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1) Title page

Age-specific function of $\alpha 5\beta 1$ integrin in microglial migration during early colonization of the developing mouse cortex

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15
16 **Main Points**

17
18 Count: 217
19 1) $\alpha 5\beta 1$ integrin regulates the migration of microglia during embryonic corticogenesis
20 2) A developmental switch of $\alpha 5\beta 1$ integrin function occurs between E13.5 and E15.5 from
21 promoting to inhibiting microglial migration

22
23 **Table of Contents Image (TOCI)**

24 see separate figure upload

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27 Embryo - Blood Vessels - Cell Adhesion Molecules - Fibronectins - Cell Migration Inhibition

2) Abstract

Microglia, the immune cells of the central nervous system, take part in brain development and homeostasis. They derive from primitive myeloid progenitors that originate in the yolk sac and colonize the brain mainly through intensive migration. During development, microglial migration speed declines which suggests that their interaction with the microenvironment changes. However, the matrix-cell interactions allowing dispersion within the parenchyma are unknown. Therefore, we aimed to better characterize the migration behavior and to assess the role of matrix-integrin interactions during microglial migration in the embryonic brain *ex vivo*. We focused on microglia-fibronectin interactions mediated through the fibronectin receptor $\alpha 5\beta 1$ integrin because *in vitro* work indirectly suggested a role for this ligand-receptor pair. Using 2-photon time-lapse microscopy on acute *ex vivo* embryonic brain slices, we found that migration occurs in a saltatory pattern and is developmentally regulated. Most importantly, there is an age-specific function of the $\alpha 5\beta 1$ integrin during microglial cortex colonization. At embryonic day (E)13.5, $\alpha 5\beta 1$ facilitates migration while as from E15.5, it inhibits migration. These results indicate a developmentally regulated function of $\alpha 5\beta 1$ integrin in microglial migration during colonization of the embryonic brain.

1 3) Text

2 **INTRODUCTION**

3 Microglia, the immune cells of the central nervous system (CNS), are renowned as the first line
4 defense during brain disease. The last decade researchers have been exploring the plentiful non-
5 immunological tasks of these cells and found them to be involved in normal brain development and
6 homeostasis, through influencing neurogenesis, axonal growth, synapse refinement, blood vessel
7 branching and clearance of dying neurons (Casano and Peri 2015; Frost and Schafer 2016). Microglial
8 cells originate from primitive myeloid progenitor cells that arise from the yolk sac at embryonic day
9 (E) 7.5 in mice. Primitive macrophages migrate to the CNS using the blood circulation and adopt a
10 microglia phenotype when they invade the brain and spinal cord parenchyma around E10.5 and
11 E11.5, respectively (Ginhoux et al. 2010; Rigato et al. 2011; Swinnen et al. 2013). Several signaling
12 pathways were recently proposed to be involved in microglia recruitment to the embryonic brain *in*
13 *vivo*, including components such as colony stimulating factor-1 receptor (CSF1R), matrix
14 metalloproteinases (MMPs), vascular endothelial growth factor receptor (VEGFR), Fractalkine
15 receptor (CX3CR1) and stromal cell-derived factor 1 (SDF-1)/CXCR4 (Arno et al. 2014; Ueno and
16 Yamashita 2014). Cortical colonization depends mainly on microglial invasion and migration since
17 only a minority of these cells proliferates within the parenchyma during this developmental period
18 (Swinnen et al. 2013). Despite the fact that parenchymal migration of microglia is essential to brain
19 colonization, the mechanisms allowing microglial dispersion have never been investigated *in vivo*
20 (Eyo and Dailey 2013; Ueno and Yamashita 2014).

21 Cell migration relies on interactions with the extracellular matrix (ECM). Cell-ECM adherence is
22 regulated through integrins, which are transmembrane heterodimeric cell adhesion receptors
23 composed of a non-covalently linked α and β subunit. Upon activation, the β subunit physically links
24 the ECM with the cytoskeleton and enables the cell to transduce forces necessary for soma
25 displacement (Vicente-Manzanares and Horwitz 2011). Twenty four different integrin heterodimers

1 exist in vertebrates with varying ligand binding properties and cell and tissue distributions. They
2 translate ligand binding signals to a broad array of cell responses, such as cytokine production,
3 proliferation, differentiation and migration (Hynes 2002). Our research group previously showed that
4 microglial migration speed changes during cortex colonization in the mouse embryo (Swinnen et al.
5 2013) which suggests the interaction of microglia with their local environment changes during the
6 course of early development. Several *in vitro* findings point to a possible functional role of microglial
7 interactions with fibronectin (an ECM protein) in migration during embryonic development.
8 Fibronectin, a heterodimeric glycoprotein abundant in most tissues, is an important component of
9 basement membranes. It is expressed in the developing mouse CNS (Lau et al. 2013; Ruoslahti 1996;
10 Sheppard et al. 1991) and is essential for normal embryonic development (Romberger 1997) where it
11 regulates cell differentiation and migration in general (Romberger 1997; Tanzer 2006). Microglia *in*
12 *vitro* express the corresponding major fibronectin receptor, $\alpha 5 \beta 1$ integrin (Milner 2009; Milner and
13 Campbell 2003) and it has been shown *in vitro* that these cells can migrate along fibronectin matrix
14 (Milner and Campbell 2003; Nasu-Tada et al. 2005). Moreover, they can interact with blood vessels
15 that are known to express fibronectin during development (De Gasperi et al. 2012; Grossmann et al.
16 2002; Milner and Campbell 2002b; Pont-Lezica et al. 2011; Sheppard et al. 1991; Stewart and
17 Pearlman 1987; Tanzer 2006).

18 Here, we explore the role of microglia-fibronectin interactions, mediated through $\alpha 5 \beta 1$ integrin,
19 when the developing embryonic neocortex is colonized by microglia. We use *ex vivo* acute brain slice
20 preparations in order to maintain the physiologic 3D brain environment, which is essential for
21 studying normal migration behavior (Doyle and Yamada 2016; Kasahara et al. 2016; Petersen and
22 Dailey 2004). We first extensively characterize microglial migration behavior at E13.5, E15.5 and
23 E17.5 using 2-photon time-lapse microscopy. We then analyze the presence of fibronectin in the
24 developing cortex using immunofluorescence and western blotting and determine the expression
25 level of $\alpha 5 \beta 1$ integrin receptor using flow cytometry on acutely isolated embryonic microglia. Finally,

1 we assess the functional importance of fibronectin- $\alpha 5\beta 1$ interactions during microglia contact with
2 blood vessels and during parenchymal migration.
3

MATERIALS AND METHODS

Animals

Wild type *mus musculus* C57BL/6 J0laHsD females (Harlan, The Netherlands) were mated overnight with CX3CR1^{eGFP/eGFP} knock-in males, obtained from the European Mouse Mutant Archive (EMMA) with the approval of Jung et al. (Jung et al. 2000). The next morning, females were checked for the presence of a copulation plug and designated E0.5. Pregnant mothers were sacrificed at E13.5, E15.5, E17.5 by cervical dislocation. Resulting CX3CR1^{+eGFP} embryos harbor green fluorescent microglia, monocytes and subsets of natural killer cells and dendritic cells, without the disadvantages of a full CX3CR1 gene deletion (Jung et al. 2000). All experiments were conducted in accordance with the European Community guiding principles on the care and use of animals and with the approval of the Ethical Committee on Animal Research of Hasselt University. Mice were maintained in the animal facility of Hasselt University in accordance with the guidelines of the Belgian Law and the European Council Directive.

Markers

The following primary antibodies were used: anti-fibronectin (1:100 for immunohistofluorescence, 1:1000 for western blotting, #Ab2413, Abcam), anti- β -actin antibody (1:10.000, #Sc47778, Santa Cruz), , anti- α 5-Phycoerythrin (clone 5H10-27 (MFR5), 1.5 μ g/ml, #557447, BD Biosciences). Isolectin GS-IB₄ from *Griffonia simplicifolia* conjugated to Alexa568 (5 μ g/ml for time-lapse imaging, 10 μ g/ml for immunohistofluorescence, #I21412, Life Technologies) was used to mark blood vessels. For time-lapse blocking experiments, anti- α 5 β 1 (clone BMC5, 10 μ l/ml, #NBP2-29788, Novus) or isotype control (clone RTK4174, 10 μ l/ml, #400710, Biolegend) were used. The following secondary antibodies were used for immunohistofluorescence: anti-rabbit-Alexa555 (1:500, #A31572, Life Technologies), anti-rabbit-Alexa647 (1:500, #A21245, Life Technologies); and for western blotting: anti-rabbit and anti-mouse-HRP (1:2000, #P0217 and 1:5000, #P0447, DAKO).

1 To label fibronectin, we used a polyclonal anti-fibronectin antibody (Li et al. 2016). Fibronectins are
2 disulphide linked heterodimeric molecules of 235–270 kDa. Fibronectin molecular isoforms arise via
3 alternative splicing of a single gene. Specificity was determined by the manufacturer by western
4 blotting on different mouse tissue lysates and a single band was obtained around 250 kDa as
5 predicted for the molecular weight of a single fibronectin subunit (Tanzer 2006). In time-lapse
6 experiments related to microglia-blood vessel contact assessments, isolectin GS-IB₄-Alexa568 was
7 added to the migration medium to visualize blood vessels (Graupera et al. 2008). It is widely used to
8 label blood vessels and microglial cells in slice cultures and does not activate microglial cells
9 (Grinberg et al. 2011). To label $\alpha 5 \beta 1$ integrin on microglial cells dissociated from cortex homogenates
10 for flow cytometry, we used a monoclonal anti- $\alpha 5$ -Phycoerythrin conjugated antibody (Cukierman et
11 al. 2001). Because the $\alpha 5$ integrin subunit (alternatively named CD49e and VLA-5) exclusively
12 associates with the $\beta 1$ subunit, we consider its presence to be in heterodimeric form with $\beta 1$ (Milner
13 and Campbell 2002c).

14 **Time-lapse imaging**

15 E13.5, E15.5 and E17.5 embryonic brains were isolated and sliced as described before (Swinnen et al.
16 2013). Slices were transferred to MilliCell organotypic inserts (Merck Millipore) in a 24-well plate
17 designed for confocal microscopy (IBIDI) and maintained in semidry conditions as described before
18 (Swinnen et al. 2013). Slicing quality was verified using the dissection microscope and slices that
19 showed aberrant morphology (ruptures, insufficient flatness) were excluded from time-lapse
20 measurements. In blocking experiments, migration medium was supplemented with either a function
21 blocking antibody specifically targeting the $\alpha 5 \beta 1$ integrin dimer (Legate et al. 2011) or with isotype
22 control. Specificity of the $\alpha 5 \beta 1$ antibody was verified by the manufacturer by the antibody's ability to
23 immunoprecipitate $\alpha 5 \beta 1$ heterodimers from (125)-I-surface labelled cells, by reciprocal depletion of
24 $\alpha 5 \beta 1$ antigen from cell lysate with antiserum against the cytoplasmatic domain of the $\alpha 5$ subunit,
25 and by immunoprecipitation of $\alpha 5 \beta 1$ integrin from cells known to express this integrin.

1 Image acquisition started after 1 h of tissue equilibration at 35°C with 5% CO₂ and within 3.5 h after
2 decapitation. During measurements humidified air with 5% CO₂ was continuously applied to the slice,
3 kept at 35°C. Per experiment (1 mother animal), six slices were imaged sequentially and this was
4 repeated every 10 min during 6 h using the '*multitime macro*' in the Zeiss LSM510 software (version
5 4.2 SP1, Zeiss) on an inverted Zeiss Axiovert 200M microscope with a Zeiss LSM 510 Meta confocal
6 laser scanning system and a 20x EC plan-Neofluar objective (NA of 0.5 and 2 mm working distance). A
7 Mai Tai DeepSee Ti:Sapphire laser (Spectra-Physics) with a central wavelength tuned at 900 nm was
8 used to visualize eGFP positive microglial cells and isolectin GS-IB₄A568 labeled blood vessels. Z-
9 stacks spanning 30 µm, with serial optical sections (voxel size 0.88 x 0.88 x 3.3 µm) were recorded
10 starting from a minimal depth of 50 µm beneath the surface of the slice to avoid cells activated by
11 slicing (Eyo et al. 2014; Schiefer et al. 1999).

12 **Migration tracking and analysis**

13 Image processing and migration tracking were performed using open source Fiji software (ImageJ
14 2.0.0-rc-643/1.50i). Time series were first corrected for 3D drift using the 3D drift correction plugin
15 and microglial migration was manually tracked in 4D using the MTrackJ plugin designed by Erik
16 Meijering (Meijering et al. 2012). Only cells remaining in the field of view for at least 100 min were
17 included in the analysis. Per experiment, at least one control and/or isotype condition were
18 performed.

19 Average migration speed (µm/h) was calculated as the total length of the travelled path divided by
20 the duration of the track. The immobile fraction was calculated as the percentage of total microglia
21 that did not migrate further than 45 µm over the total imaging time span. This threshold corresponds
22 to 3 times the average cell diameter and was applied because of small errors due to residual tissue
23 drift after 3D drift correction and manual tracking. Relative idling time was calculated using a custom
24 made Excel macro developed by Gorelik et al (Gorelik and Gautreau 2015) and is defined as the
25 percentage of time the cell spent on pausing, further designated as idling, with regard to the total
26 duration of the track. The threshold for idling was set at roughly half a cell diameter (8 µm) per 10

1 minutes and was verified by inspecting subsequent displacements of non-migratory cells in MTrackJ.
2 Instantaneous speeds of the active migration events ($v_{\text{inst. act.}} = \mu\text{m}/\text{min}$), i.e. events above the idling
3 threshold of $0.8 \mu\text{m}/\text{min}$, were calculated as the distance travelled between each time frame, divided
4 by the frame interval (10 min). Migration parameters are grouped under treatment and age. At least
5 8 slices from embryos of 5 different mothers were quantified.

6 **Fixed tissue preparation and immunohistofluorescence**

7 Pregnant mice were sacrificed and embryonic tissue was processed as described before (Swinnen et
8 al. 2013). 10-20 μm coronal sections were cut on a Leica CM3050S cryostat, mounted on Superfrost
9 Plus slides (ThermoFisher) and stored at -20°C until staining. For fibronectin stainings, sections were
10 washed and blocked during 1 h with PBS-20% NXS (Normal Goat or Donkey Serum, Chemicon). All
11 steps occurred at room temperature (RT) unless stated otherwise. Primary antibody was diluted in
12 PBS-1% NXS and incubated overnight at 4°C . For fibronectin-isolectin GS-IB₄ double labelling, the
13 isolectin was incubated at $10\mu\text{g}/\text{ml}$ together with the primary antibody overnight at RT. Sections
14 were washed 3 x 10 min in PBS and incubated 1 h with the secondary antibody diluted in PBS-1%
15 NXS. Sections were washed 3 x 5 min in PBS, submerged in distilled water and mounted using
16 vectashield including 4,6-diamidino-2-phenylindole (DAPI) (Vector, Burlingame). For negative
17 controls, primary antibodies were omitted. Secondary antibodies were centrifuged 5 min at 5000
18 revolutions per minute prior to use.

19 **Microscopy and mean grey value assessment**

20 Images of fibronectin immunostainings were acquired using a Digital sight DS-2MBWc fluorescence
21 camera adapted on a Nikon Eclipse 80i microscope. Fibronectin presence was quantified in the
22 embryonic cerebral cortex area located dorsally to the lateral ganglionic eminences (LGE) and medial
23 ganglionic eminences as previously described (Swinnen et al. 2013). Background signal was
24 determined using the line plot profile tool in Fiji for each image separately. Signal below 2.5 times
25 the background was removed. In each slice a region of interest (ROI; white dotted lines Fig. 3A) was

determined including the entire cortex but excluding the meninges. The mean grey value, defined as the average grey value of all pixels inside the ROI, was assessed using the '*Measure*' function in Fiji. These measurements resulted in a mean grey value per pixel ($0.16 \mu\text{m}^2$) automatically corrected for the surface of the ROI and therefore also for the size of the growing cortex. Slices were from 3 different embryos of at least 2 different mothers.

Images of fibronectin-isolectin GS-IB₄-A568 double immunostainings ($20 \mu\text{m}$ tissue sections) were acquired using an inverted Zeiss Axiovert 200M microscope with a Zeiss LSM 510 Meta confocal laser scanning system. An Arg-ion laser at 488 nm, a HeNe laser at 543 and 633 nm and a Mai Tai DeepSee Ti:Sapphire laser (Spectra-Physics) tuned at 710 nm were used for excitation of eGFP, Alexa568, Alexa647 and DAPI, respectively. Overview pictures (voxel size $0.44 \times 0.44 \times 1 \mu\text{m}$; 10-15 μm Z-stacks) were acquired using a 40x LD C-Apochomat/1.1 W Korr UV-Vis-IR objective (NA=1.1) with the Zeiss Laser scanning microscope LSM510 software. A 4x confocal zoom was applied to cells of interest (voxel size $0.11 \times 0.11 \times 2 \mu\text{m}$).

Western blotting

Embryonic brains were isolated as described before (Swinnen et al. 2013), the meninges were removed, cortices were excised and stored at -80°C . Cortices were lysed in cold RIPA buffer (50mM Tris pH 7.4; 150mM NaCl; 1mM EDTA; 1% NP-40; 0.25% Na-deoxycholate; protease inhibitor (#11873580000, Roche)). Protein concentrations of individual cortices were determined by the BCA protein assay kit (#23225, Thermo Fisher). Samples containing equal amount of proteins ($10 \mu\text{g}$) were separated on a 12% SDS-PAGE gel, transferred to a polyvinylidene fluoride (PVDF) membrane and blocked for 1 h with Tris buffered saline-0.1% Tween 20 (TBS-T) containing 5% milk powder (Marvel) followed by incubation overnight at 4°C in the presence of anti-fibronectin antibody. Mouse anti- β -actin antibody was subsequently incubated for 2 h followed by horseradish peroxidase-conjugated secondary antibodies incubation for 1 h. All antibodies were diluted in blocking buffer and incubations were at RT unless stated otherwise. Enhanced chemiluminescence using the Pierce ECL

1 Plus Western Blotting Substrate (#32132, Thermo Scientific) was used before imaging with the
2 ImageQuant LAS4000 mini (GE Healthcare Life Sciences). Quantification was performed using
3 ImageQuantTL.

4 **Microglia isolation and flow cytometry**

5 Cortical microglia from CX3CR1^{+/eGFP} E13.5, E15.5 and E17.5 brains were isolated as described before
6 (Smolders et al. 2015) with modifications. The tissue was mechanically homogenized in neurobasal
7 medium (Gibco, Thermo Fisher Scientific) supplemented with 2mM L-glutamine, N2 supplement, B27
8 supplement and 1% penicillin/streptomycin (all from Thermo Fisher Scientific). The homogenate was
9 centrifugated 5 min at 700 g at 4°C, the pellet was resuspended in cold PBS and stained with Fixable
10 Viability stain 620 (FVS620) (BD Biosciences) during 10 min at RT. Cell suspensions were fixed in 4%
11 PFA during 10 min, washed and dissolved in PBS. Cells were incubated for 15 min on RT with an α5-
12 Phycoerythrin conjugated antibody in PBS. After washes, cells were acquired in a FACS Fortessa (BD
13 Biosciences) and analyzed with FACS Diva 8.0.1 software (BD Biosciences). Within the living cell
14 population (FVS620 low) the eGFP positive microglia (110-4799 cells per tube) were gated. Within
15 the microglial population, the percentage of α5 positive microglia and its median fluorescence
16 intensity (MFI) were analyzed. Because α5 measurements were part of a panel not further described
17 here, Fluorescence-minus-one (FMO) controls (for justification see (Maecker and Trotter 2006)) were
18 used to gate the positive cell population. At E13.5, embryos were pooled per 2 or 3 (N=8). At E15.5
19 (N=16) and E17.5 (N=20), embryos were analyzed separately. Data were obtained from 3 different
20 mothers (M=3).

21 **Microglia-blood vessel contact analysis**

22 To quantify microglia-blood vessel interactions, we added isolectin GS-IB₄-A568 (see section ‘Time-
23 lapse imaging’) in the imaging medium 1 h prior to imaging onset to visualize blood vessels.
24 Quantification occurred on the 30 μm Z-stacks acquired from 3 h after onset of imaging, in order for
25 the GS-IB₄ labeling to sufficiently penetrate the tissue, until 6 h. Only cells located in the parenchyma

and that were visible during at least 9 subsequent time points were included in the analysis. For each cell throughout the Z-stack at each time point, the type of contact was noted as full soma, touching with a process or no contact (free). Microglia that made contact with a blood vessel (soma or process) during 1 or more frames, were considered to be in contact with blood vessels for the analysis in Fig. 4C and a percentage was calculated per slice. The percentage of time each cell spent on a particular contact (Fig. 4D) was calculated and data from cells were pooled per treatment and age. At least 7 different embryonic brain slices (N=7) of 3 different mothers (M=3) were quantified per treatment. All blood vessel contact analyses were performed blinded.

Statistical analysis

The number of analyzed cells or steps is indicated as “n”, the number of embryos or slices as “N” and the number of mothers as “M”. The #cells/#embryos or slices/#mothers is thus designated in the text as n/N/M unless stated otherwise. The reader is referred to the figure legends for details about the sample size used for statistical analysis. Data are described in the text as “median [interquartile range (IQR)]” according to standards for describing nonparametric data (Madadzadeh et al. 2015). Statistical analyses and graphs were produced using SAS JMP® Pro 12.1.0. Data are represented as box plots with whiskers to 1.5x the IQR (Tukey representation). Data distributions were assessed for normality (Shapiro-Wilk) and equality of variance (Brown-Forsythe). In case these assumptions were met for all groups, a Student *t*-test in case of 2 groups or ANOVA was performed in case of three groups followed by Tukey HSD post-hoc, correcting for multiple comparisons. In Fig. 3D and 3H, data were transformed on a log₁₀ scale to meet the equality of variance assumption, though the original scales were used for data presentation for ease of interpretation. When data distribution of least one group was non-gaussian, nonparametric tests such as Mann-Whitney in case of two groups or Kruskal-Wallis with Dunn’s multiple comparison post-hoc in case of three groups were performed. *P*-values smaller than 0.05 were considered significant with * *P*<0.05, ** *P*<0.01 and *** *P*<0.001.

1 **RESULTS**

2 **Microglial migration speed decreases with development**

3 Microglial colonization of the embryonic mouse brain cortex occurs in three phases based on
4 microglial density: an initial phase of fast increasing cell density from E10.5 until E14.5, followed by a
5 plateau phase between E14.5 and E15.5, and a third invasion phase after E15.5 (Swinnen et al. 2013).
6 During mammalian development these cells are already highly mobile, with the capacity to
7 phagocytose dying cells and scan the microenvironment as observed in the adult brain (Eyo and
8 Dailey 2013; Nimmerjahn et al. 2005; Swinnen et al. 2013; Zhang et al. 2016). Two-photon time-lapse
9 microscopy was used in this study to investigate developmental changes in microglial migration
10 within the time frame of ongoing neuronal migration (Greig et al. 2013; Swinnen et al. 2013). To this
11 end, acute brain slices obtained from E13.5, E15.5 and E17.5 CX3CR1^{+/eGFP} embryos were used,
12 representing the 3 aforementioned phases in microglial development. Migration was analyzed
13 starting from E13.5, because on E12.5 very few cells reside yet in the cortical parenchyma and
14 mobility is low (Swinnen et al. 2013). We focused our analysis on the cerebral cortex area located
15 dorsally to the lateral ganglionic eminences (LGE) and medial ganglionic eminences (MGE) (see also
16 Fig. 3A a1, b1 and c1) (Swinnen et al. 2013). Representative Z-projections of time-lapse experiments
17 with overlaid migration tracks are shown for each age in Figure 1A.

18 The cell average migration speed within one brain slice was highly variable (Fig. 1B). The median
19 average speed at E13.5 (33.4 $\mu\text{m/h}$ [IQR: 22.5-44.4]) did not differ significantly from the speed at
20 E15.5 (33.63 $\mu\text{m/h}$ [IQR: 21.2-47.4]) (Fig. 1B and C). At E17.5 average migration speed significantly
21 decreased (24.2 $\mu\text{m/h}$ [IQR: 15.7-34.3]) compared to E13.5 and E15.5 (Fig.1B and C). The decrease in
22 speed suggests that with development, microglia start to acquire their final locations. To evaluate
23 this presumption, we analyzed the percentage of immobile cells per slice from E13.5 to E17.5 and
24 found that the immobile fraction rose significantly from 0.0% [IQR: 0.0-2.1] at E13.5 to 9.1% [IQR:
25 3.1-15.1] at E17.5 (Fig.1D). To rule out that the decrease in migration speed was solely due to an

1 increase in immobile fraction, we reassessed the average migration speed of the mobile fraction. We
2 also found a significant decrease in speed with development (E13.5 vs. E17.5 or E15.5: $P < 0.001$,
3 Kruskal-Wallis with Dunn's post test) (data not shown).

4 The parallel decrease in migration speed and increase in immobile fraction suggest that microglial
5 migration speed is developmentally regulated during early colonization of the embryonic cortex.

6 **Microglia adopt a saltatory migration pattern over development**

7 In their migration process microglia first scan their environment, send out one or multiple processes,
8 and displace their soma in the direction of one of the protrusions while retracting the others.
9 Subsequently the cell idles, i.e. the migration of the cell body pauses, explores the environment again
10 and the cycle is repeated (Fig. 2A). This locomotion pattern can be described as saltatory and was
11 observed at each age tested. To visualize this saltatory behavior, the speed between two subsequent
12 time points, defined as instantaneous speed, was plotted in function of time for three microglial cells
13 that are representative for cells with high, intermediate and low average speeds (Fig. 2B). To better
14 characterize the saltatory behavior over development and to find the underlying mechanism of
15 changes in average speed, the median relative idling time and the median instantaneous speed of
16 the migration events above the idling threshold were determined (Gorelik and Gautreau 2015). For
17 example, average speed can be decreased because the cells spend more time idling, and/or because
18 when migrating, distances between subsequent time points become shorter. The relative idling time
19 was calculated as the percentage of the time the cell spends on idling with regard to the total track
20 duration (Gorelik and Gautreau 2015) and was significantly increased over development (E13.5:
21 77.0% [IQR: 64.3-88.6], E15.5: 74.3% [IQR: 63.2-86.1], E17.5: 82.8% [IQR: 72.8-91.7]) (Fig. 2C).
22 Instantaneous speed ($\mu\text{m}/\text{min}$) was calculated as the travelled distance between subsequent time
23 points, divided by the frame interval and was found to be significantly decreased only between E15.5
24 ($1.4 \mu\text{m}/\text{min}$ [IQR: 1.1-2.0]) and E17.5 ($1.3 \mu\text{m}/\text{min}$ [1.0-1.8]) (Fig. 2D and E).

Our results thus show that microglia during early colonization of the embryonic cortex migrate with jumps and that the developmental decrease in microglial average migration speed is due to the cells spending more time pausing and additionally migrating in smaller steps.

Cortical fibronectin presence decreases over development

Developmental changes in microglial migration might result from changes in the microenvironment. ECM proteins, such as fibronectin, are developmentally regulated (Sheppard et al. 1991). Nevertheless, the deposition pattern of fibronectin in the embryonic mouse brain remains controversial (De Gasperi et al. 2012; Sheppard et al. 1991; Stewart and Pearlman 1987). To bring clarity on this pattern at the ages relevant for this study, fibronectin's deposition pattern in the embryonic cortex during the microglial colonization phase from E13.5 to E17.5 was analyzed using immunostaining (Fig. 3A) and cortical mean grey value quantification (Fig. 3B). Fibronectin was localized throughout the entire cortex at E13.5 as thick aggregates (Fig. 3A, a1-a4). At E15.5 (Fig. 3A, b1-b4) and E17.5 (Fig. 3A, c1-c4), fibronectin staining was less dense when compared to staining at E13.5. Mean grey value assessment (gray value scale 0-255; immunostaining intensity) indicated that fibronectin deposition decreased (Fig. 3B) during the microglial colonization phase of the embryonic cortex. The median cortical fibronectin grey values were 13.8 [IQR: 11.7-17.2] at E13.5, 8.5 [IQR: 6.2-11.5] at E15.5 and 2.9 [IQR: 1.4-5.4] on E17.5, which all significantly differ from each other (Fig. 3B). To confirm the mean grey value measurements, the fibronectin protein content was determined on isolated embryonic cortices using western blotting (Fig. 3C). Quantification of fibronectin relative to β -actin showed that the cortical fibronectin contents at E13.5 (51.6 [IQR:33.1-77.3]) and at E15.5 (32 [IQR:22.9-37.9]) were significantly higher than at E17.5 (7.6 [IQR:4.3-17]) (Fig. 3D).

Though fibronectin was diffusely localized in the parenchyma, developing blood vessels marked by isolectin-GS-IB₄ (Fig. 3E, e4) were highly immunoreactive for fibronectin (Fig. 3E, yellow arrowheads in e1, e3 for zoom-in), as reported extensively in literature (De Gasperi et al. 2012; Milner and Campbell 2002b; Sheppard et al. 1991; Stewart and Pearlman 1987). As previously described

(Grossmann et al. 2002; Kierdorf et al. 2013; Pont-Lezica et al. 2011; Rigato et al. 2011) microglia were often found in close proximity with blood vessels (Fig. 3E).

Thus, cortical fibronectin decreases with development and it is also deposited along blood vessels.

Fibronectin receptor $\alpha 5 \beta 1$ is expressed by microglia and is developmentally downregulated

Microglia-ECM contacts are likely to be mediated by fibronectin-integrin interactions. *In vitro* adhesion of microglia to a fibronectin coated surface is regulated through fibronectin receptors, such as $\alpha 5 \beta 1$ integrin (Milner 2009; Milner and Campbell 2002a; Milner and Campbell 2003; Welser-Alves et al. 2011). This integrin heterodimer is described as the major fibronectin receptor and is well characterized on the molecular as well as the signaling level (Bachman et al. 2015; Hynes 2002). To determine the expression of $\alpha 5 \beta 1$ integrin on embryonic microglia *in vivo*, E13.5, E15.5 and E17.5 cortices were isolated and the fibronectin receptor was immediately analyzed after isolation using flow cytometry. Because the $\alpha 5$ integrin subunit exclusively associates with the $\beta 1$ subunit, a monoclonal antibody raised against $\alpha 5$ was used to identify $\alpha 5 \beta 1$ expression on microglial cells (Milner and Campbell 2002c). Microglial cells were gated based on eGFP expression, after exclusion of dead cells (Fig. 3F). At E13.5, 99.8% [IQR: 98.8-100.0] of the microglial population expressed the $\alpha 5$ subunit (Fig. 3G). This percentage did not change at E15.5 (99.9% [IQR: 99.5-100.0]), but it was significantly different at E17.5 (98.9% [IQR: 97.6-99.4]) compared to E15.5 (Fig. 3G). The median fluorescence intensity (MFI), an indicator of the expression level per cell, of the $\alpha 5$ positive microglia significantly decreased from E13.5 (12356 MFI [IQR: 11678-14330]) to E15.5 (9558 MFI [IQR: 8825-10207]) and to E17.5 (4479 MFI [IQR: 4202-4796]) (Fig. 3H).

In conclusion nearly all embryonic microglia expressed the $\alpha 5 \beta 1$ integrin receptor but the expression level decreased in the course of development.

1 **$\alpha 5\beta 1$ integrin is dispensable for microglia contact with blood vessels**

2 Microglia are often observed in contact with blood vessels in the developing and adult CNS and in
3 physiologic as well as in pathologic conditions (Arnold and Betsholtz 2013; Grossmann et al. 2002;
4 Pont-Lezica et al. 2011). However, the mechanical basis for this contact is unknown (Arnold and
5 Betsholtz 2013; Dudvarski Stankovic et al. 2016; Pont-Lezica et al. 2011). Based on our previous
6 findings, the $\alpha 5\beta 1$ integrin was suspected to mediate microglia attachment to blood vessels. Since
7 microglia were reported to migrate along blood vessels after injury in rat postnatal slice preparations
8 (Grossmann et al. 2002) and they made transient contacts with blood vessels in the developing
9 zebrafish from 6 to 10 days post fertilization (dpf) (Svahn et al. 2013), it was first determined
10 whether $\alpha 5\beta 1$ integrin could be important in the capability of microglia to migrate along blood
11 vessels. Time-lapse imaging showed that microglia can use blood vessels as substrates to migrate in
12 the cortex of the mouse embryo (Fig. 4A upper panel, Suppl. Movie 1). However, $\alpha 5\beta 1$ blockage
13 using a blocking antibody specifically targeting the $\alpha 5\beta 1$ integrin dimer did not impair this capability
14 of microglia to migrate along the surface of blood vessels (Fig. 4A lower panel, Suppl. Movie 2). To
15 confirm this observation, the percentage of microglia contacting a blood vessel was determined in
16 the time-lapse sequences starting from 3 h after application of the $\alpha 5\beta 1$ blocking. Two modes of
17 contact were observed: full soma alignment (Fig. 4B left panel) and “touching” contacts between
18 blood vessels and microglial cell processes (Fig. 4B middle panel). Because the process contact mode
19 was less frequently observed, both contact modes were grouped under “microglia-blood vessel
20 contact”. For comparison purposes, a free microglia not contacting a blood vessel is shown in Figure
21 4B (right panel). At E13.5 and E17.5, 50.0% [IQR: 36.7-81.3] and 100% [IQR: 85.1-100.0] of microglial
22 cells contacted blood vessels in the presence of the isotype and the percentage did not change after
23 $\alpha 5\beta 1$ blockage (E13.5: 50.0% [IQR: 40.0-66.7], E17.5: 86.6% [IQR: 61.1-100.0]) (Fig. 4C). The
24 percentage of microglia contacting blood vessels significantly increased with development (Fig. 4C).

1 Blocking $\alpha 5 \beta 1$ might have subtle effects on microglia behavior, such as dynamic changes in contacts
2 with blood vessels, which cannot be revealed by the analysis described above. Therefore we next
3 investigated the percentage of time that each cell spent on contacting blood vessels - either by full
4 soma contact, process contact or no contact as illustrated in Figure 4B. The percentage of time spent
5 on a particular contact was highly variable (Fig. 4D). At E13.5 in control conditions, the median
6 percentage of time microglia spent on contacting a blood vessel using their soma or using processes
7 was 0.0% [IQR: 0.0-92.1] and 0.0% [IQR: 0.0-5.3], respectively. 84.2% [0.0-100.0] of the time, the cells
8 made no contact. These values did not change after $\alpha 5 \beta 1$ blockage (5.3% [0.0-89.9], 0.0% [0.0-5.8]
9 and 84.2% [5.6-100.0], for Soma, Process or no contact, respectively). At E17.5 in control conditions,
10 the median percentage of time microglia spent on contacting a blood vessel using their soma or using
11 processes was 21.1% [IQR: 0.0-80.3] and 21.1% [IQR: 0.0-48.7], respectively. 23.7% [0.0-63.2] of the
12 time, the cells made no contact. These values did not change after $\alpha 5 \beta 1$ blockage (20.5% [0.0-59.2],
13 25.7% [0.0-54.0] and 10.5% [0.0-79.0], for Soma, Process or no contact, respectively).

14 All together, these results indicate that $\alpha 5 \beta 1$ integrin is neither essential for the capability of
15 microglia to migrate along blood vessels nor for microglia-blood vessel contact.

16 **$\alpha 5 \beta 1$ integrin blockage has opposite effects on microglia migration during the embryonic** 17 **cortical development**

18 Based on the parallel decrease in microglial average migration speed, cortical fibronectin deposition
19 and $\alpha 5 \beta 1$ integrin expression level on microglia during the developmental period analyzed, it was
20 hypothesized that the functional importance of this receptor during microglial migration would
21 diminish over time. To address this issue, the same migration parameters as analyzed for control
22 migration (Figs. 1 and 2) were assessed, but in the presence of the $\alpha 5 \beta 1$ blocking antibody or isotype
23 control in E13.5, E15.5 and E17.5 acute brain slices (Fig. 5, Suppl. Movies 3 to 8). Representative
24 time-lapse Z-projections overlaid with migration tracks are shown in Fig. 5A. There was no effect of
25 isotypes on migration (Fig. 5B; Fig. 6; Fig. 1B).

At E13.5 $\alpha 5\beta 1$ integrin blockage caused a significant reduction (~25%) of the average migration speed (23.5 $\mu\text{m/h}$ [IQR: 15.7-37.8]) when compared to isotype control (31.5 $\mu\text{m/h}$ [IQR: 19.6-44.0]) (Figs. 5B and D). Conversely, $\alpha 5\beta 1$ integrin blockage at E15.5 and E17.5 significantly increased the migration speed to 41.3 $\mu\text{m/h}$ [IQR: 27.8-56.7] (~14%) and 31.6 $\mu\text{m/h}$ [IQR: 19.7-50.9] (~17%) compared to isotype (E15.5: 36.2 $\mu\text{m/h}$ [IQR: 23.7-49.3], E17.5: 27.0 $\mu\text{m/h}$ [IQR: 17.7-37.2]) (Figs. 5B, 5D). The effect of the antibody was indeed significantly different across ages (dotted lines with asterisks, Fig. 5B). Upon $\alpha 5\beta 1$ blockage, the immobile fraction was 0.0% [IQR: 0.0-9.1] at E13.5, 0.0% [IQR: 0.0-8.3] at E15.5 and 3.1% [IQR: 0.0-8.0] at E17.5, which were not significantly different from isotype (0.0% [IQR: 0.0-13.2] at E13.5; 5.0% [IQR: 0.0-7.7] at E15.5 and 5.0% [IQR: 0.0-10.9] at E17.5) (Fig. 5C). The effect of the antibody did not differ across ages. After exclusion of the immobile fraction in the average speed analysis, we found the same significant differences between isotype and $\alpha 5\beta 1$ blockage (E13.5: $P=0.016$, E15.5: $P=0.013$, E17.5: $P<0.001$, Kruskal-Wallis with Dunn's post test) (data not shown). This confirms that $\alpha 5\beta 1$ blockage does not affect the immobile microglial population.

These results indicate that $\alpha 5\beta 1$ integrin blockage affects the microglial average migration speed in opposite ways depending on the embryonic age, without affecting the proportion of immobile microglia.

To determine whether the change in average speed was due to the cells spending more or less time idling and/or to a change in instantaneous speed, we determined the median relative idling time and the median instantaneous speed of the events above the idling threshold (Fig. 6). After $\alpha 5\beta 1$ blockage, the median relative idling time was 84.6% [IQR: 72.1-91.7] at E13.5, 67.4% [IQR: 51.9-78.9] at E15.5 and 77.0% [IQR: 60.1-86.2] at E17.5 (Fig. 6A). They were all significantly different compared to isotype (76.9% [IQR: 66.6-88.3] at E13.5, 71.8% [IQR: 60.0-83.8] at E15.5 and 80.0% [IQR: 70.6-88.9] at E17.5). After $\alpha 5\beta 1$ blockage the instantaneous velocities of migration events were 1.3 $\mu\text{m/min}$ [IQR: 1.0-1.9] at E13.5, 1.4 $\mu\text{m/min}$ [IQR: 1.1-2.0] at E15.5 and 1.4 $\mu\text{m/min}$ [IQR: 1.1-2.1] at

1 E17.5 (Fig. 6B and C). Only at E17.5, after blockage microglia migrated with a significantly higher
2 instantaneous speed compared to isotype control (1.4 $\mu\text{m}/\text{min}$ [IQR: 1.0-2.0] at E13.5, 1.5 $\mu\text{m}/\text{min}$
3 [IQR: 1.1-2.0] at E15.5, 1.4 $\mu\text{m}/\text{min}$ [IQR: 1.0-1.8] at E17.5) (Fig. 6B and C). Additionally, after $\alpha 5\beta 1$
4 blockage at all ages, microglia still migrated saltatory (see all Supplementary Movies).

5 In conclusion, $\alpha 5\beta 1$ blockage mainly affects the time the cells spend idling without affecting the
6 saltatory migration pattern.

7

1 **DISCUSSION**

2 In this study, we show that during early colonization of the embryonic cortex microglia migrate in a
3 saltatory fashion and that their average migration speed is developmentally regulated. We
4 demonstrate that the adhesion molecules fibronectin and its receptor the $\alpha 5 \beta 1$ integrin play an
5 important role in regulating embryonic microglial migration. The presence of cortical fibronectin and
6 the expression of $\alpha 5 \beta 1$ integrin on microglia decreased throughout development, but as a paradox
7 we found that $\alpha 5 \beta 1$ integrin has opposite functions in microglial migration depending on the
8 embryonic age. Blockage of the $\alpha 5 \beta 1$ integrin decreased migration speed at E13.5 while it led to an
9 increased migration speed at E15.5 and E17.5, without affecting the size of the immobile fraction.

10 **Microglia exhibit a saltatory migration behavior while speed decreases during embryonic**
11 **corticogenesis**

12 The behavior of microglial cells invading the embryonic cortex from E13.5 to E17.5 is characterized by
13 a saltatory migration pattern. This pattern consists of pausing phases during which the microglial cell
14 explores its surroundings interspersed with active phases of migration in the direction of a selected
15 protrusion. This saltatory migration pattern of microglia in the mouse embryonic brain, which is
16 maintained during the developmental period studied here, is similar to the migration behavior of
17 microglia described *in vivo* in the developing zebrafish larvae (Sieger et al. 2012), suggesting that this
18 particular behavior of microglia during brain development is evolutionary conserved over species.

19 We observed a decrease in the microglial average migration speed over embryonic development and
20 this resulted from both an increased idling time and a lower instantaneous speed. Our observations
21 are similar to what has been observed between postnatal ages P2 and P6 in the mouse hippocampus
22 (Eyo et al. 2016) and between 3.5 and 5 dpf in the zebrafish optic tectum (Svahn et al. 2013). The
23 decrease in average speed at E17.5 coincided with an increase in the immobile fraction of microglial
24 cells and could indicate that some microglial cells acquire their final locations in the cortex between
25 E13.5 and E17.5. However, we cannot exclude that the rise in immobile fraction over early
26 development reflects a long lasting transitory resting state between active migration phases, since

1 the cortical development proceeds postnatally (Finlay and Darlington 1995; Sauvageot and Stiles
2 2002). The immobile fraction was insensitive to $\alpha 5 \beta 1$ blockage from E13.5 to E17.5. This suggests
3 that $\alpha 5 \beta 1$ does not essentially contribute to the integrin-ECM interactions tightly anchoring the cell
4 in place. The decrease in microglial migration speed is likely to result from changes in the local
5 environment. We show that the fibronectin deposition and the expression of fibronectin receptor
6 $\alpha 5 \beta 1$ integrin on microglia, decrease from E13.5 to E17.5 in the cortex. Indeed, changes in ECM
7 composition alter microglial adhesion to substrates, which could impact their migration (Milner and
8 Campbell 2002c; Milner et al. 2007). Accordingly microglial migration speed was decreased in the
9 newborn rabbit brain as a consequence of *in utero* inflammation and it was suspected to result from
10 changes in adhesion molecule expression after inflammation (Zhang et al. 2016).

11 **Developmental decrease in fibronectin and microglial fibronectin receptor $\alpha 5 \beta 1$ integrin**
12 **expression in the embryonic cortex**

13 All three parameters, microglial average migration speed, the cortical fibronectin deposition and
14 microglial $\alpha 5 \beta 1$ integrin expression levels decreased from E13.5 to E17.5. This concurrent decrease
15 indicates that the interaction between fibronectin and $\alpha 5 \beta 1$ integrin might regulate microglia
16 migration speed supporting the hypothesis that the ECM plays an important role in migration during
17 early colonization of the cortex by these immune cells. Throughout development, fibronectin is
18 highly expressed by blood vessels and along radial glial processes (De Gasperi et al. 2012; Milner and
19 Campbell 2002b; Sheppard et al. 1991; Stewart and Pearlman 1987), which makes these structures
20 ideal scaffolds to guide microglial migration. Accordingly, changes in microglial migration speed
21 observed in the presence of the $\alpha 5 \beta 1$ integrin blocking antibody may result from an alteration of
22 interactions between microglia, blood vessels and/or radial glial fibers.

23

1 **No role for $\alpha 5\beta 1$ integrin in mediating microglial contact with blood vessels**

2 Contact between microglia and blood vessels during development has been reported in zebrafish,
3 quails, mice, rats as well as humans (Pont-Lezica et al. 2011). Although $\alpha 5\beta 1$ integrin is implicated in
4 the adhesion of CNS endothelial cells to fibronectin (Milner and Campbell 2002b), we did not find any
5 evidence for a role of this receptor in the dynamic interaction between microglia and blood vessels
6 during the developmental period investigated here. Neither the capability of microglia to use blood
7 vessels as guiding substrates for migration, nor the fraction of these cells contacting blood vessels,
8 nor the time spent on soma or process contact was altered in the presence of the blocking antibody.
9 Other ECM proteins, such as laminin or Intercellular Adhesion Molecule (ICAM)-1 or 2, expressed
10 along developing blood vessels might mediate contact (Dalmau et al. 1997; Rezaie et al. 1997) as
11 microglia *in vitro* do express the receptors for these ligands (Dalmau et al. 1997; Milner and Campbell
12 2003). Additionally, integrins other than $\alpha 5\beta 1$ might be involved in adhesion to fibronectin expressed
13 by blood vessels (Milner and Campbell 2003; Welser-Alves et al. 2011). Lack of effect of the blocking
14 antibody on microglia interaction with blood vessels does not preclude disturbances of microglial
15 interactions with other cell types, such as radial glia which produce and align fibronectin along their
16 processes (Sheppard et al. 1995; Sheppard et al. 1991; Stettler and Galileo 2004). Dense packing of
17 these radial glial fibers may however hamper reliable quantification of interactions with microglia in
18 the cortex.

19 **Age-specific role of $\alpha 5\beta 1$ integrin in microglial migration**

20 Although almost all microglia from E13.5 to E17.5 expressed $\alpha 5\beta 1$ integrin, its expression level
21 decreased over development. This might indicate that embryonic microglia are capable to interpret
22 changes in fibronectin deposition. This idea is supported by *in vitro* work showing that after
23 cultivation on fibronectin primary microglia upregulate $\alpha 5\beta 1$ integrin (Milner and Campbell 2003).
24 Based on the developmental decrease in both adhesion molecules, we expected that blocking $\alpha 5\beta 1$
25 would largely decrease migration speed at E13.5 while it would affect migration less at E17.5.

1 Surprisingly our experiments indicate that this is not the case. After $\alpha 5 \beta 1$ blockage, migration speed
2 indeed decreased at E13.5, but it increased at E17.5.

3 Changes in microglial migration speed observed in the presence of the blocking antibody are
4 apparently modest (14-25% changes), but they are in the range of those observed on neurons
5 migration speed after blocking glycine receptors (Avila et al. 2013) or on microglia migration after
6 blocking the CC chemokine receptor 5 (Carbonell et al. 2005). It is likely that $\alpha 5 \beta 1$ integrin is not the
7 sole integrin dimer to play a role in microglial migration (Milner and Campbell 2002b). The long term
8 consequences of defective integrin dependent microglial migration on brain development and
9 neuronal network functionality remain unknown and require further attention. This might be a
10 challenging task regarding the fact that genetically engineered integrin knock-outs can suffer from
11 functional compensation of other integrins (Schmid and Anton 2003; Zent 2010).

12 As observed here at E13.5, a decrease in migration after either $\alpha 5 \beta 1$ or general $\beta 1$ integrin blockage
13 was also reported *in vitro* in microglial chemotaxis and wound healing assays (Kim et al. 2014; Nasu-
14 Tada et al. 2005; Yao et al. 2013). Integrin blockage has led to various outcomes on migration
15 depending on the cell type, the integrin heterodimer and the environmental dimensions. For
16 example $\alpha 5 \beta 1$ depletion inhibited neuronal migration during mouse embryonic corticogenesis *in vivo*
17 (Marchetti et al. 2010). On the contrary, integrin blocking antibodies increased migration in platelets
18 (Moroi et al. 2000), neutrophils (Toyjanova et al. 2015), cancer cells (3D matrix) (Costa et al. 2013)
19 and trophoblast cells (Zhao et al. 2012) *in vitro*, as observed in our experiments at E17.5.

20 The observed opposite functions of $\alpha 5 \beta 1$ integrin during cortical development might be explained by
21 a maturation of the adhesion involving $\alpha 5 \beta 1$. Generally, cell migration involves unstable nascent
22 adhesion (physical interaction between the ECM, the integrin and the cytoskeleton) formation which
23 undergoes rapid turnover. When a protrusion rests, the nascent adhesion can “mature” into a highly
24 stable focal adhesion. This means the adhesion grows in size and stability by attraction and
25 posttranslational modifications of intracellular adaptor proteins that constitute the link between the

1 integrin and the cytoskeleton. When the cell migrates further, the ECM-cytoskeleton link is disrupted
2 (Lock et al. 2008; Vicente-Manzanares and Horwitz 2011). The stability of the adhesion is important
3 for overall cell migration speed and can be regulated at the level of the ECM, the integrin and the
4 adaptor proteins (Doyle and Yamada 2016; Fraley et al. 2010; Lock et al. 2008; Vicente-Manzanares
5 and Horwitz 2011). Interestingly, adhesion strength has a biphasic effect on migration speed: the
6 speed increases between low and intermediate adhesion strength and slows down between
7 intermediate and high adhesion strength (Barnhart et al. 2011; Vicente-Manzanares and Horwitz
8 2011). We therefore speculate that at E13.5 $\alpha 5\beta 1$ is involved in unstable adhesions which favor
9 migration while from E15.5 onwards, the integrin is linked to more stable, mature adhesions that
10 cause tighter anchoring of the cell body. When the ability to form these unstable adhesions is
11 impaired upon blockage at E13.5, the cell will not find an anchor point to transduce force in order to
12 migrate. At E17.5 $\alpha 5\beta 1$ linked adhesions would be more stable, causing a decrease in migration
13 speed. When the integrin-matrix link is disrupted by the blocking antibody, microglia could be
14 released and could be free to migrate faster using other integrins. Plausible candidates for mediating
15 migration could be $\alpha 4\beta 1$, $\alpha 6\beta 1$, $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 8$, $\alpha L\beta 2$ or $\alpha M\beta 2$, since microglia express these
16 integrins at least *in vitro* (Milner 2009; Milner and Campbell 2002a; Milner and Campbell 2003).
17 Alternatively, the fibronectin concentration could determine whether $\alpha 5\beta 1$ integrin promotes or
18 inhibits migration, as observed for cultured glioblastoma cells (Blandin et al. 2016).

19 Migrating neurons, radial glia and blood vessels in the developing brain are known to express the
20 $\alpha 5\beta 1$ integrin (Marchetti et al. 2010; Milner and Campbell 2002b; Yoshida et al. 2003) which could
21 indirectly interfere with the alteration of microglia migration we observed in the presence of the
22 blocking antibody. This is unlikely to be the case at the blood vessel level since we did not observed
23 any difference in microglia-blood vessels interactions in the presence of the blocking antibody.
24 Knock-down of this integrin in neural precursors resulted in a decreased radial migration and
25 affected their morphology and differentiation capacity (Marchetti et al. 2010). To our knowledge it is
26 yet unknown if neuronal migration can affect microglia mobility. So far it has been shown that SDF-1-

expressing basal progenitors in the ventricular/subventricular zone (VZ/SVZ) promotes microglia recruitment into the SVZ (Arno et al. 2014). Nevertheless, we cannot completely exclude that interactions between developing neurons and microglia might be altered after blockage. Finally $\beta 1$ integrins in radial glia control the morphological differentiation of both glia and neurons (Belvindrah et al. 2007) but the alterations of these processes by impairing $\beta 1$ integrin expression occur in a time scale that is incompatible with the time scale observed for microglia behavior alteration.

Conclusions

This study is the first to dig deeper into molecular mechanisms of physiologic migration of microglia during development. A limitation of the use of brains slices in this study might be that microglial migration in slice preparations does not reflect the true physiologic behavior during development. Microglia at the slice surface could be activated in terms of phagocytosis and velum-like pseudopod formation as observed in slices of rat facial nucleus following peripheral axotomy (Schiefer et al. 1999). Nevertheless, microglia within the tissue depth did not show such behaviors. This indicates that deep tissue imaging, as performed in our study, is likely to allow analyzing behavior of the microglial population close to physiologic conditions (Eyo and Dailey 2013; Eyo et al. 2016; Schiefer et al. 1999). It is important to note that microglial mobility in the *in vivo* developing zebrafish was also high (Sieger et al. 2012; Svahn et al. 2013). Finally, an *in utero* embryonic brain imaging (Yokota et al. 2007; Yuryev et al. 2015), although challenging, would be required to fully confirm that the intense microglial migration behavior we observed in slices truly reflects the microglial behavior in the developing brain of the intact embryo.

In conclusion, our results strongly indicate that $\alpha 5\beta 1$ integrin regulates the microglial migration process during embryonic microglial colonization of the mouse cortex, without taking part in contact with blood vessels. We report for the first time opposing age-dependent functions of the $\alpha 5\beta 1$ integrin. At E13.5 the $\alpha 5\beta 1$ integrin promotes while at E15.5 and E17.5 it inhibits microglial migration. We hypothesize that during development, the stability of the $\alpha 5\beta 1$ -linked adhesion

- 1 changes and therefore blockage of the fibronectin receptor leads to different outcomes. What causes
- 2 microglial migration to decrease and how changes in $\alpha 5 \beta 1$ integrin function are molecularly
- 3 regulated - cell intrinsically and/or environmentally - are questions that require further investigation.

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6) Figure Legends

Fig. 1. Microglial migration is developmentally regulated. Microglia movement was recorded in acute brain slices during 6 h using 2-photon time-lapse imaging and cell somas were manually tracked. **(A)** Representative microglial migration tracks in different colors at E13.5 (left panel), E15.5 (middle panel) and E17.5 (right panel) (eGFP/microglia, green). The meninges is located at the top of the image and the ventricle at the bottom (not visible at E15.5 and E17.5). **(B)** Microglial average migration speed v_{av} ($\mu\text{m}/\text{h}$) decreased significantly over development (Kruskal-Wallis with Dunn's, E17.5 vs E13.5 and E15.5, $P < 0.001$). **(C)** Cumulative probability plots of migration speed with matching colors for the ages in (B). E17.5 migration speed distribution shows a shift to lower average speeds. **(D)** The percentage of immobile microglia significantly rose from E13.5 to E17.5 (Kruskal-Wallis with Dunn's, $P = 0.014$). Sample size (A-C) as $n = \text{cells} / N = \text{slices} / M = \text{mothers}$ at E13.5: 160/18/12; E15.5: 170/10/8; E17.5: 341/14/9. n (cells) was used as sample size in statistical tests. Sample size (D) as $N = \text{slices} / M = \text{mothers}$ at E13.5: 18/12; E15.5: 10/8; E17.5: 14/9. N (slices) was used as sample size in statistical tests. Scale bar = 100 μm .

Fig. 2. Microglial migration behavior. Experimental set-up see Fig. 1. **(A)** Z-projections (30 μm) of representative time-lapse sequences showing characteristic microglial jumping behavior during migration at E13.5. The microglial soma (arrowheads) translocates in jumps. They first scan their environment (=idling, frames with red arrowheads) by sending out and retracting multiple processes and then migrate in the direction of one process (indicated by yellow arrows). The cell soma then displaces in the direction of that process (=active migration, frames with blue arrowheads) followed by a stationary phase during which the cells explores its environment again (=idling, frames with red arrowheads). The action of the cell towards the next time frame (interval = 10 min) determines the color of the arrowhead. Zoom-ins see Suppl. Fig. 1. **(B)** Representative instantaneous velocity in function of time plots of a cell migrating at high (left panel), intermediate (middle panel) and low (right panel) speed. Plots show phases of active migration interspersed with idling, defined as an instantaneous speed lower than the threshold of 0.8 $\mu\text{m}/\text{min}$ (dotted line). **(C)** Relative idling time

1 increased significantly over development (Kruskal-Wallis, E17.5 vs E13.5 and E15.5, $P=0.004$ and
 2 $P<0.001$). **(D)** Instantaneous speed of the active migration events ($v_{\text{inst. act.}}$) decreased over
 3 development (Kruskal-Wallis, E15.5 vs E17.5, $P<0.001$). **(E)** Cumulative probability plots of the data
 4 presented in (D) showing a shift to lower instantaneous speeds at E17.5. Sample size (C) as
 5 $n=\text{cells}/N=\text{slices}/M=\text{mothers}$ at E13.5: 150/18/12; E15.5: 170/10/8; E17.5: 349/14/9. n (cells) was
 6 used as sample size in statistical tests. Sample size (D) as $n=\text{steps}$ from cells in (C) at E13.5: $n=832$;
 7 E15.5: $n=972$; E17.5: $n=1740$. n (steps) was used as sample size in statistical tests. Scale bar=30 μm .

8 **Fig. 3. Cortical fibronectin and fibronectin receptor on microglia decrease over development. (A)**
 9 E13.5 (a1-4), E15.5 (b1-4), E17.5 (c1-4) coronal brain sections (DAPI, blue) with insets zooming in on
 10 the cortex (a2, b2, c2), indicating ROIs for analysis (white dotted lines in a3, b3, c3) and fibronectin
 11 staining (red, a4, b4, c4). Fibronectin was detectable as dense aggregates at E13.5 and with lower
 12 density at E15.5 and E17.5. **(B)** Mean grey value (MGV, 0-255) quantifications of the fibronectin
 13 immunostainings in the cortical areas marked in a3, b3 and c3. Fibronectin presence was significantly
 14 higher at E13.5 than at E15.5 ($P=0.041$) and E17.5 ($P<0.001$), while presence at E15.5 was significantly
 15 higher than at E17.5 ($P<0.001$) (Kruskal-Wallis with Dunn's). **(C)** Representative western blotting for
 16 fibronectin deposition in the cortex at E13.5, E15.5 and E17.5 with β -actin as loading control. **(D)**
 17 Fibronectin western blotting quantification relative to β -actin. Fibronectin deposition was
 18 significantly higher at E13.5 compared to E17.5 ($P<0.001$), while deposition at E15.5 was significantly
 19 higher compared to E17.5 ($P=0.001$) (ANOVA with Tukey HSD on log10 transformed data). **(E)** Laser
 20 scanning microscopy images (Z-projections) showing E13.5 cortex (e1) with microglia (eGFP, green,
 21 e2), nuclear staining (DAPI, blue), fibronectin (greys, e3) and blood vessels (GS-IB₄, red, e4). Microglia
 22 are frequently observed in the vicinity of blood vessels (inset zoom) and blood vessels show high
 23 fibronectin reactivity (e5, yellow arrowheads in e1). **(F)** Flow cytometry gating strategy to assess $\alpha 5$
 24 integrin (fibronectin receptor) expression on microglial cells in panels G and H. **(G)** The percentage of
 25 $\alpha 5$ positive microglial cells subtly, however significant, decreased from E15.5 to E17.5 (Kruskal-Wallis
 26 with Dunn's, $P<0.001$). **(H)** The expression level (median fluorescence intensity, MFI) of the $\alpha 5$

1 positive population significantly decreased from E13.5 to E15 to E17.5 (ANOVA with Tukey HSD on
2 log10 transformed data, all $P<0.001$). At E13.5 embryos were pooled per 2-3 and at E15.5-E17.5
3 individual embryos were analyzed. Sample size (B) as $n=\text{slices}/N=\text{embryos}/M=\text{mothers}$ at E13.5:
4 28/3/3; E15.5: 21/3/3; E17.5: 39/3/2. n (slices) was used as sample size in statistical tests. Sample
5 size (D) as $N=\text{embryos}/M=\text{mothers}$ at E13.5: 8/3; E15.5: 9/3; E17.5: 9/3. N (embryos) was used as
6 sample size in statistical tests. Sample size (G,H) as $N=\text{embryos}/M=\text{mothers}$ at E13.5: 8/3; E15.5:
7 16/3; E17.5: 20/3. N (embryos) was used as sample size in statistical tests. FN, Fibronectin. Scale bar
8 (A)=500 μm in a1, b1, c1; 100 μm in insets (all other panels). Scale bar (E) =50 μm in e1, 20 μm in e5.

9 **Fig. 4. $\alpha 5\beta 1$ integrin is not essential for microglia–blood vessel contact. (A)** Time-lapse sequences
10 (30 μm Z-projections) at E17.5 showing microglial cells (eGFP, green) capable to migrate
11 (arrowheads) along the surface of blood vessels (GS-IB₄, red) in control as well as after $\alpha 5\beta 1$ integrin
12 blockage in acute brain slices. Frame interval=10 min. **(B)** Modes of microglia–blood vessel contact:
13 Full soma (left panel), touching with a process (middle panel) and free or no contact (right panel). **(C)**
14 The percentage of microglia that made contact with a blood vessel during time-lapse recordings (3-6
15 hours after blocking onset) was not significantly different after $\alpha 5\beta 1$ integrin blockage compared to
16 isotype at E13.5 (Student t -test, $P=0.813$) nor at E17.5 (Mann-Whitney, $P=0.169$). The percentage of
17 microglia that made contact with a blood vessel rose significantly from E13.5 to E17.5 (Mann-
18 Whitney, $P=0.003$). **(D)** The percentage of time spent per cell on a particular contact was not
19 significantly affected by $\alpha 5\beta 1$ blockage (Mann-Whitney, $P=0.683$; 0.802; 1.000 for % Soma; %
20 Process; % Free at E13.5 and $P=0.173$; 0.343; 0.974 for % Soma; % Process; % Free at E17.5,
21 respectively). Sample size (C) as $N=\text{slices}/M=\text{mothers}$ at E13.5: 9/7 (Iso) and 7/3 (Ab); E17.5: 10/7
22 (Iso) and 12/3 (Ab). N (slices) was used as sample size in statistical tests. Sample size (D) as
23 $n=\text{cells}/N=\text{slices}/M=\text{mothers}$ at E13.5: 41/9/7 (Iso) and 33/7/3 (Ab); E17.5: 86/10/7 (Iso) and 82/12/3
24 (Ab). n (cells) was used as sample size in statistical tests. Scale bar (A,B)= 30 μm .

25

1 **Fig. 5. $\alpha 5\beta 1$ integrin blockage at E13.5 decreases while at E15.5 and E17.5 it increases microglial**
 2 **migration speed.** Microglia movement was recorded in acute brain slices in the presence of an $\alpha 5\beta 1$
 3 blocking antibody or isotype control during 6 h using 2-photon time-lapse imaging. Cell somas were
 4 manually tracked. **(A)** Representative microglial (eGFP, greys) migration tracks in different colors at
 5 E13.5, E15.5 and E17.5. The meninges is located at the top of the image and the ventricle at the
 6 bottom (not visible at E15.5 and E17.5). **(B)** At E13.5 $\alpha 5\beta 1$ integrin blockage significantly diminished
 7 microglial migration speed v_{av} ($\mu\text{m/h}$) compared to isotype ($P=0.017$) while it caused an increase in
 8 migration speed at E15.5 ($P=0.009$) and at E17.5 ($P<0.001$) (all Kruskal-Wallis with Dunn's). The effect
 9 of the blocking antibody was significantly different across ages (dotted lines; E13.5 vs. E15.5:
 10 $P<0.001$; E13.5 vs. E17.5: $P<0.001$; E15.5 vs. E17.5: $P=0.001$; all Kruskal-Wallis with Dunn's). **(C)**
 11 Immobile fractions after $\alpha 5\beta 1$ integrin blockage at E13.5, E15.5 and E17.5 did neither differ
 12 significantly from isotype (all ages $P=1.000$), nor from control (E13.5 $P=1.000$; E15.5 $P=0.525$; E17.5
 13 $P=0.146$) (all Kruskal-Wallis with Dunn's). The effect of the blocking antibody did not differ across
 14 ages (all ages $P=1.000$; all Kruskal-Wallis with Dunn's). **(D)** Cumulative probability distributions
 15 (control in black, isotype in grey and $\alpha 5\beta 1$ Ab in red) of average migration speed data in $\alpha 5\beta 1$
 16 blockage conditions show clear shifts from isotype and control distributions. Sample size as
 17 $n=\text{cells}/N=\text{embryos}/M=\text{mothers}$ at E13.5: 135/15/8 (Ab), 128/16/11 (Iso); E15.5: 227/11/6 (Ab),
 18 180/11/7 (Iso); E17.5: 213/11/6 (Ab), 246/10/5 (Iso). n (cells) was used as sample size in statistical
 19 tests. For sample size control condition see Fig. 1. Isotypes did not affect normal (control) migration.
 20 Scale bar=100 μm .

21 **Fig. 6. $\alpha 5\beta 1$ integrin blockage affects idling and instantaneous speed.** Experimental set-up see Fig.
 22 7. **(A)** $\alpha 5\beta 1$ blockage at E13.5 significantly increased relative idling time compared to isotype, while
 23 at E15.5 and E17.5 it significantly decreased idling (Kruskal-Wallis with Dunn's, E13.5 $P=0.013$; E15.5
 24 $P=0.015$; E17.5 $P<0.001$). **(B)** Instantaneous speed of the active migration events ($v_{\text{inst. act.}}$) significantly
 25 increased at E17.5 after blockage compared to isotype (Kruskal-Wallis with Dunn's, $P<0.001$). **(C)**
 26 Cumulative probability plots (control in black, isotype in grey and $\alpha 5\beta 1$ Ab in red) of the data

1 presented in (B) at E13.5, E15.5 and E17.5 showing a shift to higher instantaneous speeds at E17.5
2 after $\alpha 5\beta 1$ blockage. Sample size (A) as $n=\text{cells}/N=\text{slices}/M=\text{mothers}$ at E13.5: 121/15/8 (Ab),
3 134/16/11 (Iso); E15.5: 228/11/6 (Ab), 178/11/7 (Iso); E17.5: 222/11/6 (Ab), 247/10/5 (Iso). n (cells)
4 was used as sample size in statistical tests. Sample size (B) as $n=\text{steps}$ from cells in (C) at E13.5: 605
5 (Ab), 795 (Iso); E15.5: 1749 (Ab), 1076 (Iso); E17.5: 1399 (Ab), 1338 (Iso). n (steps) was used as
6 sample size in statistical tests. For sample size control condition see Fig. 1. Isotypes did not affect
7 normal (control) idling and instantaneous speed.

8 **Suppl. Fig 1. Microglial morphology changes during saltatory migration in the cortex at E13.5.**
9 Zoom-ins from the microglial cell in Fig. 2. Yellow arrows point to the process that is chosen to
10 initiate directive migration. Frame interval=10 min. Scale bar=15 μm .

11 **Suppl. Movie 1. Microglial migration along the developing vasculature at E17.5 after isotype**
12 **application (see Fig. 4C).**

13 **Suppl. Movie 2. Microglial migration along the developing vasculature at E17.5 after $\alpha 5\beta 1$ antibody**
14 **application (see Fig. 4C).**

15 **Suppl. Movie 3. Microglial migration and tracking at E13.5 after $\alpha 5\beta 1$ antibody application (see Fig.**
16 **5A).** Each cell migration track is indicated with a number and different color.

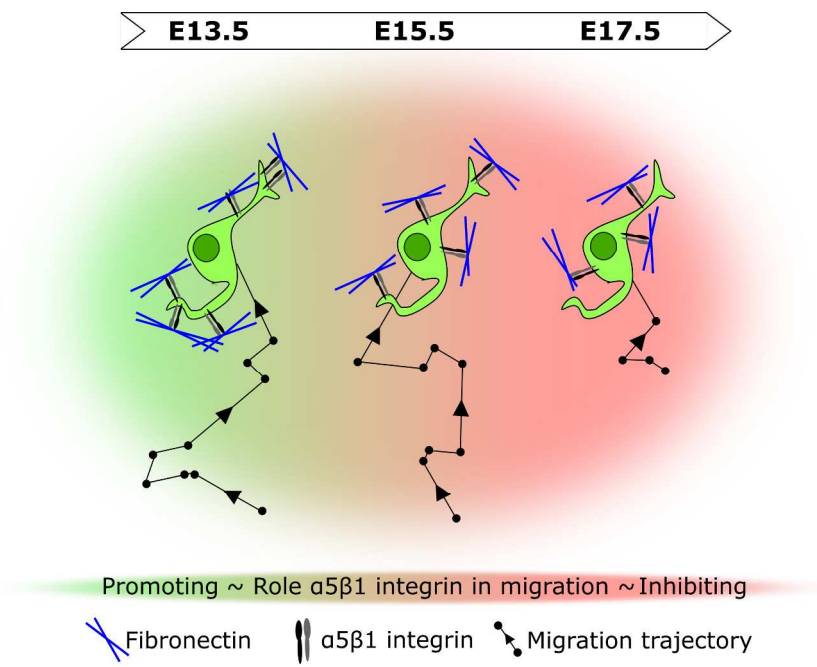
17 **Suppl. Movie 4. Microglial migration and tracking at E15.5 after isotype application (see Fig. 5A).**
18 Each cell migration track is indicated with a number and different color.

19 **Suppl. Movie 5. Microglial migration and tracking at E15.5 after $\alpha 5\beta 1$ antibody application (see Fig.**
20 **5A).** Each cell migration track is indicated with a number and different color.

21 **Suppl. Movie 6. Microglial migration and tracking at E17.5 after isotype application (see Fig. 5A).**
22 Each cell migration track is indicated with a number and different color.

- 1 **Suppl. Movie 7. Microglial migration and tracking at E17.5 after $\alpha 5\beta 1$ antibody application (see Fig.**
- 2 **5A).** Each cell migration track is indicated with a number and different color.
- 3 **Suppl. Movie 8. Microglial migration and tracking at E17.5 after $\alpha 5\beta 1$ antibody application (see Fig.**
- 4 **5A).** Each cell migration track is indicated with a number and different color.
- 5

Microglia in cortical development



TOCI (Graphical abstract)
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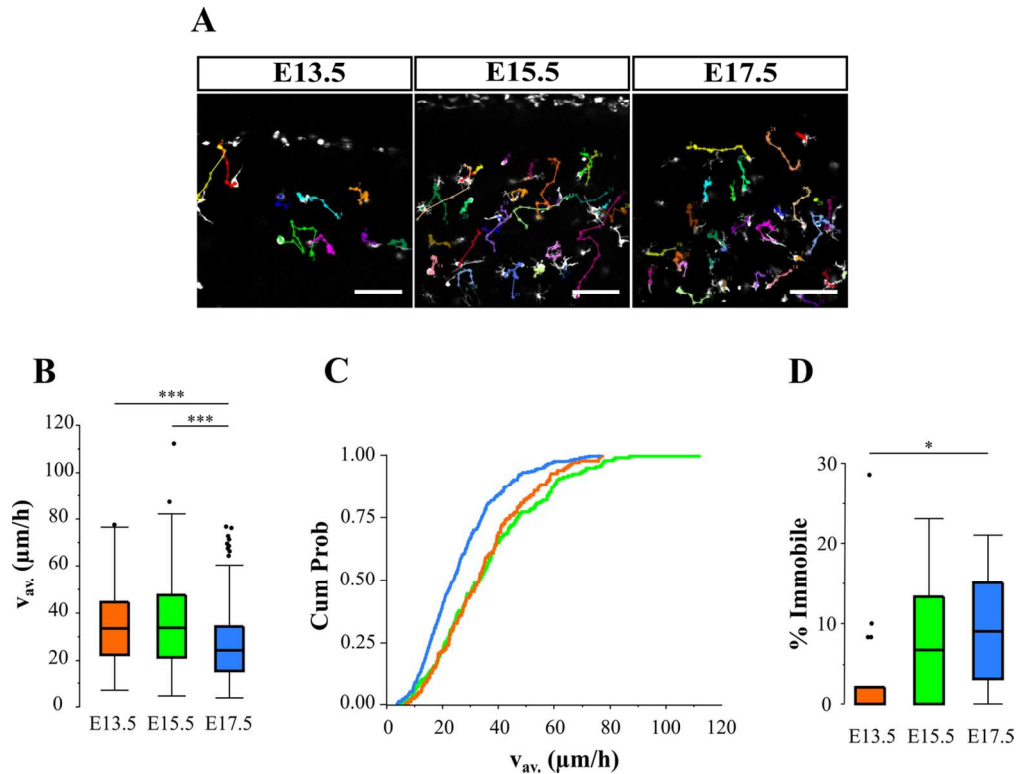


Fig. 1. Microglial migration is developmentally regulated. Microglia movement was recorded in acute brain slices during 6 h using 2-photon time-lapse imaging and cell somas were manually tracked. **(A)** Representative microglial migration tracks in different colors at E13.5 (left panel), E15.5 (middle panel) and E17.5 (right panel) (eGFP/microglia, green). The meninges is located at the top of the image and the ventricle at the bottom (not visible at E15.5 and E17.5). **(B)** Microglial average migration speed v_{av} ($\mu\text{m/h}$) decreased significantly over development (Kruskal-Wallis with Dunn's, E17.5 vs E13.5 and E15.5, $P < 0.001$).

(C) Cumulative probability plots of migration speed with matching colors for the ages in (B). E17.5 migration speed distribution shows a shift to lower average speeds. **(D)** The percentage of immobile microglia significantly rose from E13.5 to E17.5 (Kruskal-Wallis with Dunn's, $P = 0.014$). Sample size (A-C) as $n = \text{cells} / N = \text{slices} / M = \text{mothers}$ at E13.5: 160/18/12; E15.5: 170/10/8; E17.5: 341/14/9. n (cells) was used as sample size in statistical tests. Sample size (D) as $N = \text{slices} / M = \text{mothers}$ at E13.5: 18/12; E15.5: 10/8; E17.5: 14/9. N (slices) was used as sample size in statistical tests. Scale bar = 100 μm .

Fig. 1

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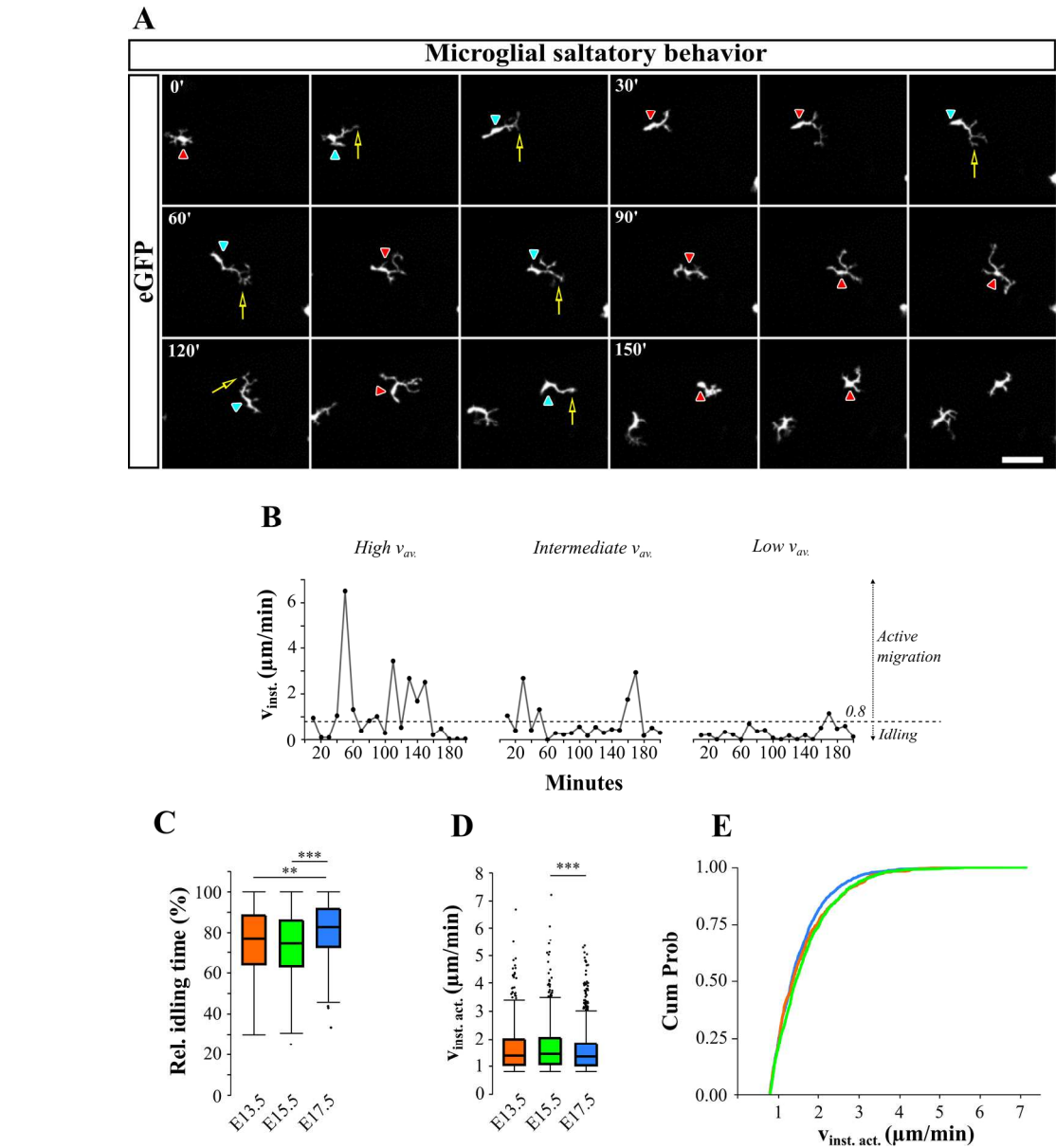


Fig. 2. Microglial migration behavior. Experimental set-up see Fig. 1. **(A)** Z-projections (30 μm) of representative time-lapse sequences showing characteristic microglial jumping behavior during migration at E13.5. The microglial soma (arrowheads) translocates in jumps. They first scan their environment (=idling, frames with red arrowheads) by sending out and retracting multiple processes and then migrate in the direction of one process (indicated by yellow arrows). The cell soma then displaces in the direction of that process (=active migration, frames with blue arrowheads) followed by a stationary phase during which the cells explores its environment again (=idling, frames with red arrowheads). The action of the cell towards the next time frame (interval=10min) determines the color of the arrowhead. Zoom-ins see Suppl. Fig. 1. **(B)** Representative instantaneous velocity in function of time plots of a cell migrating at high (left panel), intermediate (middle panel) and low (right panel) speed. Plots show phases of active migration interspersed with idling, defined as an instantaneous speed lower than the threshold of 0.8 $\mu\text{m}/\text{min}$ (dotted line). **(C)** Relative idling time increased significantly over development (Kruskal-Wallis, E17.5 vs E13.5 and E15.5, $P=0.004$ and $P<0.001$). **(D)** Instantaneous speed of the active migration events ($v_{\text{inst. act.}}$) decreased over

development (Kruskal-Wallis, E15.5 vs E17.5, $P < 0.001$). **(E)** Cumulative probability plots of the data presented in (D) showing a shift to lower instantaneous speeds at E17.5. Sample size (C) as $n = \text{cells}/N = \text{slices}/M = \text{mothers}$ at E13.5: 150/18/12; E15.5: 170/10/8; E17.5: 349/14/9. n (cells) was used as sample size in statistical tests. Sample size (D) as $n = \text{steps}$ from cells in (C) at E13.5: $n = 832$; E15.5: $n = 972$; E17.5: $n = 1740$. n (steps) was used as sample size in statistical tests. Scale bar = 30 μm .

Fig. 2

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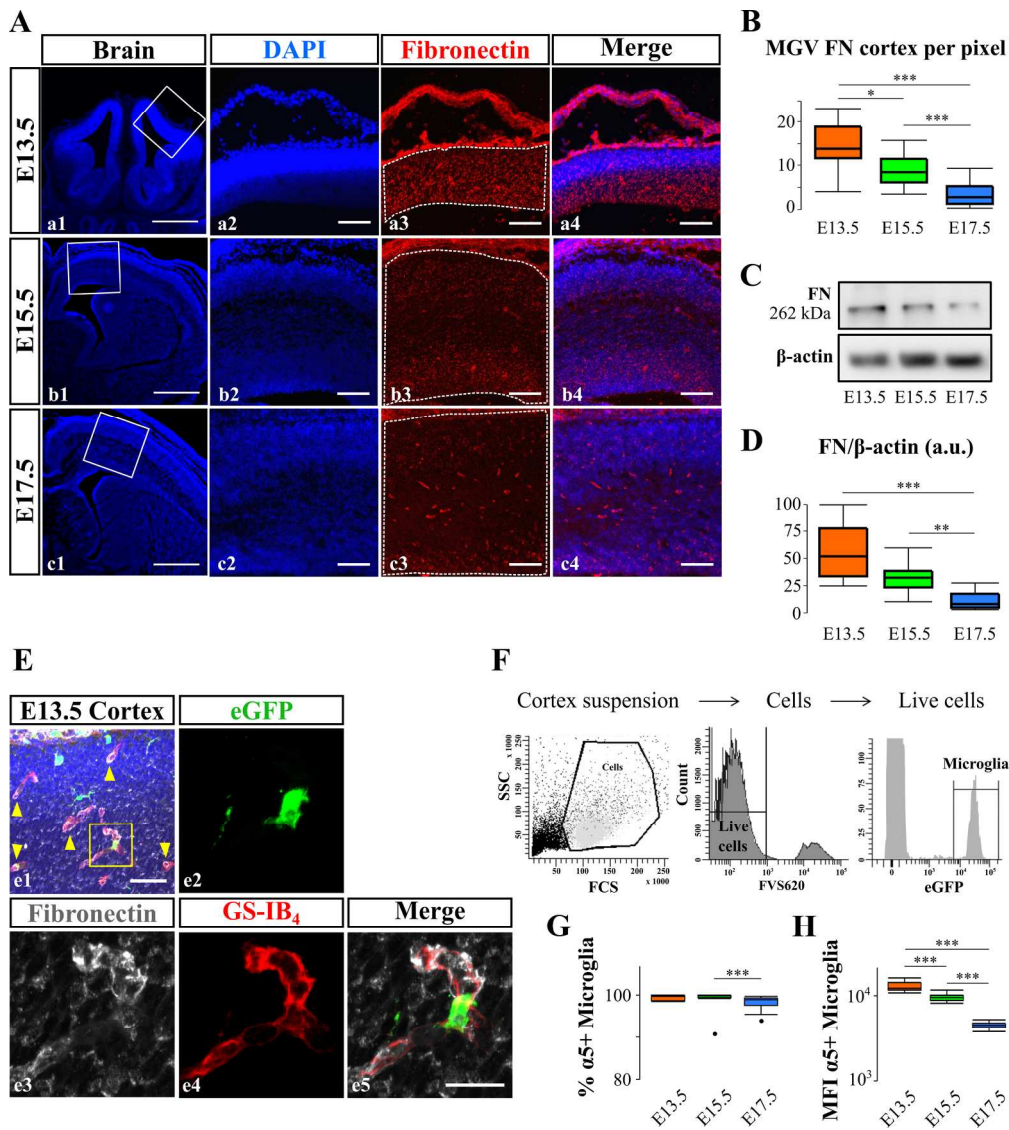


Fig. 3. Cortical fibronectin and fibronectin receptor on microglia decrease over development. (A) E13.5 (a1-4), E15.5 (b1-4), E17.5 (c1-4) coronal brain sections (DAPI, blue) with insets zooming in on the cortex (a2, b2, c2), indicating ROIs for analysis (white dotted lines in a3, b3, c3) and fibronectin staining (red, a4, b4, c4). Fibronectin was detectable as dense aggregates at E13.5 and with lower density at E15.5 and E17.5. (B) Mean grey value (MGV, 0-255) quantifications of the fibronectin immunostainings in the cortical areas marked in a3, b3 and c3. Fibronectin presence was significantly higher at E13.5 than at E15.5 ($P=0.041$) and E17.5 ($P<0.001$), while presence at E15.5 was significantly higher than at E17.5 ($P<0.001$) (Kruskal-Wallis with Dunn's). (C) Representative western blotting for fibronectin deposition in the cortex at E13.5, E15.5 and E17.5 with β -actin as loading control. (D) Fibronectin western blotting quantification relative to β -actin. Fibronectin deposition was significantly higher at E13.5 compared to E17.5 ($P<0.001$), while deposition at E15.5 was significantly higher compared to E17.5 ($P=0.001$) (ANOVA with Tukey HSD on log10 transformed data). (E) Laser scanning microscopy images (Z-projections) showing E13.5 cortex (e1) with microglia (eGFP, green, e2), nuclear staining (DAPI, blue), fibronectin (greys, e3) and blood vessels (GS-IB₄, red, e4). Microglia are frequently observed in the vicinity of blood vessels (inset zoom) and blood vessels show high fibronectin reactivity (e5, yellow arrowheads in e1). (F) Flow cytometry gating strategy to assess $\alpha 5$ integrin (fibronectin receptor) expression on microglial cells in panels G and H. (G) The

percentage of $\alpha 5$ positive microglial cells subtly, however significant, decreased from E15.5 to E17.5 (Kruskal-Wallis with Dunn's, $P < 0.001$). **(H)** The expression level (median fluorescence intensity, MFI) of the $\alpha 5$ positive population significantly decreased from E13.5 to E15 to E17.5 (ANOVA with Tukey HSD on log10 transformed data, all $P < 0.001$). At E13.5 embryos were pooled per 2-3 and at E15.5-E17.5 individual embryos were analyzed. Sample size (B) as $n = \text{slices} / N = \text{embryos} / M = \text{mothers}$ at E13.5: 28/3/3; E15.5: 21/3/3; E17.5: 39/3/2. n (slices) was used as sample size in statistical tests. Sample size (D) as $N = \text{embryos} / M = \text{mothers}$ at E13.5: 8/3; E15.5: 9/3; E17.5: 9/3. N (embryos) was used as sample size in statistical tests. Sample size (G,H) as $N = \text{embryos} / M = \text{mothers}$ at E13.5: 8/3; E15.5: 16/3; E17.5: 20/3. N (embryos) was used as sample size in statistical tests. FN, Fibronectin. Scale bar (A)=500 μm in a1, b1, c1; 100 μm in insets (all other panels). Scale bar (E) =50 μm in e1, 20 μm in e5.

Fig. 3

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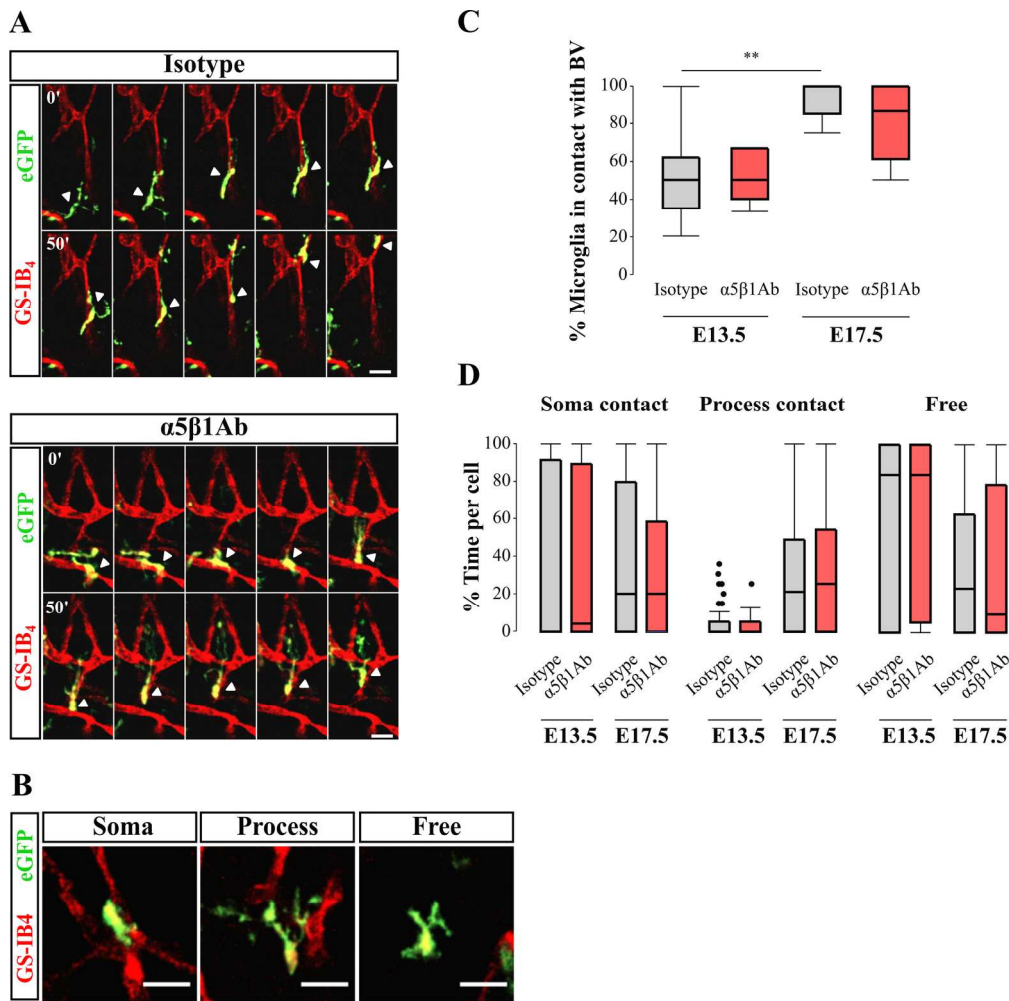


Fig. 4. $\alpha 5 \beta 1$ integrin is not essential for microglia–blood vessel contact. **(A)** Time-lapse sequences (30 μ m Z-projections) at E17.5 showing microglial cells (eGFP, green) capable to migrate (arrowheads) along the surface of blood vessels (GS-IB₄, red) in control as well as after $\alpha 5 \beta 1$ integrin blockage in acute brain slices. Frame interval=10 min. **(B)** Modes of microglia–blood vessel contact: Full soma (left panel), touching with a process (middle panel) and free or no contact (right panel). **(C)** The percentage of microglia that made contact with a blood vessel during time-lapse recordings (3–6 hours after blocking onset) was not significantly different after $\alpha 5 \beta 1$ integrin blockage compared to isotype at E13.5 (Student t-test, $P=0.813$) nor at E17.5 (Mann-Whitney, $P=0.169$). The percentage of microglia that made contact with a blood vessel rose significantly from E13.5 to E17.5 (Mann-Whitney, $P=0.003$). **(D)** The percentage of time spent per cell on a particular contact was not significantly affected by $\alpha 5 \beta 1$ blockage (Mann-Whitney, $P=0.683$; 0.802; 1.000 for % Soma; % Process; % Free at E13.5 and $P=0.173$; 0.343; 0.974 for % Soma; % Process; % Free at E17.5, respectively). Sample size (C) as N =slices/ M =mothers at E13.5: 9/7 (Iso) and 7/3 (Ab); E17.5: 10/7 (Iso) and 12/3 (Ab). N (slices) was used as sample size in statistical tests. Sample size (D) as n =cells/ N =slices/ M =mothers at E13.5: 41/9/7 (Iso) and 33/7/3 (Ab); E17.5: 86/10/7 (Iso) and 82/12/3 (Ab). n (cells) was used as sample size in statistical tests. Scale bar (A, B)= 30 μ m.

Fig. 4

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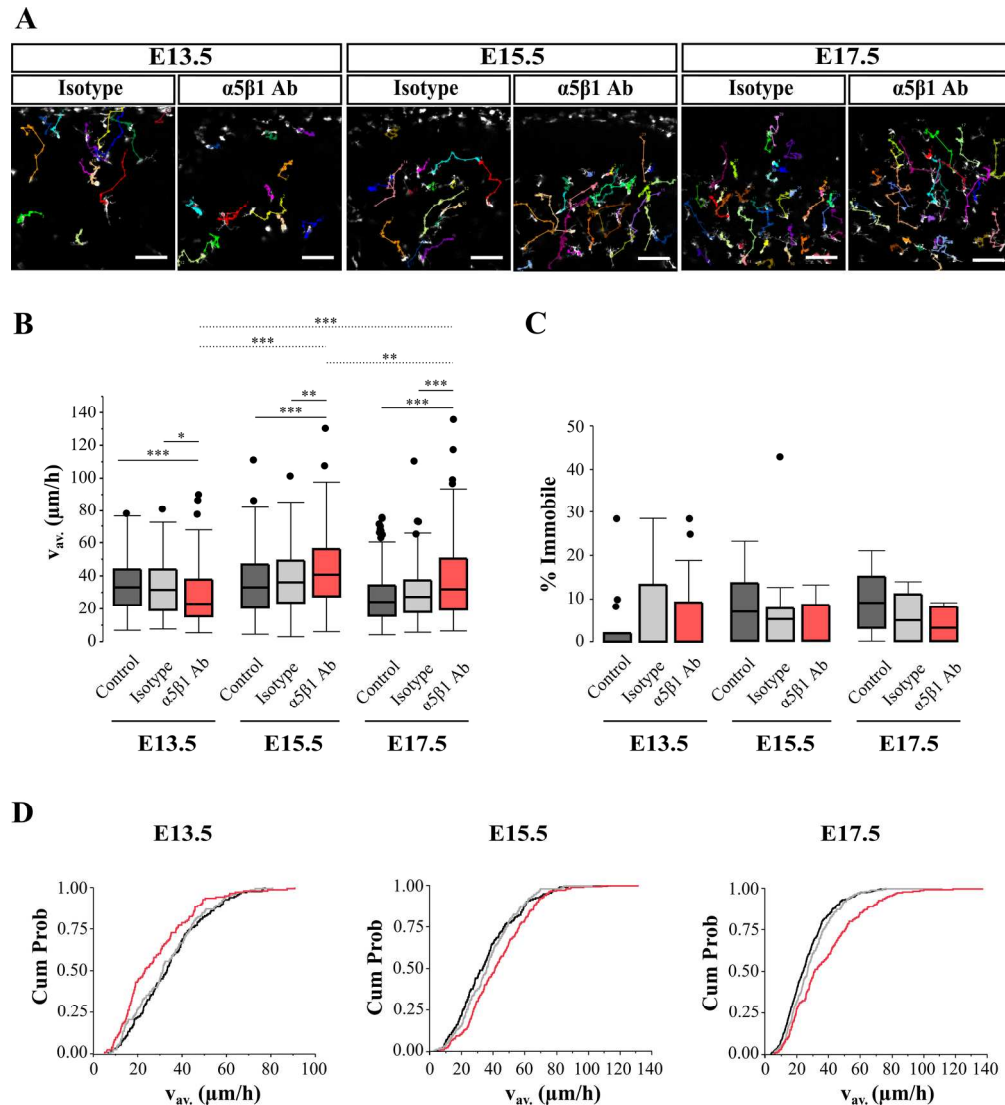


Fig. 5. $\alpha 5\beta 1$ integrin blockade at E13.5 decreases while at E15.5 and E17.5 it increases microglial migration speed. Microglia movement was recorded in acute brain slices in the presence of an $\alpha 5\beta 1$ blocking antibody or isotype control during 6 h using 2-photon time-lapse imaging. Cell somas were manually tracked. **(A)** Representative microglial (eGFP, greys) migration tracks in different colors at E13.5, E15.5 and E17.5. The meninges is located at the top of the image and the ventricle at the bottom (not visible at E15.5 and E17.5). **(B)** At E13.5 $\alpha 5\beta 1$ integrin blockade significantly diminished microglial migration speed v_{av} ($\mu\text{m}/\text{h}$) compared to isotype ($P=0.017$) while it caused an increase in migration speed at E15.5 ($P=0.009$) and at E17.5 ($P<0.001$) (all Kruskal-Wallis with Dunn's). The effect of the blocking antibody was significantly different across ages (dotted lines; E13.5 vs. E15.5: $P<0.001$; E13.5 vs. E17.5: $P<0.001$; E15.5 vs. E17.5: $P=0.001$; all Kruskal-Wallis with Dunn's). **(C)** Immobile fractions after $\alpha 5\beta 1$ integrin blockade at E13.5, E15.5 and E17.5 did neither differ significantly from isotype (all ages $P=1.000$), nor from control (E13.5 $P=1.000$; E15.5 $P=0.525$; E17.5 $P=0.146$) (all Kruskal-Wallis with Dunn's). The effect of the blocking antibody did not differ across ages (all ages $P=1.000$; all Kruskal-Wallis with Dunn's). **(D)** Cumulative probability distributions (control in black, isotype in grey and $\alpha 5\beta 1$ Ab in red) of average migration speed data in $\alpha 5\beta 1$ blockade conditions show clear shifts from isotype and control distributions. Sample size as $n=\text{cells}/N=\text{embryos}/M=\text{mothers}$ at E13.5: 135/15/8 (Ab), 128/16/11 (Iso); E15.5: 227/11/6 (Ab), 180/11/7 (Iso); E17.5: 213/11/6 (Ab), 246/10/5 (Iso). n (cells) was used as sample size in statistical

tests. For sample size control condition see Fig. 1. Isotypes did not affect normal (control) migration. Scale bar=100 μ m.

Fig. 5

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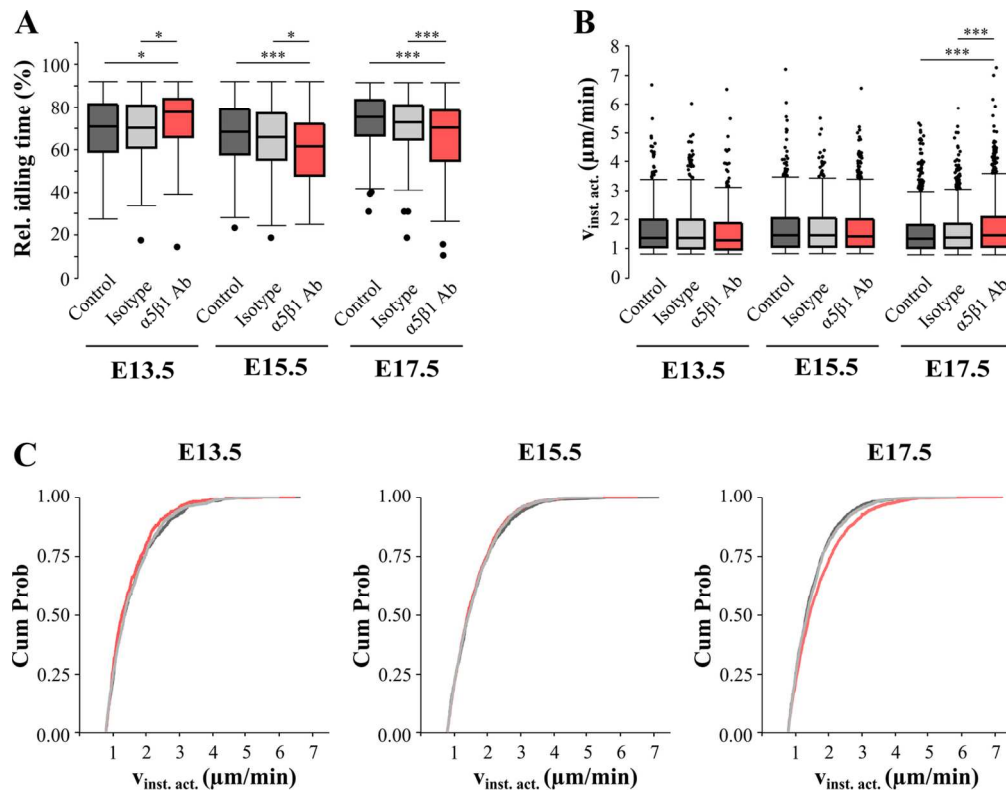
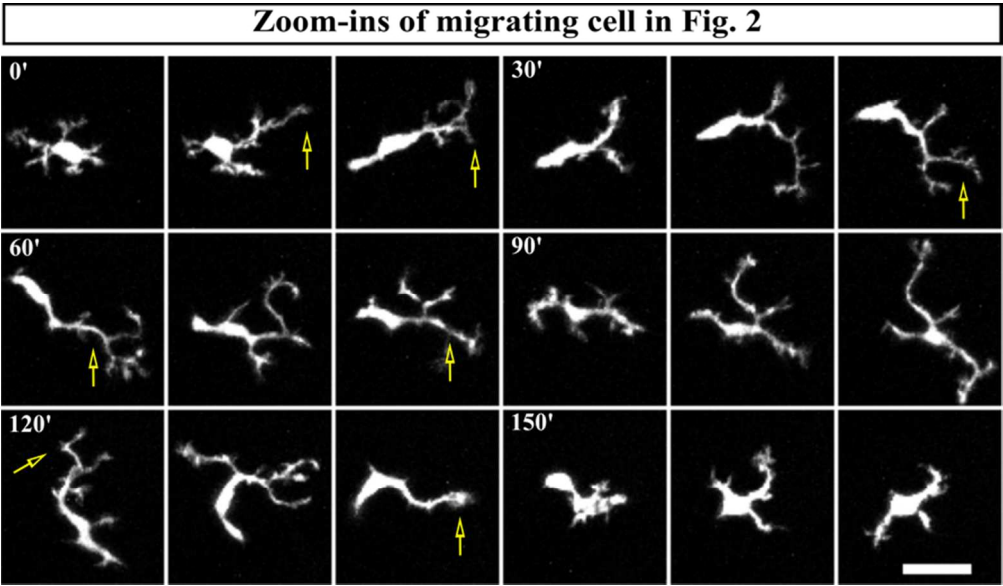


Fig. 6. $\alpha 5 \beta 1$ integrin blockage affects idling and instantaneous speed. Experimental set-up see Fig. 7. **(A)** $\alpha 5 \beta 1$ blockage at E13.5 significantly increased relative idling time compared to isotype, while at E15.5 and E17.5 it significantly decreased idling (Kruskal-Wallis with Dunn's, E13.5 $P=0.013$; E15.5 $P=0.015$; E17.5 $P<0.001$). **(B)** Instantaneous speed of the active migration events ($v_{inst. act.}$) significantly increased at E17.5 after blockage compared to isotype (Kruskal-Wallis with Dunn's, $P<0.001$). **(C)** Cumulative probability plots (control in black, isotype in grey and $\alpha 5 \beta 1$ Ab in red) of the data presented in (B) at E13.5, E15.5 and E17.5 showing a shift to higher instantaneous speeds at E17.5 after $\alpha 5 \beta 1$ blockage. Sample size (A) as $n=cells/N=slices/M=mothers$ at E13.5: 121/15/8 (Ab), 134/16/11 (Iso); E15.5: 228/11/6 (Ab), 178/11/7 (Iso); E17.5: 222/11/6 (Ab), 247/10/5 (Iso). n (cells) was used as sample size in statistical tests. Sample size (B) as $n=steps$ from cells in (C) at E13.5: 605 (Ab), 795 (Iso); E15.5: 1749 (Ab), 1076 (Iso); E17.5: 1399 (Ab), 1338 (Iso). n (steps) was used as sample size in statistical tests. For sample size control condition see Fig. 1. Isotypes did not affect normal (control) idling and instantaneous speed.

Fig. 6

128x100mm (300 x 300 DPI)



Suppl. Fig 1. Microglial morphology changes during saltatory migration in the cortex at E13.5.
Zoom-ins from the microglial cell in Fig. 2. Yellow arrows point to the process that is chosen to initiate
directive migration. Frame interval=10 min. Scale bar=15 μ m.
Suppl. Fig. 1
75x43mm (300 x 300 DPI)