskeleton remodeling in microglia, causing a shift from stable lamellipodia protrusions to more dynamic, 'scanning' filopodia. This shift may underlie the migratory deficits observed in *Disc1*$^{LI/LI}$ microglia (42,68). However, given the extensive range of DISC1-protein interactions, it remains challenging to isolate a single definitive signaling pathway (69). Therefore, we aim to identify potential interaction partners of DISC1 in the future.

Besides protrusion dynamics, microglial migration is further controlled by cell adhesion mechanisms, including the formation of FAs (29). Integrin-based FAs enable microglia to anchor to the ECM, facilitating traction during migration (70). At the leading edge of the lamellipodium, integrin clustering gives rise to nascent adhesions, which are transient structures that initiate extracellular signal transduction (71). A subset of these matures into FAs through recruitment of adaptor proteins such as α-actinin, myosin II, RhoA, and tensin, enabling linkage to the actin cytoskeleton. These adhesion sites coordinate signaling pathways that regulate cytoskeletal dynamics essential for directed microglial migration (72). Therefore, FAs are essential for effective microglial migration and reduction in their formation could contribute to the migratory deficits observed in our *Disc1*$^{LI/LI}$ model (73). To explore this, we examined the role of DISC1 in microglial FA formation. Our data indicate that *Disc1*$^{LI/LI}$ microglia form fewer adhesions relative to their cell size. However, adhesions that do form are of comparable size to those in *Disc1*$^{WT/WT}$ cells. Mechanistically, this reduction in FA density may stem from underlying defects in actin cytoskeletal organization. As it remains unclear whether DISC1 directly regulates FA-associated proteins, pinpointing the precise mechanism underlying the observed reduction in FA formation is challenging. However, the observed reduction in FA formation in *Disc1*$^{LI/LI}$ suggests that DISC1 may play an upstream regulatory role in FA formation. Previous studies have shown that DISC1 upregulates β1-integrin protein levels in neuronal cells, which is a crucial component of FAs that mediate cell–matrix interactions (74). Similarly, it is possible that DISC1 dysfunction in microglia leads to reduced β1-integrin expression, thereby contributing to decreased FA formation and density. The relationship between FAs and cell migration is biphasic: insufficient adhesion fails to generate the traction forces necessary for movement, whereas excessive adhesion can hinder cell detachment and limit forward progression (75). This balance is critical for efficient migration and may be disrupted in *Disc1*$^{LI/LI}$ microglia, where reduced FA density could impair traction generation and thus lead to migratory deficits (76).

Building on these findings, we next explored whether changes in protrusion dynamics and adhesion formation observed in *Disc1*$^{LI/LI}$ microglia could be explained by disruptions in actin polymerization. We observed a significant increase in the F/G-actin ratio in *Disc1*$^{LI/LI}$ microglia, driven by a reduction in G-actin levels, while F-actin levels

remained unchanged compared to *Disc1*$^{WT/WT}$ microglia. This indicates a reduction in the total actin pool, driven by decreased levels of G-actin without a corresponding increase in F-actin. Our previous transcriptomic data revealed upregulation of *Pfn2*, which promotes G-actin delivery to barbed ends, and downregulation of *CapG*, which caps barbed ends to limit filament growth (42,77,78). Together, these changes would be expected to enhance actin polymerization and reduce the available pool of G-actin monomers (79). This is consistent with our observation of reduced G-actin levels in *Disc1*$^{LI/LI}$ microglia. However, F-actin levels remained unchanged, indicating that increase in polymerization does not lead to a net gain in F-actin. Alternatively, the observed reduction in G-actin and stable F-actin levels may reflect impaired recycling of actin monomers (80). Here, actin filaments may form and persist, but turnover is inefficient, leading to depletion of the monomer pool (81). Altogether, these findings suggests that DISC1 dysfunction disrupts actin turnover, which may underlie altered protrusion dynamics and reduced adhesion formation in *Disc1*$^{LI/LI}$ microglia. However, interpretation is limited by our small sample size, and further replication and experiments are needed to confirm whether this reflects true biological processes.

CONCLUSION

Taken together, our findings provide evidence that DISC1 plays a critical role in regulating microglial actin cytoskeleton dynamics, affecting both protrusion formation and cell adhesion essential for effective migration during neurodevelopment. Altered lamellipodia and filopodia dynamics, reduced FA density and disrupted actin polymerization all point toward a cytoskeletal network that is destabilized in the absence of functional DISC1. However, while these data offer important insights into the cellular consequences of DISC1 dysfunction, precise molecular mechanisms remain unknown. Given the multifunctional nature of DISC1 and its extensive network of interacting proteins, further studies are necessary to map its specific molecular partners in microglia. This will help us understand how DISC1 influences microglial function in development and disease.

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