TABLE OF CONTENTS

ACKNOWLEDGDEMENTS

Over the last six months, I had the opportunity to perform my senior internship at the department of Physiology at BIOMED. When looking back, this was a fantastic experience with six very interesting and educational months. I arrived in a very close and enthusiastic team and all of you made me feel very welcome.

In the first place I would like to thank my promotor prof. dr. Bert Brône for the opportunity to perform my senior internship in the group of Physiology and to introduce me in the intriguing world of microglia. His experience, enthusiasm and critical mind really helped me to succesfully finish this project.

Ofcourse, I would like to thank my daily supervisor drs. Sophie Smolders for her daily advice, support, and enthusiasm. Thanks to her, I was able to develop a critical look and to gain experience in a real research project. I would like to thank her for all her support, encouragements, efforts and interesting ideas. I also like to thank her for the confidence and the opportunity to have input in the project. Her guidance and encouraging support have really helped me in becoming a more skilled and critical minded researcher.

Also I would like to thank my second supervisor, dr. Annelies Bronckaers for all her suggestions, helpful discussions and her critical mind. Besides, I would like to thank her for providing us the blockers.

Further, I would also like to thank drs. Silke Smolders, Sofie Kessels, Ruth Mazon and ofcourse drs. Sophie Smolders and prof. dr. Bert Brône for their weekly attention and helpful discussions during our microglia meetings.

Last but not least, I would like to thank my parents. They encouraged me for the past 17 years of my school career and gave me the opportunity to perform higher studies. They were always there to support me.

Kaline Arnauts,

09/05/2016

ABBREVIATIONS

Akt/PKB	Protein kinase B
ASD	Autism spectrum disorder
BSA	Bovine serum albumin
BP	Bandpass
CNS	Central nervous system
CSF-1	Colony stimulating factor 1
Cx3cR1	Chemokine (C-X3-C motif) receptor 1 or fractalkine receptor
Cxcl12	C-X-C motif chemokine 12
CxcR4	C-X-C chemokine receptor type 4
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
E	Embryonic day
ECM	Extracellular matrix
eGFP	Enhanced green fluorescent protein
ERK	Extracellular signal–regulated kinases
FBS	Fetal bovine serum
FN	Fibronectin
GDP	Guanine diphosphate
GTP	Guanine triphosphate
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1	Human type 1 immunodeficiency virus
HS	Horse serum
ICAM-1	Intercellular Adhesion Molecule 1
IL-34	Interleukin 34
IP3	Inositol 1,4,5 triphosphate
JNK	c-Jun N-terminal kinases
LFA-1	Lymphocyte function-associated antigen 1
MAPK	Mitogen-activated protein kinases
MFI	Median fluorescence intensity
MIF	Migration inhibitory factor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	Phosphate-buffered saline
PLC	Phospholipase C
PFA	Paraformaldehyde
PM	Primary microglia
P/S	Penicillin Streptomycin
PyK2	Protein-tyrosine kinase 2
P38	P38 mitogen-activated protein kinases
Rpm	Revolutions per minute
RNA	Ribonucleic Acid
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
S	Supplementary
SDF-1	Stromal cell-derived factor 1
SEM	Standard error of the mean
VEGFR-1	Vascular endothelial growth factor receptor 1
`	Minutes

ABSTRACT

Introduction: Recently, insight in the migration pattern of microglia, the immune cells of the central nervous system (CNS), in embryonic development has been expanded. However, the molecular mechanisms regulating this migration are still unidentified. Chemoattractant Cxcl12 (SDF-1α) and its receptor CxcR4 are involved in microglia recruitment. Additionally, preliminary data indicate a decreased migration speed after blockage of integrin β1 in brain slices at embryonic day (E) 13. Further, Cxcl12 increases migration/invasion and adhesion in cancer cells by the use of integrin β1. Therefore, we hypothesize that "The Cxcl12/CxcR4/β1 axis stimulates microglia migration in embryonic development."

Materials & methods: Expression levels of CxcR4 on microglial cells were examined by immunohistochemistry on E13.5-15.5-17.5 and flow cytometry on E13.5 and E17.5. Transwell migration assays with the microglial BV-2 cell line were performed using Cxcl12 as a chemoattractant in the presence of CxcR4, β1, β2, PI3K or MEK1/2 blockers. Additionally, adhesion assays were performed to exclude an effect of the blockers on adhesion. Migration was also studied in acute embryonic brain slices of E13.5 and E17.5 using time lapse microscopy with blockers for β1, CxcR4, PI3K and MEK1/2. Furthermore, the active conformation of β1 was analyzed by flow cytometry upon Cxcl12 stimulation.

Results: Microglial cells express CxcR4 at all indicated embryonic ages in brain slices. Transwell migration assays showed increased migration towards Cxcl12 in the presence of fibronectin, which was fully abolished after administering a CxcR4 blocker. Also blockage of integrin β1 and the intracellular PI3K and MEK1/2 declined migration levels towards control levels, whereas blockage of β2 did not affect migration. In acute brain slices of E13.5, PI3K and MEK1/2 blockage both resulted in a decreased migration speed, whereas CxcR4 blockage did not have an effect. Surprisingly, at E17.5 β1 blockage resulted in an increased migration speed whereas PI3K blockage resulted in a decreased migration speed. CxcR4 and MEK1/2 blockage did not result in an altered migration speed. Further, stimulation with Cxcl12 suggested higher levels of active β1 conformation, this effect was abolished in the presence of the CxcR4 blocker.

Discussion & conclusions: We showed microglial cells migrate towards Cxcl12 in the presence of fibronectin, dependent on integrin β1. Additionally, we demonstrated involvement of PI3K and MEK1/2 in Cxcl12 stimulated migration. Remarkably, whereas β1 integrins stimulate migration at E13.5, they seem to decrease migration speed at E17.5. This suggest different functions of integrins during embryonic development. Our results contribute to the unraveling of migratory processes and will result in a better understanding of microglia migration and the function of microglia in general in embryonic development.

SAMENVATTING

Inleiding: Recentelijk is er meer inzicht verkregen in het migratie patroon van microglia, de immuuncellen van het centraal zenuwstelsel, tijdens de embryonale ontwikkeling. Echter zijn de moleculaire mechanismes betrokken in dit proces nog niet duidelijk. Chemoattractant Cxcl12 en de bijhorende CxcR4 receptor zijn betrokken in de aantrekking van microglia. Daarnaast tonen preliminaire data in hersenslices een verlaagde migratiesnelheid aan na toevoeging van een β1 blokker op embryonale dag (E) 13. Verder is er beschreven dat Cxcl12 zorgt voor een verhoogde migratie/invasie en adhesie in kankercellen door het gebruik van integrine β1. Om deze redenen hypothetiseren wij dat "De Cxcl12/CxcR4/β1 as migratie van microglia stimuleert in de embryonale ontwikkeling."

Materiaal & methoden: Het expressieniveau van CxcR4 werd geanalyseerd op microglia door middel van immunohistochemie op E13.5, E15.5, E17.5 en flow cytometry op E13.5 en E15.5. Transwell migratie essays met de microglia BV-2 cellijn werden uitgevoerd met Cxcl12 als chemoattractant in de aanwezigheid van blokkers voor CxcR4, β1, β2, PI3K en MEK1/2. Bijkomend werden er ook adhesie essays uitgevoerd om een effect van de blokkers op de adhesie uit te sluiten. Migratie werd ook bestudeerd in embryonale hersenslices van E13.5 en E17.5 door middel van time lapse microscopie na toevoeging van blokkers voor β1, CxcR4, PI3K en MEK1/2. Daarnaast werd met flow cytometrie de actieve conformatie van β1 geanalyseerd na stimulatie met Cxcl12.

Resultaten: Expressie van CxcR4 op microglia cellen was aanwezig op alle aangegeven embryonale leeftijden. Transwell migratie essays duiden op een verhoogde migratie naar Cxcl12 in de aanwezigheid van fibronectine. Deze migratie werd volledig teniet gedaan door toevoeging van een CxcR4 blokker. Ook het blokkeren van integrine β1 en het intracellulaire PI3K en MEK1/2 zorgden voor een afname tot op controle niveau, terwijl integrine β2 blokkering geen effect had. Blokkering van PI3K en MEK1/2 in acute hersenslices op E13.5 resulteerden in een gedaalde migratie snelheid, in tegenstelling tot CxcR4 blokkering wat geen effect opleverde. Verwonderlijk, op E17.5 zorgde het blokkeren van β1 voor een verhoogde migratiesnelheid terwijl PI3K inhibitie zorgde voor een verlaging. Het blokkeren van CxcR4 en MEK1/2 had geen effect op de migratie snelheid op E17.5. Verder sugereerde stimulatie met Cxcl12 ook nog voor een verhoogd niveau van integrine β1 in de actieve conformatie, wat tenietgedaan werd door toevoeging van de CxcR4 blokker.

Discussie & conclusie: We toonden aan dat microgliale cellen naar Cxcl12 migreren in de aanwezigheid van fibronectine, met behulp van integrine β1. Verder werd de de betrokkenheid van PI3K en MEK1/2 in Cxcl12 gestimuleerde migratie aagetoond. Terwijl integrines migratie stimuleren op E13.5, zorgen ze op E17.5 net voor een verminderde migratiesnelheid. Dit suggereert een verandering in de functie van integrins tijdens de embryonale ontwikkeling. Onze resultaten zullen bijdragen aan het beter begrijpen van migratieprocessen tijdens de embryonale ontwikkeling en dit zal helpen het volledige migratie proces en het belang van microglia tijdens de ontwikkeling bloot te leggen.

1 Introduction

1.1 Functions of microglia

Microglia are well-known as the resident macrophages of the central nervous system (CNS). Being widely spread all over the brain and spinal cord the population accounts for 5-20 % of all cells in the CNS (1). Microglial cells possess two main functional aspects, the first key feature is immune defense in the CNS in either health and disease. Microglia fulfill similar functions in the CNS as macrophages throughout the rest of the body. By constantly scanning the environment with their long branching processes, signals of danger can be detected immediately. These signals can originate from both invading pathogens or internal signals of cells in the brain itself. The detection of uncommon signals turns microglial cells into their activated state, resulting in the clearance of pathogens or debris. Aberrant activation of microglia is described in Alzheimer's and Parkinson's disease and will have destructive functions on the brain (1). Excessive and undesired chronically activation is also described to result in inflammation and devastation of the brain (1). Hence, proper functioning of this population of glial cells is essential for a healthy and well functioning brain (2). The second key function is the maintenance of brain homeostasis. This feature includes multiple aspects like controlling proliferation and differentiation of neurons and synaptic pruning (2, 3). In the postnatal brain, microglial cells are essential for the construction of a well functioning neural circuit by involvement in synaptic pruning (4, 5). Paolicelli et al. showed that microglia play an active role in synaptic pruning in postnatal development by actively engulfing and remodeling developing synapses (4). These results suggest that proper functioning of microglia is essential for the construction of a good neuronal network by playing a major role in synaptic pruning. In the adult brain, neurogenesis persists in particular zones of the brain, especially in these areas where neural stem cells are present, namely the subventricular zone and the hippocampal subgranular zone (6). In these regions, microglia take charge of the clearance of excessive neurons. Once the main neuronal circuits are constructed, microglia are still responsible for the fine-tuning of the synaptic connections. In addition, microglia are also able to influence neuronal activity (7, 8). Recent studies showed that microglia can actively induce apoptosis in neuronal cells by the excretion of specific growth factors, or in Purkinje cells in the cerebellum by respiratory burst (7, 8). In addition it is shown that microglia can assist in neurotransmission (7, 8).

All together, research into microglial behavior in the adult CNS in widely performed and involvement of these cells in various neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease is widely described. In the past, the focus on microglia has defined the complex functions of microglia as immune cells in health and disease. By contrast, the field of microglia in development is relatively new and contains many intriguing aspects waiting to be discovered. These aspects become gradually clarified, starting with the origin of microglia.

1.2 Microglia in development

Recent work implicates the importance of microglial cells during developmental processes in the CNS. Among others, control of neuronal cell fate, spatial patterning and the formation and fine-tuning of neuronal networks are suggested to be modulated by microglia (3). In contrast to other immune cells in the body, microglia migrate to the brain during embryonic development and persist there permanently, maintaining their population throughout life by self-renewal (1, 9-11). Microglia are present in the brain just before the onset of neurogenesis, hence at the right time to participate in critical processes (3).

1.2.1 The origin of microglia

For a long time the origin of microglia was highly debated. Multiple theories about their developmental origin have been proposed. Originally, the "mononuclear phagocyte system" by Metchnikoff and Ehrlich in 1969 was generally accepted. In this system, all high phagocytic mononuclear cells and their precursors were included, based on similarities in their morphology, function and origin (1, 10, 12). Later on, the mesodermal origin of microglia was claimed by Rio-Hortega. Ginhoux et al. presented exclusive evidence of the mesodermal origin of microglia, this by fate mapping of microglia in the brain (9).

In vivo experiments in which microglia were traced demonstrated microglia as a distinct population derived from primitive myeloid progenitors (9). In mouse embryos, the first primitive hematopoietic cells appear in the embryonic yolk sac between embryonic day (E) 7.0 and E9.0 (13, 14). Genetic targeting revealed that microglia are specified in the yolk sac between E7.0 and E7.5 (2, 9). Cells expressing the microglia markers CD11b, F4/80 and CX3CR1 were present in the brain at E9.5, simultaneously with the development of blood vessels. In mice lacking a functional blood circulation, levels of progenitor cells remained the same in the yolk sac but microglia were absent in the brain, suggesting the necessity of blood circulation for this migration. In addition, the phenotype of the microglial cells in the brain matches the phenotype of the macrophages present in the yolk sac during embryonic development. Injection of newborns with donor hematopoietic precursors confirmed that donor cells do not contribute to the microglial population. When evaluating levels of microglia three months after injection, still 95 % of microglia were of host origin, confirming again the microglia population is a distinct population which migrates towards the brain during embryogenesis (15). The yolk sac derived origin is described in multiple animals models including birds, fish and mammals (15-17).

1.2.2 Migration and differentiation of microglia

For the differentiation and development towards microglia, the cells are strongly dependent on the colony stimulating factor 1 (CSF-1) and its receptor (CSF-1R). Absence of CSF-1R leads to an almost complete absence of microglia. CSF-1R blocker administration during adulthood evoked the same effect, indicating the necessity of CSF-1R for the maintenance of a healthy microglial population (18-20). Since blockage of the receptor affects the microglia population more than absence of CSF-1, a second ligand is expected to be involved. Indeed, interleukin-34 (IL-34) is shown to interact with

CSF-1R (21). The expression pattern of IL-34 and CSF-1 are different, indicating a double function of CSF-1R in the embryonic and adult stage (22). Next to CSF-1R, also the presence of transcription factor PU.1 is indispensable in microglial development. PU.1 is a factor crucial in the development of B cells and macrophages and is also involved in the differentiation of neutrophils and T-cells (23).

Microglial cells are shown to be important cells in the developing brain. Microglia possess a highly dynamic behavior during embryonic development, this in contrast to the adult brain in which they are relatively stationary cells. They migrate intensively during embryonic development to reach their desired location. They are present in the brain before the start of differentiation of other CNS cell types, thus at the right time to participate in important processes during development (3). In mice, microglia enter the brain at E9.5 by invading the leptomeninges and lateral ventricles (16). Once arrived in the brain, the migration pattern in the brain comprises three phases. Microglial cells start to invade the cortex at E11.5, which is the beginning of neuron differentiation and synaptogenesis and this migration process continues until E17.5 (16, 24, 25). In the first phase, from E10.5 until E14.5, there is a gradual increase in the amount of microglial cells. At E10.5 there are almost no microglial cells present in the cortex. At E12.5, microglia were still rarely observed although their number doubled compared to E11.5. From E12.5 until E14.5, the number of microglia remained stable. Initially, there is no clear pattern in the distribution of the microglial cells in the cortical wall, accumulation of the cells is only visible at the ventricular wall and the pia. The second migration wave follows at E14.5-E15.5 and is typified by an enormous increase in the absolute number and the density of microglia in the developing brain. At E15.5 a shift takes place in the distribution of the cells by a rearrangement of the cortex. The development of different cortical layers results in three different zones, a ventricular zone, an intermediate zone and a cortical plate zone. Cells will spread through the cortical wall whereas the cortical plate zone contains almost no microglia. Finally, the third phase from E15.5-E17.5 is defined by a small increase in the number of microglia. Besides the change in the number of microglia, there is also a change in morphology. Around E12.5, the cells have a more amoeboid form which shifts towards a more ramified form during development (16).
Whereas insight into the migration pattern of microglia towards and inside the developing brain has been expanded, up to now little information is established about the mechanisms involved in the migration, recruitment and positioning of microglia. One of the latest discoveries of Arno et al. exposed an important function for chemokine Cxcl12 in the attraction and migration of microglial cells during embryonic development (15).

1.3 Chemokine Cxcl12

Chemokine CXC ligand 12 (Cxcl12) or stromal cell-derived factor 1 (SDF-1) is a small chemotactic cytokine that belongs to the CXC subfamily of chemokines. The classification of the subfamilies is based on the construction of the two cysteine residues next to the N-terminus on the receptor (26, 27). In the CXC subfamily an amino acid is present between the two cysteine residues. Cxcl12 functions as a ligand for the G-protein coupled chemokine receptor 4 (CxcR4) (27). This receptor contains seven transmembrane domains and is highly conserved during evolution (28, 29). CxcR4 is unique among chemokine receptors because it exclusively interacts with chemokine Cxcl12. However, it is described that migration inhibitory factor (MIF) can also compete for binding with

CxcR4 (30). For a long time, Cxcl12 was considered to interact only with CxcR4 until recently CxcR7 was revealed as an alternative receptor for Cxcl12 (31, 32). Furthermore, CxcR4 functions as a co-receptor for the entry of the T-trophic human type 1 immunodeficiency virus (HIV-1) in T-cells. For this reason the receptor is often targeted in treatment for HIV-1 by blocking the entrance of the virus into the cells (33, 34). Besides its involvement in HIV-1, CxcR4 has a broad range of functions in the organism including involvement in neuromodulation, haematopoiesis and cancer (29, 34). Also in embryonic development Cxcl12 is already implicated in multiple processes.

1.3.1 Cxcl12 in embryonic development

Messenger RNA of the chemokine receptor is detectable in multiple tissues by RT-PCR at different embryonic stages. Analysis demonstrated CxcR4 as the predominant chemokine receptor, especially during the earlier stages of embryonic development (E7.5-E8.5). CxcR4 and its ligand Cxcl12 are essential during organogenesis in the cardiac, vascular, hematopoietic and matter of course the neuronal system (35). Mutations in the genes of Cxcl12 or CxcR4 lead to perinatal lethality in mice as a result of deficiency in neurogenesis, angiogenesis and cardiogenesis (36). During embryogenesis, expression of these genes is also shown to be related with cell migration. Migrating cells express CxcR4 and are attracted by cells in the ectoderm which express the chemokine Cxcl12 (29, 36). Next to regulating cell migration throughout the body, CxcR4 is involved in brain development. The migration and guidance of neuronal precursors and the formation of neuronal circuits in the developing brain is regulated by CxcR4-Cxcl12 interaction (29, 37). Aside from its importance in the migration of neural progenitor cells, the CxcR4-Cxcl12 axis has extended functions in the developing brain. Arno et. al. (15) showed the importance of this axis in microglia recruitment in embryonic development. They showed that basal progenitor cells are involved in the recruitment of microglia towards the ventricular/ subventricular zone through the secretion of Cxcl12 (15). The density of microglial cells declines in the absence of Cxcl12, whereas microglial cell density is highly enriched in regions with high concentrations of Cxcl12. Blockage of the CxcR4 receptor, present on microglia resulted also in a decrease in the density of microglia. The general pathway following upon Cxcl12-CxcR4 interaction is described in multiple processes.

1.3.2 Cxcl12-CxcR4 pathway

The CxcR4-Cxcl12 pathway is described to be involved in among others migration, proliferation, inflammation, tissue homeostasis, embryogenesis and cancer (29, 38-40). Interaction of Cxcl12 with the CxcR4 receptor results in the activation of multiple downstream pathways with a broad range of functional responses (Figure 1). CxcR4 is a G-protein-coupled receptor, which means that the receptor is directly coupled to an intracellular heterotrimeric G-protein consisting out of an α, β and γ subunit. In its basal state this heterotrimer is bound to GDP, which is exchanged for GTP upon activation. This exchange results in dissociation towards the GTP subunit bound with the α monomer and a βγ dimer. The α monomer will activate PLC, resulting in generation of DAG and IP3 and this will result in intracellular Ca^{2+} release and the activation of protein kinases like PKC. Besides, ligand binding also induces activation of PI3K, resulting in the activation of more downstream targets such as PyK2, Akt and the downstream NF-κB pathway. These pathways are involved in multiple processes such as chemotaxis, transcription, proliferation and survival. Likewise, several MAPKs pathways are

activated including the ERK, JNK and p38 pathway. All together, the subunits can activate different intracellular pathways involved in migration, proliferation, inflammation, tissue homeostasis, embryogenesis and cancer (29, 38-40). The Cxcl12-CxcR4 pathway has been described multiple times in cancer cells in relation to migration and invasion, especially by activation of β1 and β3 integrins (36, 38, 41, 42). Since molecular mechanisms are often preserved in different cell types, we believe migration of microglial cells can be regulated by the Cxcl12-CxcR4 axis by means of integrin activation.

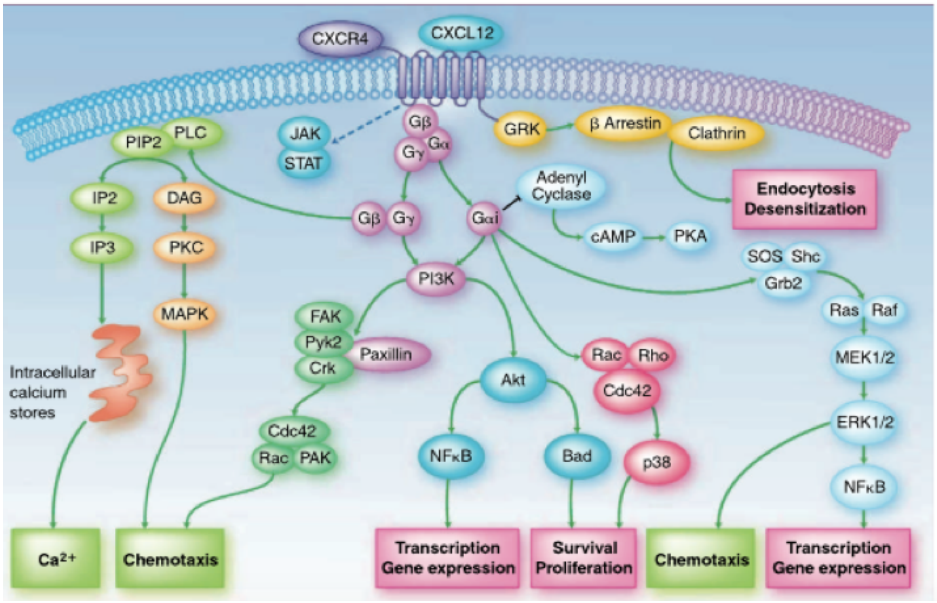

Figure 1 : Cxcl12-CxcR4 pathway described in cancer (Teicher, 2010) (38).

1.4 Integrins

Integrins are transmembranic cell adhesion receptors, which are responsible for the interaction of cells with the extracellular matrix (ECM) by forming a bridge between the extracellular and intracellular environment. Integrins consist of an alpha (α) and a beta (β) subunit which are non-covalently linked. In mammals the complete integrin family exist of 18 α and 8 β subunits, which can combine into 24 different integrin heterodimers. Dependent on their pairing each heterodimer interacts with different types of ECM (eg. collagen, fibronectin, laminin) (43, 44).

Integrins consist of three domains, a large extracellular domain, a transmembranic domain and a small intracellular domain with a cytoplasmic tail. The extracellular domain, also referred to as the legs, can adopt different conformational states dependent on the activation status of the integrin which is mainly regulated by inside-out signaling (Figure 2). Binding of cytokines, chemokines or other antigens to the cell initiates intracellular signals or pathways which will lead to a conformational change in the integrin, resulting in increased adhesiveness for the extracellular matrix (45). Next to inside-out signaling, also outside-in signaling is related to the conformational change of integrins. Ligand binding or the proximity of a ligand will transduce signals towards the intracellular part of the integrin and this will also result in increased adhesiveness (45). X-ray crystal structures revealed that in the inactive or low-affinity conformation both legs are bent, with the head directed towards

the cell membrane. Ligand binding or the inducement of the active conformation is associated with a rearrangement of both legs towards an extended conformation (45-47). Integrin ß1 is the most important integrin subunit and interacts with the majority of the alpha subunits. ß1 heterodimers interact mainly with fibronectin, laminin and collagen (43, 44). Not surprisingly, knockout of ß1 leads to embryonic lethality in mice in preimplantation development. Integrin ß1 is required for normal development and has a major function in morphogenesis and the growth and survival of cells. (48-50).

1.4.1 Integrins on microglial cells

Multiple integrins are present on microglial cells, including the α4-, α5- and α6β1 integrins (51, 52) (Table 1). Preliminary data of our research group showed that integrin α5β1, which interacts with fibronectin, is involved in the migration of microglia in embryonic brain slices. Blockage of α5β1 integrins on brain slices of E13.5, led to a decreased migration speed. Moreover, the presence of β1 blockers resulted into an even higher decrease in migration speed of the microglial cells. The presence of the β1 ligand fibronectin is also shown in different animal models in the brain during embryonic development. In the embryonic chicken brain fibronectin was found to be expressed in a pattern associated with radial cells (56). Those cells are acknowledged to guide neuronal cells during development (53). Fibronectin was localized around these radial glial cells and found to be expressed by these cells in the developing optic tectum in a pattern complementary to neuronal migration (54). Also in cats, the presence of fibronectin is demonstrated at fetal and postnatal ages. There, the presence of fibronectin in the brain was limited to the subplate and the marginal zone, in the cortical plate fibronectin could not be detected (55). De Gasperi et al. showed the presence of fibronectin in embryonic brain slices of mice at E15.5. The intensity of fibronectin seems to be strongly related to vascular structures (56). Additionally, also in mice the proximity of fibronectin and glial cells was shown. After culturing glial cells, these cells showed intense reaction for fibronectin and it was shown that fibronectin was endogenously produced by these cells (57). Also immunostainings performed by our lab (unpublished results) showed the presence of fibronectin in the embryonic mice brain.

Table 1: Integrins present on microglial cells (51, 52).

Integrin	Ligand
α4β1	Fibronectin, VCAM-1
α5β1	Fibronectin
α6β1	Laminin
αLβ2 (LFA-1)	ICAM-1, ICAM-2
αMβ2 (Mac-1)	ICAM-1
αVβ3	Vitronectin
αVβ5	Vitronectin

1.5 The link between Cxcl12 and integrin ß1

All together, Cxcl12 and integrin β1 are both suggested to be involved in the migration of microglial cells. Arno et al. (15) described that Cxcl12 has an important function in the attraction of microglial

cells in the brain during embryonic development. The absence of Cxcl12 or the CxcR4 receptor resulted in decreased levels of microglial cells in the brain. These data suggest an important role for Cxcl12 in microglial recruitment. However, which mechanisms are involved in this attraction towards Cxcl12 remained unclear.

We possess preliminary data indicating the involvement of integrin ß1 in migration of microglial cells. Blockage of integrin ß1 on brain slices of embryonic day 13.5 resulted in decreased migration speed of microglial cells. Additionally, the presence of the ß1 ligand, fibronectin is shown in the embryonic brain. Immunohistochemical stainings of our lab (unpublished results) revealed fibronectin as the most appropriate ligand. Fibronectin showed a relatively homogenenous expression pattern in time and place whereas the expression of laminin was more spatiotemporal. Thus, both Cxcl12 and integrin ß1 are suggested to be involved in migration of microglial cells.

Furthermore, a link between Cxcl12 and integrin ß1 is described in relation with increased adhesion and migration upon Cxcl12 binding in multiple types of cancer cells. Among others, in acute lymphoblastic leukemia blasts increased migration towards bone marrow fibroblasts is shown by ß1 integrins induced adhesion upon Cxcl12 stimulation (58). Also in ovarian cancer cells and prostate cancer cells the Cxcl12/ ß1 integrins axis is described to enhance invasion, thus migration, of cancer cells by upregulation of ß1 integrins (59-61). Besides, in human lung cancer cells it is described that Cxcl12 increases the migration and also the expression of integrins ß1 and ß3 by regulation of the ERK and NF-κB dependent pathway (62).

This linked is showed in multiple cell types and molecular mechanisms are often conserved between different cell types, besides both Cxcl12 and β1 are described in relation with migration of microglial cells. For those reasons, a link between Cxcl12 and integrin ß1 can be involved in the migration of microglial cells during development. The pathway involving PI3K and MEK1/2 is named multiple times, and will therefore also be investigated during our project (38, 62).

1.6 Goals and experimental approach

All together, this results in the following combination; (1) Cxcl12 attracts microglial cells during development, (2) our preliminary data indicate involvement of integrin β1 in microglia migration, and (3) the link between Cxcl12 and integrin β1 is described in migration of cancer cells. These facts led us to hypothesize that "**migration of microglia in embryonic development is stimulated by the Cxcl12/CxcR4/β1 axis.**" This hypothesis will be verified by three main objectives, first of all the presence and expression levels of CxcR4 *in situ* will be analyzed at different embryonic ages, namely E13.5, E15.5 and E17.5. Therefore, immunostaining on brain slices and flow cytometry of the embryonic cortex at the aforementioned ages will be performed. Secondly, we will investigate whether the migration is regulated via Cxcl12 and integrin β1. To this end, we will perform transwell migration assays, using a microglial cell line and primary microglia, in which Cxcl12 will be used as a chemoattractant. The effect of CxcR4 blockage and β1 blockage will be investigated in migration towards Cxcl12. Besides, migration will be analyzed in acute embryonic brain slices using time lapse microscopy in the presence of CxcR4 and β1 blockers. Additionally, we will investigate whether Cxcl12

can induce the active β1 conformation which is possibly involved in facilitating adhesion/migration. Our third goal will include unraveling the intracellular pathway between Cxcl12 and integrin β1. For this aim, transwell migration assays and time lapse imaging in acute embryonic brain slices will be performed with intracellular pathway blockers.

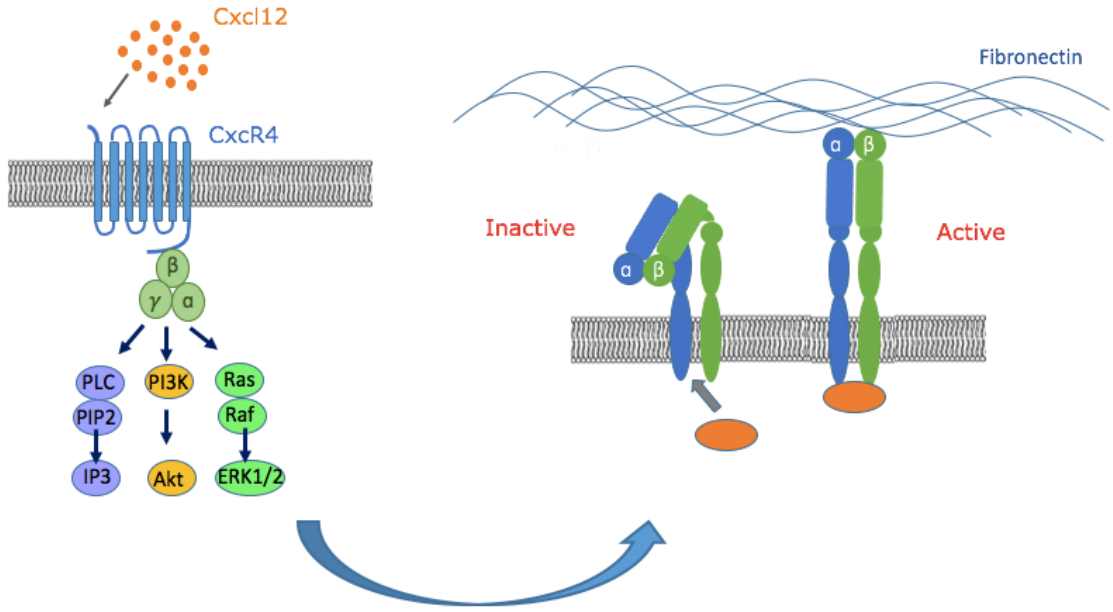

Figure 2: Suggested Cxcl12- β1 axis. Cxcl12 will bind to its receptor, CxcR4. This will lead to the activation of intracellular pathways which will recruit complexes to the intracellular part of the integrins and will induce the active conformation.

Our results will lead to a better understanding of microglial cell migration in the cortex during embryonic development. Until now, there is still a gap in literature regarding microglia in embryonic development, especially about factors involved in recruitment and regulation of their migration. It is widely accepted by now that microglial cells are involved in synaptic pruning. This process is essential for the development of well functioning neural circuits and impairment will result in disturbed functioning circuits. As a result of this, neurodevelopmental disorders like autism spectrum disorder (ASD) can be developed (5, 63, 64). To conduct this functions properly, the presence of microglial cells at the right timing and place is essential. Increased insight concerning the molecular mechanism in the migration pattern is essential to expand the fundamental principles before further steps regarding dysfunctioning of microglia during development can be attempted.

2 Material and methods

2.1 Cell culture

2.1.1 BV-2 cell line

The immortalized murine microglial BV-2 cell line was cultured in Dulbecco's Modified Eagle's medium (DMEM (D5796), Sigma, Saint Louis, MO, USA) supplemented with 10 % fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA) and 1 % penicillin-streptomycin (P/S, Invitrogen, Carlsbath, CA, USA) at 37° C in a humidified atmosphere with 5 % CO2. Confluent cell cultures were passaged by trypsin-EDTA treatment (T3924, Sigma) after rinsing with 1x Phosphate Buffered Saline (PBS, Lonza, Basel, Switserland). The trypsin reaction was stopped by the addition of supplemented DMEM. Cells were used until passage 10. Cells were placed on serumfree medium (DMEM 5796) supplemented with 1 % P/S overnight preceding an experiment.

2.1.2 Primary microglia

Primary microglia were obtained from brain isolation of C57BL/6 wildtype pups at postnatal day 2. Briefly, pups were decapitated and brains were isolated in ice-cold HBSS buffer (Gibco) supplemented with 7 mM HEPES buffer solution (Gibco). Brains were transferred to DMEM D5796 with 1 % P/S and kept on ice. Next, the tissue was titruated in 1 ml DMEM supplemented with 1% P/S using glass pipets, which were precoated with horse serum (HS, ThermoFisher, Massachusetts, USA) until a homogeneous suspension remained. The cell suspension was filtered with a 70 µm cell strainer and centrifugated for 5' (minutes) at 400G at 4°C. Cell pellets were resolved in DMEM supplemented with 10 % FBS, 10 % HS and 1 % P/S (DMEM 10.10.1) preheated at 37°c and seeded in poly-D-lysine (PDL, 20µg/ml, Sigma) precoated T175 flasks, at a concentration of 2 brains/flask. Cells were cultured for 7-10 days in DMEM 10.10.1 in a humidified atmosphere with 5 % CO_2 at 37° C. After 10 days, the medium was changed for fresh medium supplemented with 10 ml conditioned colony stimulating factor 1 (CSF-1) medium. CSF-1 was produced in our lab, as described below (Point 2.1.3 Colony stimulating factor 1). After 3-5 days, a shake-off was performed for 3 hours at 230 rpm at 37° C. After 3 hours, medium containing detached cells was collected and filtered with a 70 µm cell strainer. The cell suspension was centrifugated for 10' at 300G, resuspended in 37°C preheated DMEM 10.10.1, counted with trypan blue and seeded in a 24 well plate precoated with PDL at 2.10^5 cells/well or immediately used for experiments.

2.1.3 In-house Colony stimulating factor 1 production

The mouse fibroblast cell line L929 was cultured in DMEM supplemented with 10 % FBS, 1 % P/S, 1 % non-essential amino acids (NEAA, M7145, Sigma-Aldrich) and 1 % L-glutamine (G7513, Sigma-Aldrich). Cells were cultured in T175 as a confluent monolayer and excreted CSF-1. The medium containing CSF-1 was collected after 10-11 days of culturing, filtered with a 20 µm filter and stored at -20°C.

2.2 Animals

All protocols for animal experiments were conducted following the European Community guiding principles about 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 the Hasselt University in accordance with the guidelines of the Belgian Law and the European Council Directive. Animals used in the experiments were embryos obtained by mating transgenic Cx3cR1-eGFP knock-in mice (65) with C57BL/6 wild type females. The transgenic mice were obtained from the European Mouse Mutant Archive (EMMA) with the approval of Stephen Jung. In these knock-in mice, Cx3cR1 is exchanged for eGFP, hence all monocyte-derived cells of our heterogeneous animals, including microglial cells, express eGFP (65). In order to obtain primary microglia homogeneous wildtypes C57BL/6 mice were used.

Mice were mated overnight and vaginal plugs were checked every morning, considering the day of conception as embryonic day (E) 0.5. Mice were considered pregnant after the presence of a vaginal plug and weight gain of at least 3 grams after 10 days of pregnancy. Pregnant mice were sacrificed by means of cervical dislocation on the required day of pregnancy and embryos were isolated.

2.3 Cryosectioning and immunostaining

Embryonic brains of E13.5, E15.5 and E17.5 were decapitated in ice-cold PBS-glucose (pH 7.4, 25 mM) immediately after sacrifice of the mother by cervical dislocation. Heads were immediately transferred to 4 % paraformaldehyde (PFA) and fixed during 3 hours (E13.5-E15.5) or 5 hours (E17.5) at 4°C. Subsequently, heads were washed in PBS and cryoprotected overnight in PBS-30% sucrose at 4 °C. When the tissue had sunk, it was embedded in Frozen section compound 22 (Leica, Richmond, IL, USA), frozen in liquid nitrogen and stored at -80°C. Coronal sections of 10 and 20 µm were cut with a Leica CM3050S cryostat, mounted on Superfrost Plus glasses (ThemoFisher) and stored at -20°C until staining. Before staining, brain sections were thawed and washed 3x5’ with PBS. Next, sections were incubated overnight at 4°C with the CXCR4 antibody (1:100) (Anti-mouse, Alexa 647 coupled, 146503, Biolegend) in 0.05 % Triton X100 (Sigma). The next morning, sections were washed 3x10’ with PBS and incubated for 60’ with the secondary Goat-Anti Rat Alexa 647 labeled antibody (1:500) (714287, Invitrogen) in 0.05 % Triton X100 to enhance the signal. Subsequently, slices were washed 2x10’ with PBS and stained for 10’ with DAPI. Finally, slices were washed with PBS and distilled water. A coverslip was mounted using Dako Fluorescent mounting medium (Dako, Heverlee, Belgium). Slices without primary antibody were used to check unspecific binding of the secondary antibody, whereas slices without primary and secondary antibody were used to check for autofluorescense. For each age, slices of at least three different embryos (n=3) from at least three mothers (M=3) were stained. All steps were conducted in the dark as much as possible.

2.4 Wide field fluorescence microscopy

Images of the immunostainings were obtained using the Nikon Eclipse 80i microscope, coupled to the Digital sight DS-2MBWc fluorescence camera. Pictures were made with a Nikon Plan Fluor 20x/0.5 objective and Nikon Plan Fluor 40x/0.75 objective in Nis-Elements 4.00.06 software. 40x pictures were used to quantify the amount of positive cells. Cells were considered positive in case a clear deposition was visible corresponding to the microglia cell surface. Three pictures per cortex were

made in at least six slices per embryo. Pictures were further modified for brightness and contrast and analyzed in Fiji (NIH, USA; http://fiji.sc/#download).

2.5 Adhesion assay

Cells were maintained overnight on DMEM 5796 with 1 % P/S, from now on referred to as serumfree medium. The next morning cells were harvested by trypsinisation, centrifugated for 10' at 400G, washed with PBS, centrifugated and resuspended in serumfree medium. A flat bottom-96 well plate was uncoated or precoated during 60' at 37 °C with fibronectin (10 µg/ml, Sigma), ICAM-1 (10 µg/ml, Sino Biological, Beijing, China) or 1 % BSA. Wells were washed twice with PBS after coating. Cells were seeded at a concentration of 5.10^4 cells in 100 µl serumfree medium. The assay was performed whether or not in the presence of SDF-1α or Cxcl12 (100 ng/ml) (Peprotech, Rocky Hill, NJ, USA) in combination with either AMD3100 (4 µM, A5602, Sigma), a ß1 blocker (5 µg/ml, 102210, Biolegend, Fell, Germany) or ß1 isotype antibody (5 µg/ml, 400916, Biolegend). Cells were allowed to attach for 6 hours. Non-adherent cells were washed away with PBS, adherent cells were fixed for 5' with 4 % PFA. After 2' staining with 0.05 % crystal violet, pictures were taken at 4x magnification with the Zeiss Primovert microscope and Axiocam camera. Pictures were tresholded using the Default threshold in Fiji and the mean grey value of the total picture was calculated using the measure function in Fiji. Grey values were calculated relative to adhesion to the fibronectin coating condition, set as 1.0 adhesion. The assay was performed in triplo (n=1).

2.6 Transwell migration assay

2.6.1 BV-2 cells

Cell migration assays were performed using Corning transwell membrane filters (8 µm pore size, Corning Costar, New York, USA). BV-2's were placed overnight on serumfree medium and harvested the next morning as described before. If mentioned, inserts were precoated with fibronectin (10 µg/ml) for 1 hour at 37 °C, before the start of the assay. After 60' inserts were washed twice in PBS and placed in a 24 well plate, containing 400 µl serumfree medium. Cells were seeded at a density of 1.10^5 cells/insert in 100 µl serum free medium with or without blockers and isotypes. Cells were preincubated for 30' with the following blockers or the accompanying isotype: AMD3100 (4 µM), ß1 blocker (5 µg/ml), ß1 isotype (5 µg/ml), ß2 (5 µg/ml), ß2 isotype (5 µg/ml), LY294002 (10 µM, Sigma) or U0126 (10 µM, Sigma). After 30', serumfree medium in the bottom well was changed for medium containing the chemoattractant SDF-1α (100 ng/ml). Cells were allowed to migrate for 6 hours at 37°C in a humidified atmosphere with 5 % CO_2. Thereafter, cells were fixed for 5' in 4 % PFA, washed with PBS and stained with for 2' with 0,05 % crystal violet. Migrated cells were present at the bottom side of the insert. Upper sides of the inserts were cleaned with cotton buds. From each insert pictures were taken on 20 x magnification in black and white modus at three different locations on the insert using a Zeiss Primovert microscope. Pictures were tresholded using the Default threshold in Fiji and the mean grey value of the total picture was calculated using the measure function in Fiji. Grey values were calculated relative to migration in the negative control. Migration assay were performed in duplo unless stated otherwise. From each insert three pictures were made,

resulting in a total of six pictures/condition, considered as n=1. N=3 in uncoated conditions, n=6 on fibronectin coating. The following transwells (n=5 for ß2, ß2 isotype, LY294002 and U0126) were performed in mono.

2.6.2 Primary microglia

Migration assays with primary microglia were performed similarly as described above, microglia were seeded immediately after shake-off on inserts precoated with fibronectin (10 µg/ml) in DMEM (D5796) supplemented with 10 % FCS, 10 % horse serum (HS) and 1 % P/S. Migration assays were conducted for 24 hours at 37°C in a humidified atmosphere with 5 % CO_2. Migration to the bottom of the insert was analyzed as described above by measuring the mean grey value considering the negative control as 100 % migration. From each insert three pictures were made, resulting in a total of six pictures/condition, considered as n=1. Each experiment was performed in duplo and repeated 4 times (n=4).

2.7 Flow cytometry

2.7.1 BV-2

BV-2 cells were seeded overnight in a 24 well plate precoated with 1 % BSA, fibronectin (10 µl/ml), ICAM-1 (10 µl/ml) or uncoated at a density of 2.10^5 cells/well in serumfree medium. The next morning, cells were stimulated for 2 hours with SDF-1α (100 ng/ml), in combination with the following blockers; AMD3100 (4 µM), LY294002 (10 µM) or U0126 (10 µM). Next, cells were fixed for 20' with 4 % PFA. After fixation, cells were detached by scraping the bottom of the plate with a 200 µl yellow tip and transferred to a 96 well plate. Cells were washed with PBS and centrifugated for 5' at 400 G, 4°C. The cell pellet was stained for 25' at room temperature (RT) with the primary 9EG7 antibody 50 µl/well (1:1000, 553715, BD) or isotype control (Rat IgG2a) in FACS buffer (PBS, 2% FCS, sodium azide). Cells were stimulated for 2 hours with RGD (1mM, Sigma) as a positive control) or 30' with Ca^{2+} (12.5mM) as a negative control. After 25' incubation cells were centrifugated, washed with FACS buffer, centrifugated and incubated for 15' with the secondary antibody (1:600, Goat-anti rat Alexa 488, 11006, Life Technologies) at RT in FACS buffer (50 µl/well). After a wash step, cells were stained for 30' on ice with the monoclonal anti-mouse antibodies CD29 and CD184 (table 1) in FACS buffer (PBS, 2% FCS, sodium azide) (50 µl/well). Finally, a wash step was performed and the pellet was resolved in FACS buffer. Cells were analyzed with the FACS Aria II and the FACS Diva 6.1.3 software (BD Biosciences). N=number of experiments performed in mono.

2.7.2 Primary microglia

Primary microglia were seeded after shake-off on a 24 well plate, precoated with fibronectin (10 µg/ml) or Poly-D-lysine (5 µg/ml). Cells were stimulated for 2 hours with 100 ng/ml SDF-1α. After removing the medium, cells were fixed for 20' on ice with 4 % PFA. Next, cells were detached with a 200 µl yellow tip and transferred to a 96 well plate. Cells were stained with the aforementioned antibodies and analyzed with the FACS Aria II.

2.7.3 Whole embryonic brain suspension

Embryonic brains were isolated at embryonic day 13.5 or 17.5. Briefly, embryos were decapitated, the skin, skull and meninges were removed and cortici were isolated in ice-cold PBS-glucose. The tissue was transferred to neurobasal medium (Gibco 12348, Life Technologies) supplemented with 2mM L-glutamine, N2 supplement, B27 supplement and 1 % P/S (all from Invitrogen) and kept on ice. Next, the tissue was incubated for 30' in medium containing papaïne (48 U/ml, Sigma) at 30°C and gently shaken each 5'. Next, the tissue was mechanically dissociated with a 1000 µl pipet and centrifugated during 5' at 400G. The pellet was resuspended in 30 % Percoll (GE Healthcare, Little Chalfont, United Kingdom) and centrifugated for 10' at 700G without brake. Next, the cell suspension was resuspended in PBS and filtered through a 35 µm cell strainer. Cells were counted and fixed in 4 % PFA at a concentration of 1.10^5cells/ml on ice during 20'. After fixation, cells were washed with 1x PBS and transferred to a v-bottom shaped 96 well plate. Cells were incubated for 25' at room temperature with the antibody cocktail mix in FACS buffer (50 µl/well) as described in table 1. Next, cells were washed with FACS buffer and transferred to FACS tubes in 150 µl FACS buffer. M= number of mothers, tissue of two embryos was combined as N=1, events were considered as n.

Table 2: Antibodies and accompanying isotypes used for flow cytometry.

Antibody	Fluorescent label	Dilution BV-2	Dilution embryo	Company AB & isotype	Cat. nr.	Isotype	Cat.nr. Isotype
9EG7		1:1000	1:250	BD	553715	Rat IgG2a	553922
CD29 (β1)	APC.Cy7	1:1000	1:250	Biolegend	102225	Ham IgG C	400927
CD184 (CxcR4)	Alexa Fluor 647	1:100	1:25	Biolegend	146503	Rat IgG2b	400626

2.8 Time lapse imaging

Pregnant mice were sacrificed by cervical dislocation at E13.5, E15.5 or E17.5. Embryos were decapitated and brains were isolated in ice-cold PBS-glucose (pH 7.4, 25 mM), paying special attention to not harm the telencephalon. Next, the dissected brains were embedded in 4 % low melting point agarose with the olfactory bulb pointing downwards, manipulating the positioning with the rhombencephalon. The embedded tissue was placed on ice for 15' to complete agarose polymerization. Coronal slices at a thickness of 300 µm were made by the use of a Leica VT1200S vibratome. Slices were transferred to MilliCell organotypic inserts (Merck Millipore, Massachusetts, USA) in a 24-well plate and maintained in semidry conditions at 37°C and 5 % CO2 in neurobasal medium (Gibco) supplemented with 2mM L-glutamine, N2 supplement, B27 supplement and 1 % P/S (all from Invitrogen). Medium was supplemented with AMD3100 (40 µM), LY294002 (20 µM), U0126 (20 µM) or ß1 blocking antibodies (10 µg/ml). The tissue was able to equilibrate for 30' at 37° C and 30' during the set up of the time lapse movies before imaging and the slices were rotated with the pia towards the top of the imaging field. During the complete imaging period, the microscope chamber was provided with humidified air with 5 % CO2 at 35°C. The eGFP-positive microglia were excited by the Mai Tai DeepSee Ti:Saphire pulsed laser (Spectra-Physics) with a wavelength of 900 nm and visualized with a BP 495-545 nm filter. Z-stacks of 30 µm, with serial optical sections every 3 µm, were recorded every 10' for a total duration of 6 hours, starting from a minimal depth of 50 µm beneath the surface of the slice. A 20x EC plan-Neofluar objective (NA of 0.5 and 2 mm working

distance) (Zeiss), equivalent to a field of vision of 450 x 450 µm, was used to record the time lapse series. Displacement of eGFP-positive microglia, was tracked manually using the ImageJ plugin MTrackJ developed by Meijering et al. (66). Migration speed (µm/h) was calculated as the total length of the traveled path divided by the duration of the track. The average migration speed was calculated from all cells in slices with the same treatment, considering n=the number of cells, M= number of mothers.

2.9 Statistics

Statistical analysis were performed using GraphPad Prism (version 6.2) or SAS JMP PRO 12 for time lapse imaging. All values were shown as mean ± standard error mean, with n as the number of experiments or cells if indicated. Normality was tested in all experiments. Multiple comparison Kruskal-Wallis tests were performed to compare different groups. In case isotype controls were used, Mann-Withney tests were performed. P-values smaller than 0.05 were considered as significantly different. Asterisks were used to indicate the level of significance * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3 Results

The goal of this project was to reveal if microglial cells migrate towards Cxcl12 using β1 integrins during embryonic development in mice. The first aim was to confirm the presence of the receptor for Cxcl12 on microglial cells, namely CxcR4, by immunohistochemistry and flow cytometry. Next, we investigated if microglial cells migrate towards Cxcl12 through the use of β1 integrins. To this end, *in vitro* transwell migration assays and *ex vivo* time lapse imaging experiments in embryonic brain slices were performed. Additionally, the active conformation of integrin β1 analyzed upon Cxcl12 stimulation. Finally, the involved intracellular pathway was determined in migration assays and in embryonic brain slices at embryonic day (E) 13.5 and 17.5.

3.1 The CxcR4 receptor is present on microglial cells

The first aim to verify our hypothesis was to check if microglial cells had the possibility to migrate towards Cxcl12. Therefore, the presence of the desired CxcR4 receptor on microglial cells in the embryonic brain was required. Arno et al. were the first to demonstrate expression levels at E15.5, showing approximately 13 % CxcR4 positive microglia in the cortex (15), expression levels of other embryonic ages remained unrevealed until now. To determine expression levels on other embryonic ages, immunohistochemistry was performed on brain slices. Embryonic brain slices of E13.5-E15.5-E17.5 containing eGFP positive microglia were stained for CxcR4 (M=3, n=3) (Figure 3). At E13.5 and E15.5, respectively 36.2 ± 2.5 and 35.3 ± 5.2 % of the total amount of microglial cells present in the cortex expressed CxcR4 (Table 3). At E13.5 the average number of microglial cells in the cortex was 18 cells/cortex while at E15.5 this was 28 cells/cortex. This increase in cells is due to the migration waves described in Swinnen et al. (67). At E17.5 the average amount of microglial cells increased towards 38 cells/cortex, whereas the level of CxcR4 positive cells decreased towards 16.5 ± 1.6 %.

To verify our immunostainings, expression levels of microglial CxcR4 were analyzed in dissociated whole embryonic cortices tissue at E15.5 and E17.5 by flow cytometry (M=1, table 3). On E15.5, CxcR4 (N=3, n= ± 500) was expressed on 16.6 ± 2.4 % of the microglial cells in the cortex. At E17.5 these expression levels decreased towards 8.3 ± 4.9 % (N=2). However, variation is substantial between embryos at E17.5 (13 % (n=192) and 3.1 % (n=1410)).

Table 3: Percentage CxcR4 positive microglial cells at different embryonic ages. Values are mean ± SEM.

	Immunohistochemistry (%)	Flow cytometry (%)
E13.5	36.2 ± 2.5	
E15.5	35.3 ± 5.2	16.6 ± 2.4
E17.5	16.5 ±1.6	8.3 ± 4.9

Furthermore, CxcR4 expression is described on both the BV-2 cell line and primary microglia (15). In addition, we demonstrated using flow cytometry that 93 ±1.5 % of the BV-2 cells (n=5) and 82.2 ± 3.8 % of primary microglial cells (n=2) showed CxcR4 expression (Table 4). Also a relative

upregulation of CxcR4 (1.1 ± 0.1, n=2) and β1 expression (1.1 ± 0.0, n=2) was showed upon stimulation with Cxcl12 on 1 % BSA coating by flow cytometry.

Table 4: Levels of CxcR4 positive BV-2 and primary microglial (PM) cells.
Values are mean ± SEM.

	CxcR4 positive microglia (%)
BV-2	93.0 ± 1.5
PM	82.2 ± 3.8

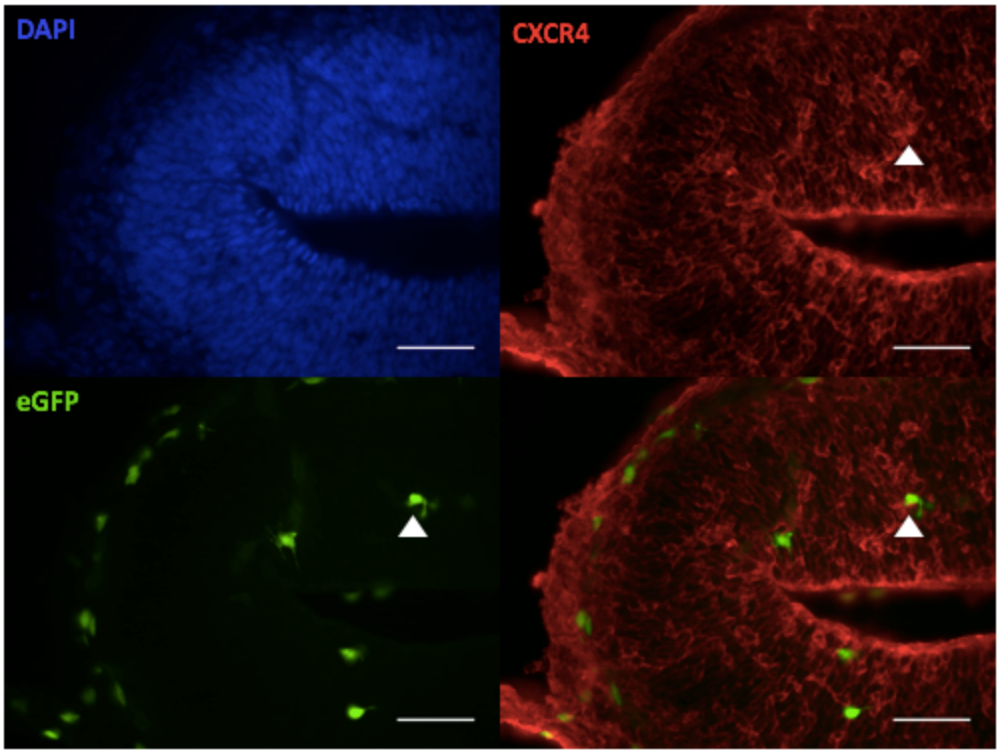

Figure 3: CxcR4 expression on embryonic brain slices of E13.5. Microglial cells are eGFP fluorescent. CxcR4 staining in red and DAPI in blue. CxcR4 expression on microglial cell is marked with arrows. Scale bar is 50 µm.

3.2 Microglial cells migrate towards Cxcl12 upon CxcR4 binding

After confirming the presence of the CxcR4 receptor, migration of microglial cells towards Cxcl12 was investigated by different methods. First of all, transwell migration assays were performed in the presence of Cxcl12 using the immortalized murine microglial BV-2 cell line and primary microglia. Migration towards Cxcl12 was analyzed in the presence of different blockers. To validate the accuracy of the transwell migration assay and to check if differences in migration were not related to decreased adhesion by the blockers, adhesion assays were performed as well.

Transwell migration assays with BV-2 cells were performed in the presence of Cxcl12. Migration without chemoattractant Cxcl12 was used as control considering migration levels as 1.0. On non-coated inserts (n=3), BV-2 cells showed a basal migration that was neither increased when cxcl12 (1.1 ± 0.1) was present nor decreased when AMD was added (1.0 ± 0.1) (Figure 4B). Subsequently, migration was analyzed on fibronectin coated inserts. The presence of Cxcl12 (n=6) increased the level of migrated cells to 1.6 ± 0.2 compared to control (Figure 4C). Administering the CxcR4 blocker,

AMD3100, declined migration towards 0.9 ± 0.1. Thus, in the presence of fibronectin, Cxcl12 resulted in significantly increased migration ($p<0.01$), whereas AMD3100 reduced migration significantly towards control levels ($p<0.001$).

Transwell migration assays were also performed with primary microglia. In case inserts were not precoated with fibronectin, cells did not migrate at all after 24 hours (data not shown). On fibronectin coating (n=4), an increased migration of 1.8 ± 0.5 towards Cxcl12 was observed (Figure 4D), whereas the AMD3100 blocker decreased migration towards 1.1 ± 0.3. Because of high variation levels, the significant effect in BV-2 cells could not be confirmed in primary microglia.

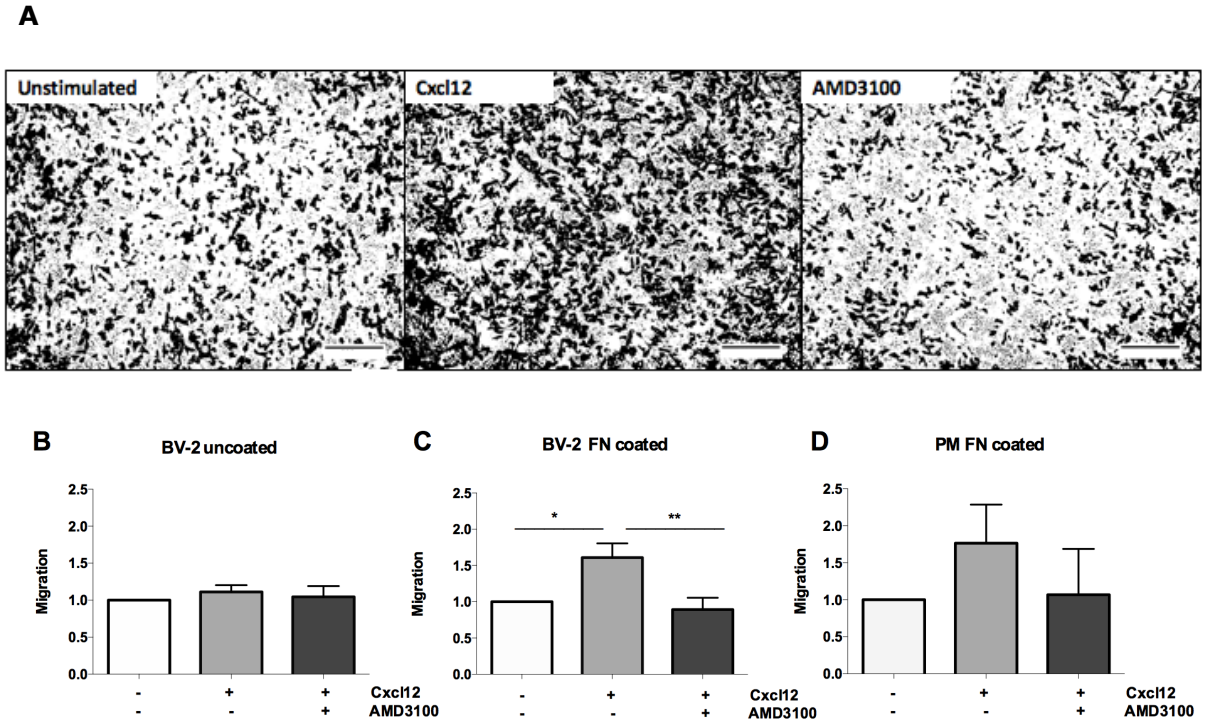

Figure 4: Cxcl12 attracts microglial cells in the presence of fibronectin. BV-2 cells or primary microglia (PM) were incubated on uncoated or fibronectin (FN) coated inserts in the presence of Cxcl12 and AMD3100. Migration in the negative control (unstimulated) was considered as 1.0. **(A)** Pictures of migrated BV-2 cells on the bottom side of the filter in different conditions; unstimulated, Cxcl12, Cxcl12 and AMD3100. **(B)** BV-2 cells were seeded on uncoated inserts (n=3). Migration was not significantly different. **(C)** BV-2 cells were seeded on inserts precoated with fibronectin (n=6). In the presence of Cxcl12, migration increased significantly compared to control, while administration of AMD3100 reversed migration significantly. **(D)** Primary microglial cells (n=4) were seeded on fibronectin coated inserts. In the presence of Cxcl12 migration increased while administration of AMD3100 returned migration to control levels. Migration was not significantly different. (*, $p<0.05$; **, $p<0.01$). Values are mean ± SEM. Scale bar is 300 µm.

3.3 CxcR4 and β1 blockers do not affect adhesion

By performing migration assays we aimed to verify whether blockers do not affect adhesion. In case the administered blockers induce decreased adhesion, we can not conclude if decreased migration in transwell assays is not a consequence of decreased adhesion. Cells were incubated on fibronectin coating in the presence of Cxcl12 in combination with AMD3100, which is a CxcR4 blocker, β1 blocking antibodies or isotype control (Figure 5). 1% BSA was used as a negative control. Adhesion was compared to unstimulated cells on fibronectin coating, set as 1.0. Adhesion on different coatings resulted in the following values; 1 % BSA (n=3) 0.26 ± 0.09, uncoated (n=3) 1.01 ± 0.14, FN 1.00 ± 0.00, Cxcl12 0.88 ± 0.08, AMD3100 0.82 ± 0.10, β1 0.78 ± 0.09, isotype β1 0.85 ± 0.13 (all

n=4). Except for 1 % BSA ($p<0.001$) no significant difference was present, indicating that decreased migration levels in our migration assays are not due to an effect on adhesion.

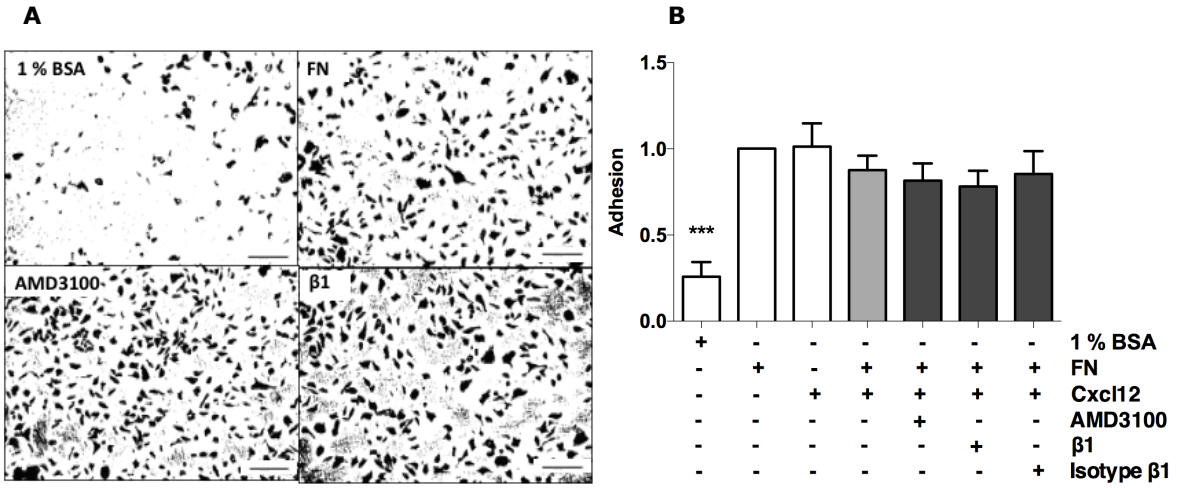

Figure 5: CxcR4 and β1 blockers do not affect adhesion. BV-2 cells were incubated with Cxcl12 in combination with AMD3100, a β1 blocker or isotype control. Wells were precoated with fibronectin (FN) unless stated otherwise. The fibronectin coated condition was considered as 1.0. **(A)** Pictures of different conditions after fixation. 1 % BSA, fibronectin and fibronectin with respectively the AMD3100 and a β1 blocker. The mean grey value was measured per picture and was used to calculate relative adhesion. **(B)** Relative adhesion. All conditions were compared to unstimulated cells on fibronectin. Adhesion was not significantly different, except for the negative control. Values are mean ± SEM. (*** $p<0.001$.) Scale bar is 300 µm.

3.3 Migration towards Cxcl12 is β1 dependent

To functionally validate the dependency on integrin β1 in Cxcl12 directed migration, migration assays were performed using β1 and β2 blockers (n=5). Indeed, administering β1 blockers decreased migration towards control levels (0.9 ± 0.2) while the isotype control did not cause any effect (2.0 ± 0.1) (Figure 6A). To confirm whether Cxcl12 induced migration was solely dependent on β1, migration was analyzed in the presence of a β2 blocker. The presence of β2 blockers (1.6 ±0.2) and the β2 isotype (2.3 ± 0.1), did not significantly affect migration towards Cxcl12 (Figure 6A), confirming the β1 dependency.

3.4 Migration towards Cxcl12 is PI3K and MEK1/2 dependent

The link between Cxcl12 and integrin β1 has been described multiple times in cancer cells, including the intracellular pathway involving PI3K and MEK1/2 (3, 4). To validate if this pathway regulates migration towards Cxcl12 in microglial cells, blockers acting upon PI3K and MEK1/2, respectively LY294002 and U0126, were administered in transwell migration assays (n=5). Indeed, the presence of the intracellular pathway blockers attenuated the migration towards Cxcl12 significantly, indicating the involvement of this pathway (Figure 6B). LY294002 resulted in decreased migration levels of 0.8 ± 0.1 whereas U0126 resulted in 1.0 ± 0.1.

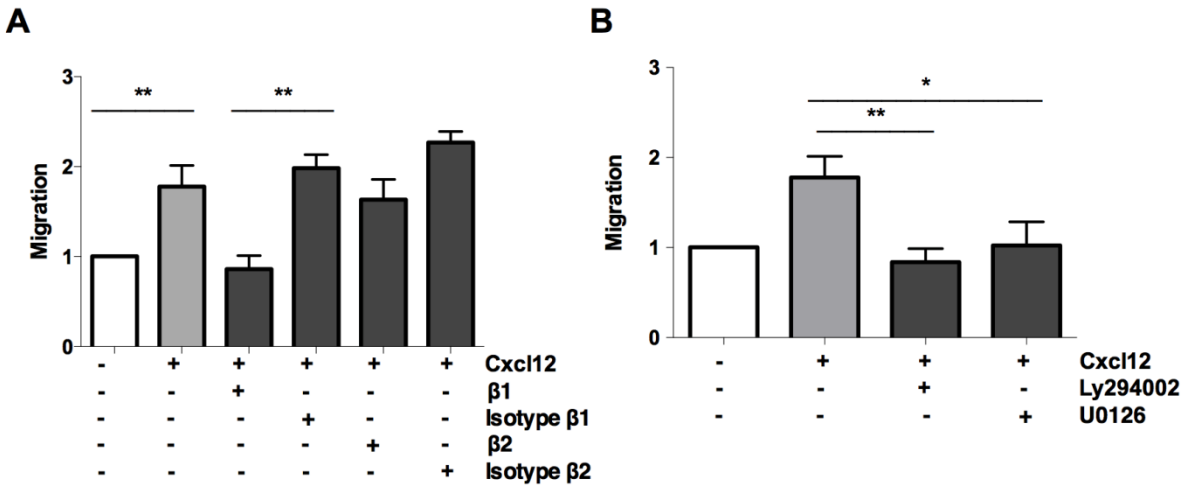

Figure 6: β1, PI3K and MEK1/2 blockers affect migration towards Cxcl12. BV-2 cells were seeded on fibronectin coated inserts in the presence of Cxcl12 and different blockers. **(A)** β1 blockers reduced migration significantly whereas β2 blockers did not affect migration (p=0.056). **(B)** Treatment with the PI3K blocker LY294002 and the MEK1/2 blocker U0126 both resulted in decreased migration compared to Cxcl12. (*, p<0.05), (**, p<0.01). Values are mean ± SEM.

3.5 Cxcl12 induces the active conformation of integrin β1

The link between Cxcl12 and integrin β1 is described in multiple cancer cells, in which Cxcl12 will stimulate migration and adhesion by affecting integrin β1 (41, 62, 68). Furthermore, integrins can adopt different conformations, related to their activation or affinity state (45). In the active conformation, heterodimers of the integrin change from a bended towards a stretched position. The 9EG7 antibody which recognizes the 9EG7 epitope, only exposed in the active conformation, enabled us to observe an increase or decrease in the levels of active conformation (69). By means of flow cytometry we analyzed the active conformation upon Cxcl12 stimulation and we aimed to verify whether Cxcl12 is able to induce the active conformation of integrin β1 in microglial cells. The experiment was performed on different coatings, enabling us to analyze the effect of the different coatings, and the associated binding of the cells, on the levels of active conformation.

Adhesion of the cells on the different coatings was determined by an adhesion assay. BV-2 cells were allowed to adhere on an uncoated surface, fibronectin, ICAM-1 or 1% BSA coating (n=1) (Supplementary (S) Figure 1). Fibronectin interacts with β1 while ICAM-1 interacts with β2 (44). Relative values were calculated, considering the uncoated condition as 1.0. Adhesion increased 1.2x on fibronectin coating. ICAM-1 coating and 1 % BSA resulted respectively in adhesion levels of 0.1x and 0.3x. Next, the active conformation levels of integrin β1 were compared upon seeding on the different coatings (n=1). The median fluorescence intensity (MFI) or expression levels of the active conformation reached absolute levels of 615 on the uncoated surface while expression increased towards 905 (x1.5) on fibronectin coating and decreased towards 345 (x0.6) on ICAM-1 coating (Figure S2).

For the effective detection of the active β1 conformation, cells were stimulated with Cxcl12 in combination with AMD3100 on the aforementioned coatings. Stimulation with RGD was described to induce the active β1 conformation and used as a positive control (69). The MFI value of unstimulated cells was considered as 1.00. On all coatings, levels of active β1 conformation increased upon Cxcl12 stimulation compared to unstimulated cells, while the CxcR4 blocker mainly reversed this effect.

Cxcl12 stimulation on an uncoated surface (n=1, Figure 7A), resulted in a 4.0x increase of the MFI compared to control, whereas CxcR4 blockage returned levels towards 2.6x. When cells were seeded on fibronectin, which resulted in strong adhesion (Figure S1), the active conformation levels rose 2.5x upon Cxcl12 stimulation compared to unstimulated cells (Figure 7B). CxcR4 blockage resulted in a decrease towards 2.2x. Likewise, the active conformation was analyzed after stimulation on ICAM-1 coating, on which cell adhesion was weak (Figure S1). Stimulation with Cxcl12 resulted in a 6.0x increase of the active conformation, and CxcR4 blockage reduced levels towards 0.8x. Additionally, this experiment was repeated on 1 % BSA (n=2) (Figure 7D). Next to a positive control (RGD stimulation), a negative control in which Ca^{2+} will inhibit the inducement of the active conformation, was included following description in Bazonni et al (69). Since cells adhere weakly on 1 % BSA (Figure S1), we expected the negative control to be equal to the unstimulated condition. The negative control resulted even in levels of 0.7x compared to the unstimulated condition. The presence of Cxcl12 increased the levels 1.4x, while administration of AMD3100 decreased the active conformation towards 1.1x. Additionally, the effect of blockage of PI3K and MEK1/2 on the active conformation was assessed. The intracellular pathway blockers for PI3K - LY29004 and MEK1/2 - U0126 resulted in respectively 1.2x and 1.1x levels of active conformation, suggesting this pathway is involved in the regulation of the active β1 conformation. However, sample size was not high enough to perform statistics.

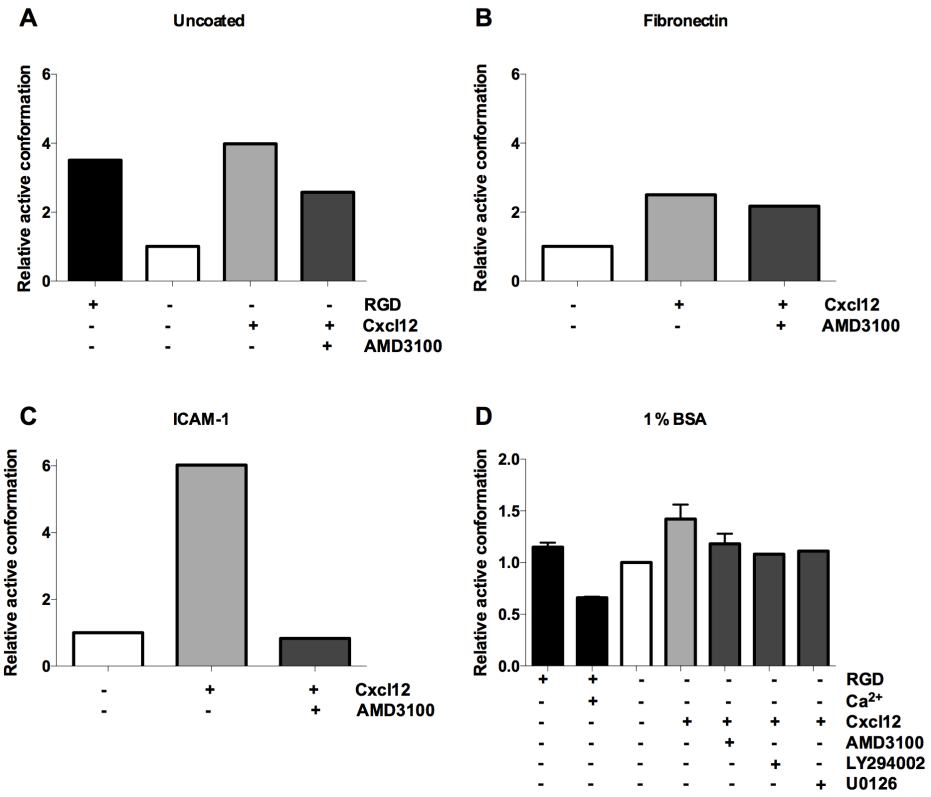

Figure 7: Cxcl12 induces the active conformation of integrin β1. BV-2 cells were stimulated with Cxcl12 on different coatings in the presence of AMD3100, LY294002 or U0126. RGD was used as a positive control, Ca^{2+} as a negative control. Relative MFI values were compared to the MFI of unstimulated cells. In all conditions Cxcl12 resulted in an increased active conformation. Levels decreased upon AMD3100 administration. **(A)** Uncoated (n=1). Cxcl12 induces the active conformation to similar levels as RGD. **(B)** Fibronectin coating (n=1). **(C)** ICAM-1 (n=1) **(D)** 1 % BSA (RGD, Cxcl12 and AMD3100 n=2, all others n=1). RGD induces the active conformation, this effect is reversed upon Ca^{2+} administration. AMD3100, LY294002 and U0126 all decreased the active conformation compared to Cxcl12 stimulation.

3.6 In situ time lapse imaging of microglia

We showed *in vitro* migration of microglial cells towards Cxcl12, dependent on integrin β1. Also the involvement of PI3K and MEK1/2 in the intracellular pathway was suggested. To confirm our results in more physiological conditions, the migratory behavior of microglial cells on different embryonic ages *ex vivo* was examined. By the use of Cx3cR1 transgenic mice, the migratory behavior of microglial cells was studied in embryonic brain slices. For this purpose, we used 300 µm-thick coronal brain slices from heterozygote Cx3cR1 transgenic embryos of embryonic day (E) 13.5 and E17.5 and recorded microglial migration inside the slice during 6 hours by means of multi-photon excitation microscopy. The brain slices were incubated with different blockers to analyze the effect on migration. The analysis was performed on the area of the cerebral cortex located dorsally to the lateral and medial ganglionic eminences. The migratory tracks of the microglial cells were analyzed by MTrackJ in ImageJ and the resulting migration speed was calculated. N is the number of tracked cells. Representative migration trackes are presented in Figure 8A.

Previous experiments in our lab determined the migration speed in control conditions at E13.5 and E17.5. Shown values are median ± SEM. At E13.5 the median migration speed was 33.5 ± 1.3 µm/h (n=157) (Figure 8B), while at E17.5 this speed decreased until 24.2 ± 0.8 µm/h (n=350) (Figure 8C). At E13.5 the presence of a β1 function blocking antibody decreased migration speed from 33.5 ± 1.3 µm/h (n=157) in control conditions towards 19.1 ± 1.2 µm/h (n=82). In contrast, at E17.5 migration speed increased upon the presence of the β1 blocker from 24.2 ± 0.8 µm/h (n=381) towards 26.4 ±1.1 µm/h (n=211). We wanted to confirm the effect of integrin β1 in migration by the blockage of other factors involved in the Cxcl12-β1 pathway, in particular CxcR4 and the intracellular PI3K and MEK1/2.

3.6.1 Migration speed decreases at E13.5 following β1, PI3K and MEK1/2 blockage

At E13.5 slices were incubated in the presence of the CxcR4 blocker (40 µM AMD3100). Migration speed in the CxcR4 blocker conditions did not differ significantly from control; the mean migration speed in the presence of the CxcR4 blocker remained 29.5 ± 2.4 µm/h, compared to 33.5 ± 1.3 µm/h in control conditions (Figure 8D). Additionally, slices were incubated with the LY294002 and U0126 blocker, counteracting the activation of PI3K and MEK1/2, respectively. Application of LY294002 resulted in a median migration speed of 16.9 ± 1.5 µm/h whereas U0126 resulted in a speed of 19.0 ± 1.6 µm/h. Both blockers significantly reduced the migration speed at E13.5 (***, $p<0.001$).

3.6.2 Migration speed increases at E17.5 following β1 but not upon CxcR4, PI3K and MEK1/2 blockage

At E17.5 the migration speed in control condition had decreased towards 24.2 ± 0.8 µm/h (n=379). In the presence of the CxcR4 blocker migration speed remained 22.8 ± 1.5 µm/h (n=95), whereas LY294002 and U0126 resulted respectively in 18.8 ± 1.3 µm/h (n=96) and 27.0 ± 1.8 µm/h (n=98) (Figure 8E). Only a significant decline was present in the LY294002 condition (***, $p<0.001$).

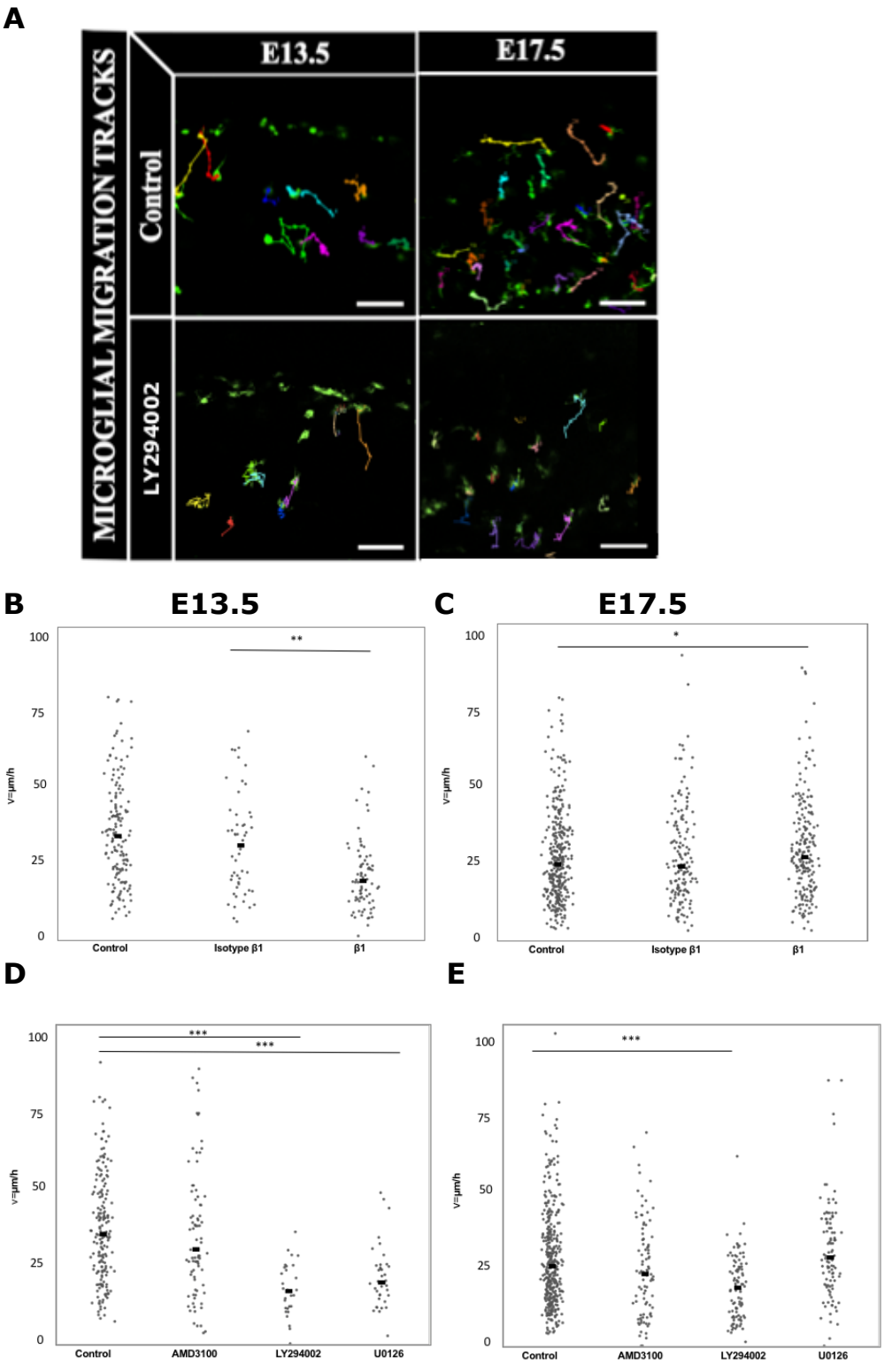

Figure 8: Migration speed differs upon β1, PI3K and MEK1/2 blockage. (A) Tracked cells in ImageJ at E13.5 and E17.5 in control condition and upon LY294002 treatment. Tracks are recorded by manual cell tracking with MTrackJ. Scale bar is 100 µm. **(B)(D)** E13.5. Migration speed decreases significantly upon β1 treatment compared to isotype β1. Treatment with LY294002 and U0126 both resulted in significantly decreased migration compared to control. AMD3100 did not have an effect on migration speed **(C) (E)** E17.5. β1 blockage resulted in increased migration speed compared to control. LY294002 resulted in a significantly decreased migration speed while U0126 did not have an effect, neither did AMD3100. P<0.05 (*), p<0.01 (**), p<0.001 (***). Dots are individual mean migration speed, ▬ =median migration speed.

4 Discussion

Despite the progress of research into migration of microglial cells during development, the molecular mechanisms involved in this migration remain still unknown. This gap in knowledge, in combination with other recent publications and our preliminary data, made us wonder about mechanism involved in this migration. These facts led us to hypothesize that "**migration of microglia in embryonic development is stimulated by the Cxcl12/CxcR4/β1 axis.**" In this present study, we showed that microglial cells express the desired CxcR4 receptor and migrate towards Cxcl12 in the presence of fibronectin. By performing migration assays, we showed the dependency on integrin β1. Moreover, we gained insight in the involved intracellular pathway and the migratory behavior of microglial cells on different embryonic ages.

It has been reported previously that CxcR4 is expressed on microglial cells (70). However, expression levels of this receptor during embryonic development has never been examined. Thus, expression of CxcR4 was analyzed on different time points during embryonic development. CxcR4 has shown to be essential in multiple developmental processes like neurogenesis, cardiogenesis and angiogenesis (35). Arno et al. were the first to determine expression of CxcR4 on microglial cells at the age of E15.5 by analysis of flow cytometry (15). Their analysis showed expression of CxcR4 on 13 % of the embryonic microglial population, corresponding to our flow cytometry results at E15.5, namely 16.7 %. We further performed immunohistochemical staining on brain slices of embryonic days 13.5, 15.5 and 17.5 and manually quantified the percentage of positive microglia. Microglial cells located in the neocortex and in the proximity of the ventricular zone were analyzed regarding the expression pattern of Cxcl12 and the migration pattern of microglial cells (15, 67). Expression levels of CxcR4 varied notably during development; immunostainings of E13.5 and E15.5 showed the presence of CxcR4 on approximately 36.2 and 35.3 % of the cells, whereas this expression decreased towards 16.5 % at E17.5. For the reason that flow cytometry is a more accurate technique as immunohistochemistry and the cell count is substantially higher in flow cytometry, we are tempted to consider our flow cytometry results as more reliable. Additionally, CxcR4 is expressed in neuronal tissue during embryonic development and therefore creates a lot of background in our immunohistochemical staining (67), making the immunostainings less clear. However, both techniques indicate a decrease in the CxcR4 expression during embryonic development. A possible explanation for the observed decline in expression levels can be found in the microglial migration pattern and migration speed during this time period. Swinnen et al. described three different phases in the microglial colonization of the brain; while invasion of the brain starts at E10.5, the majority of the brain is colonized at E17.5 (67). This indicates that the majority of the cells has arrived at their desired location and eventually downregulate the receptor to remain at their provided location. This idea is supported by our data which show a decreased migration speed from E13.5 (33.5 μm/h) towards E17.5 (24.2 μm/h). It is described that the CxcR4 receptor can be upregulated again upon activation of microglial cells (70). However, it should be investigated whether a link is present between cells with higher expression levels and their migration speed. Although, mRNA expression of CxcR4 is studied in mice starting from E9.5 (71) and the presence of Cxcl12 is demonstrated in the subventricular and ventricular zone of the embryonic brain at E16.5 (15), the exact expression pattern of Cxcl12 on the other embryonic ages is not defined in mice. An increase in the expression

levels of CxcR4 has been found on microglial cells upon Cxcl12 stimulation *in vitro*. Unraveling a possible link between the exact Cxcl12 expression pattern on different embryonic ages and the CxcR4 expression, and eventually the correlation with migration speed would be elucidating.

After confirming the presence of the desired receptor, we determined whether microglial cells migrate towards Cxcl12 by the use integrin ß1. Transwell migration assay with the microglial BV-2 cell line showed indeed increased migration towards Cxcl12 in the presence of fibronectin. Remarkably, in the absence of fibronectin, Cxcl12 did not induce increased migration of BV-2 cells. Since integrin ß1 interacts with fibronectin, migration along fibronectin is the first indication of ß1 involvement. Blockage of the CxcR4 receptor by AMD3100 restored migration to control levels, indicating that interaction of Cxcl12 is completely regulated via CxcR4. This in contrast to earlier reports mentioning CxcR7 as an alternative receptor (70). However, effects induced by the activation of CxcR4 or CxcR7 were abrogated upon CxcR4 blockage, indicating CxcR7 can not compensate for the loss of CxcR4 (70). Primary microglia seeded on uncoated inserts did not migrate at all. In contrast, migration of primary microglia on fibronectin coated inserts towards Cxcl12 seemed to result in increased migration levels, abolished by administering the CxcR4 blocker. However, due to low sample size and considerable variation levels, results were not significantly different. All these results imply the necessity of fibronectin for migration towards Cxcl12 and are the first indication of involvement of integrin ß1.

Next, we aimed to reveal whether migration is completely dependent on integrin ß1. To this end, we showed, using transwell assays, that blockage of ß1 in combination with Cxcl12 resulted in a complete abolishment of migration, indicating the complete dependency on integrin ß1. Additionally, migration was evaluated in the presence of ß2 blockers which did not affect migration towards Cxcl12. These results suggest again the necessity of ß1 and not of ß2 in this Cxcl12 directed migration. However, interaction of integrin ß1 with fibronectin in this migration does not elucidate which subtypes of ß1 are involved. Microglial cells express the α4, α5, and α6 integrins which dimerize with ß1 and can interact with fibronectin and laminin (5). Previous immunostainings executed by our lab showed fibronectin as the most appropriate ligand for ß1 interaction in the brain (unpublished results). Expression patterns of fibronectin and laminin showed very different results. The involvement of laminin is not very likely since the expression pattern of laminin is spatiotemporal and overall the distribution pattern is not uniform, hence the involvement of α6ß1 is not evident but neither excluded. Although fibronectin was also indicated as a convenient ligand by another group, the demonstrated expression pattern is not equal. De Gasperi (56) performed immunohistochemical stainings on E15.5 brain slices and showed prominent staining of fibronectin on the blood vessels, whereas stainings of our lab displayed a more homogeneous pattern. Next to vascular structures, which were also detected by our group, additional expression correlated to the pattern of radial glial cells was shown. A possible explanation can be found in the use of different antibodies, resulting in a higher or weaker specificity. Since the lack of a functional blood circulation resulted in the absence of microglial cells in the developing brain (9), migration via blood vessels would be a plausible explanation. The vascular route is indeed described as a possible entry route for microglial cells. Additionally, microglia are also described to enter the brain via the meninges through the pial surface and for that reason also migration along the radial cells is likely (72).

After showing migration of microglial cells towards Cxcl12 using integrin ß1, we aimed to elucidate the involved intracellular pathway. The pathway connecting Cxcl12 and integrin ß1 is described in multiple types of cancer cells (38, 58, 68). PI3K and MEK1/2 were often described to regulate the activation of integrins (38, 58, 68), thus leading to increased migration or adhesion in cancer cells and therefore became our selected targets. Transwell migration assays showed decreased migration towards Cxcl12 after blockage of PI3K and MEK1/2 with respectively the LY294002 and the U0126 inhibitors (38). These results suggest the involvement of this pathway in ß1 dependent migration, although effects on other factors influencing migration can not be excluded since PI3K and MEK1/2 are associated with multiple pathways (73). For example, an effect of PI3K on the cytoskeleton and the accompanying migration is described (74). In addition, the PI3K/Akt pathway is also described to regulate migration during embryonic development (75).

As described before, integrins can adopt different conformational states, a bended – low-affinity conformation – or the so called, active – stretched conformation. The 9EG7 antibody recognizes the 9EG7 epitope which is only exposed in the active conformation of integrin ß1 (69). Our results suggest that stimulation with Cxcl12 indeed resulted in increased expression levels per cell of the active conformation, while this increase was mainly reversed in the presence of the CxcR4 blocker. Ligand binding can partly induce the active conformation of integrins (45). Indeed, we observed a higher basal active conformation level of ß1 in cells which were coated on fibronectin and also the effect of CxcR4 blockage was remarkably smaller. Since a higher percentage of the integrins is already in active conformation, and Cxcl12 will probably only strengthen this effect, it is possible CxcR4 can reverse this effect only substantial. In contrast, cells on ICAM-1 coating, which is a ligand for ß2 integrin, and on which BV-2 cells do not adhere well, clearly showed lower levels of active conformation. In case cells were seeded on an uncoated surface, active conformation levels were rather high. These results suggest that not only the presence of the ligand, but also the adherent state of the cells can induce the active conformation. Further, the active conformation was analyzed on 1 % BSA coating, on which cells adhere weakly. A negative control was included in which Ca^{2+} will prevent the appearance of the active conformation. Incubation with Ca^{2+} resulted in even less active conformation than in unstimulated condition, indicating even on 1 % BSA some integrins are in active conformation. Additionally, PI3K and MEK1/2 inhibitors caused the active conformation expression to drop almost towards levels in unstimulated conditions. Despite the low sample size our results suggest that PI3K and MEK1/2 are essential in inducing the active conformation of integrin ß1 upon Cxcl12 stimulation. Since basal expression levels of active ß1 conformation in BV-2 cells were often high, we did not always detect an increase after Cxcl12 stimulation. An explanation can be found in the durability of our BV-2 cells; it is possible that our frozen vials already possess a high passage number. Besides, EDTA is able to induce the active conformation of integrin ß1 (69), while the detachment protocol of BV-2 cells included trypsin-EDTA treatment. However, the EDTA concentration to induce the active conformation is in the mM range while the trypsin-EDTA is in the µM range (69). Further, the use of a cell scraper instead of trypsin-EDTA did not make any difference. Additionally, the 9EG7 antibody recognizes the 9EG7 epitope only exposed in particular active conformations. For example, both manganese and magnesium treatment induce the active or extended conformation of ß1, whereas the 9EG7 antibody only recognizes the conformation induced by manganese (69). Possibly, the epitope is not always easily accessible and the antibody does not

recognize all ß1 integrins in active conformation induced by Cxcl12. Overall, an increase in the expression levels of the active conformation of integrin ß1 is observable after stimulation with Cxcl12, which is mainly attenuated upon CxcR4 administration, irrespective of the used coating. These results are in line with our previous results, namely Cxcl12 regulates migration by the use of integrin ß1 via the intracellular pathway involving PI3K and MEK1/2 activation. However, due to a low sample size statistics were not possible and the experiment needs to be repeated prior to firm conclusions.

In accordance with the previous report of Arno et al. (15), we believe not only Cxcl12 expression by basal progenitor cells is involved in microglial recruitment. Arno et al. observed that microglial recruitment was not completely abolished after suppressing Cxcl12, they hypothesized that other cells, such as Cajal-Retzius cells, are contributing to the expression of Cxcl12 (15). Although their hypothesis is reasonable, we are convinced more factors then Cxcl12 are involved. Since only 35 % of the cells express CxcR4, probably not all microglial cells are attracted by Cxcl12. Indeed, the involvement of other factors is described in microglia recruitment. However, CSF-1R is demonstrated to attract microglial cells into the CNS, the receptor is also implicated in migration inside the brain. CSF-1R shows synergy with the vascular endothelial growth factor receptor 1 (VEGFR1) in microglial recruitment towards the subventricular zone of the brain (72). Additionally, Xavier et al. suggested migration of microglial cells by similar mechanism as neural progenitor cells, including migration over radial glial cells and attraction by growth factors (71).

Furthermore, we studied the microglial migration in acute live brain slices, which is a more physiological condition compared to microglia in culture. Previous results of our group already showed a decline in migration speed from E13.5 towards E17.5. At E13.5 the average migration speed was 33.5 µm/h and dropped towards 24.2 µm/h at E17.5. Remarkably, blockage of integrin ß1 resulted in a decreased migration speed at E13.5, while at E17.5 on the contrary this led to an increase. For this reason, we suspect different functions of integrins during embryonic development. The decreased migration after ß1 blockage at E13.5 supports the idea that migration is stimulated by ß1 integrins. The increased migration speed upon ß1 blockage at E17.5 implicates a shift in integrin functioning. At E17.5 the general migration speed decreases, suggesting the majority of the microglial cells has reached their desired location. Although, postnatally the mobility and density of microglial cells increases again, which was expected to be the result of postnatal neuronal apoptosis and the accompanying clearance of the cells. However, the absence of apoptosis did not decrease the density or mobility of microglial cells (76). Independent of this postnatal increase in migration speed, the focus of integrin functions can be shifted towards a mainly adhesive function by increasing adhesive strength and maintain cells at their location. However, it is not yet completely clear which molecular mechanisms regulate adhesive strength, the involvement of focal adhesion complexes associated with the intracellular part of the integrins is possible (77). A focal adhesion complex contains more than 150 associated molecules and therefore the exact mechanism is hard to reveal (77). Whereas these adhesion complexes are relatively well studied in 2D, the opposite holds for 3D. In 3D structures, these complexes seem to disappear or become undetectable (78). Additionally, functions of proteins in 2D completely differ from those in 3D, so data from 2D cannot be extrapolated. Plausible candidates to regulate this adhesion strengthening are vinculin and talin, since recruitment of these adhesion proteins is shown to be involved in adhesion strengthening(79).

Also zyxin is one of these candidates in the focal adhesion complexes since reduced zyxin levels resulted in weaker adhesion and an increased migration speed in 3D (78, 80). However, since focal adhesion complexes only regulate 30 % of the adhesion strength also other factors such as integrin density should be involved in this adhesion (79). A presumable clarification would be a rearrangement in the distribution of the integrins on the cell surface. Probably, the expression pattern or density of integrins varies between different embryonic ages. However, a higher integrin expression does not necessarily correlate with higher binding. It is demonstrated that clustering of integrin α5ß1 can result in up to six fold stronger adhesion of cells (81). Next to clustering, especially the proximity of both other integrins on the cell surface and other ligands are important in increased adhesive strength (80, 82). Furthermore, also the available adhesive area seems to be implicated in regulating the strength of adhesion and modulates the focal adhesion assembly and the integrin binding (79). During cell migration adhesions are spatiotemporal, new adhesion are already formed on the front side of the cell while other adhesion are still disconnecting. The involvement of among others talin and the FAK and Src family are described to regulate the assembly and disassembly of these adhesions. Subsequently, these factors can induce prolonged adhesions and decreased disassembly resulting in a more stationary state of the cells (83). In conclusion, a lot of factors are involved in the modulation of adhesive strength but a shift in the function/binding strength of integrins during development is very plausible.

CxcR4 blockage did not affect migration speed at E13.5 nor at E17.5. It could be possible that the CxcR4 blocker could not penetrate the tissue sufficiently and thus not reach the desired location. Furthermore, an alternative function of CxcR4 regarding microglia migration inside the brain is possible. Arno et al. (15) showed migration of microglial cells towards Cxcl12 in the developing embryonic brain and a decrease in the density of microglia upon CxcR4 blockage. Injection of the CxcR4 blocker, AMD3100, at E12.5 resulted in a decreased number of microglial cells in a time span of 20 hours. Also in E14.5 CxcR4 knock-out embryos, a decrease in the density of microglia was present, whereas also downregulation of Cxcl12 resulted in reduction of microglia. All together, the authors of Arno et al. analyzed the amount of microglial cells present in the cortex, thus in viable embryos in which microglial cells are still entering the brain. Therefore, it is plausible that the studied effect on Cxcl12/CxcR4 interaction is an effect on the recruitment of microglial cells into the brain. This in contrast to the brain slices we use, in which migration speed can only be analyzed of already present microglial cells. In that case it may be possible that blockage of the CxcR4 receptor does not have an immediate effect on the migration speed. Once microglial cells have arrived in the neocortex and integrin ß1 is already activated, interaction with fibronectin can maintain the activated state and lead these microglial cells to their desired location. Further it would be interesting to evaluate the angle in which microglial cells migrate upon CxcR4 blockage. Microglial cells can be guided in a particular direction towards Cxcl12, while they can lose direction upon CxcR4 blockage. Finally, the exact expression pattern of Cxcl12 during all embryonic ages is not explored yet. Overall, CxcR4 can still have an effect but this does not per se result in a decreased migration speed.

Blockage of PI3K at E13.5 resulted in a significant decreased migration speed, similarly to the effect of ß1 blockage. Additionally, PI3K blockage decreased migration towards Cxcl12 in transwell assays and our preliminary data suggest PI3K blockage decreases active ß1 conformation levels. All

together, we may assume PI3K is involved in the activation of integrin ß1. Besides, PI3K is shown to be involved in the regulation of migration speed in neutrophils and in zebrafish during development (84, 85). Also an effect of PI3K on the cytoskeleton and the accompanying migration is described (74). Therefore, a decrease in migration speed upon PI3K blockage at E13.5 is a likely result. Surprisingly, whereas ß1 blockage resulted in an increased migration speed at E17.5, PI3K blockage resulted in the opposite effect, a decrease in migration speed. Although these results are contradictory, they do not exclude a correlation of PI3K and integrin ß1. PI3K is also described in relation with migration in cancer cells (38, 62) and in embryonic development (75). Besides, PI3K is demonstrated to be involved in actin cytoskeletal reorganization and migration (74). Additionally, blockage of PI3K can also affect other cells present, like radial glial cells or neuronal progenitor cells in the developing brain and therefore indirectly impede migration of microglial cells. Thus, even though blockage of integrin ß1 results in a higher migration speed at E17.5, the function of PI3K in cytoskeleton dynamics, can be more important for cell migration than the function of ß1 integrin and thus when blocked, overrule the effect of integrin ß1 blockage leading to a decreased migration speed.

Additionally, the role of the MEK1/2 pathway was investigated on E13.5 and E17.5. At E13.5, similar to the previous data of ß1 blockage, a significant decrease in the migration speed was observable upon MEK1/2 blockage, indicating the involvement of MEK1/2 in the migratory process of microglial cells at E13.5. We suggested also that MEK1/2 is involved in the active conformation of ß1 by flow cytometry and in Cxcl12 directed migration by transwell migration assays. An increase in migration speed at E17.5 was present upon ß1 blockage and therefore it seemed logical to expect the same effect upon MEK1/2 blockage. In contrast, at E17.5 no significant difference was present after blockage of MEK1/2. The most reasonable explanation is similar to the one described for PI3K blockage. This includes indeed an increased migration speed by blockage of MEK1/2 but this effect can eventually be overruled by the influence of MEK1/2 blockage on other mechanism involved in migration, thus compensating for the increased migration speed. Probably the influence of PI3K is higher than the effect of MEK1/2 and therefore we observed neither an increase nor a decrease in migration speed upon MEK1/2 blockage.

CONCLUSION & SYNTHESIS

The functions and migration pattern of microglial cells during embryonic development has remained unclear for a long time. Ginhoux et al. showed that microglial cells are a distinctive population that originate from myeloid progenitor cells in the yolk sac which arise already at embryonic day 7.5. Swinnen et al. revealed the complex migration pattern of microglial cells in the embryonal cortex. Also the participation of microglial cells in synaptic pruning during development has been shown. However, insight into the molecular mechanisms regulating this migration remained unclear. Arno et al. described chemoattractant Cxcl12 as an important factor in the recruitment of microglial cells during embryonic development. Additionally, preliminary data of our lab showed presumably the involvement of integrin β1 in the migration process. A link between Cxcl12 and integrin β1 was described multiple times in cancer cells regarding an increased adhesion/migration by integrin β1 upon Cxcl12 binding. Therefore, we hypothesized that "the Cxcl12/CxcR4/β1 axis stimulates microglia migration in embryonic development."

In this study, we showed that microglial cells migrate towards Cxcl12, upon CxcR4 binding in the presence of fibronectin. The necessity of fibronectin, which is a β1 ligand, is our first indication for the β1 dependency in this migration. The requirement of integrin β1 was confirmed by the administration of a β1 function blocking antibody, which resulted in a declined migration towards Cxcl12 without an effect on adhesion, whereas β2 blockage did not affect migration. In addition, we partly revealed the intracellular pathway connecting Cxcl12 and integrin β1. Different pathways were described in cancer, however in microglial cells this pathway was never investigated in relation to migration. Transwell migration assays, interfering with PI3K and MEK1/2 resulted in decreased migration levels towards Cxcl12. Our results suggested also an increase in the active conformation of integrin β1 upon Cxcl12 stimulation. This effect was abolished in the presence of PI3K and MEK1/2 blockers, indicating the involvement in the induction of the active conformation. However, these results need to be repeated to validate the effect. Further, it would be interesting to reveal whether Cxcl12 affects clustering of integrins or the proximity of other integrins or integrin ligands. Ascertaining these clustering or integrin spreading requires the use of specialized equipment and techniques such as nanopatterned surfaces and scanning electron microscopy.

Here, the involvement of Cxcl12 and β1 integrin in microglial migration was verified in more physiological conditions. Microglial cells were tracked in brain slices of E13.5 and E17.5 and the migration speed was compared upon β1, CxcR4, PI3K and MEK1/2 blockage. Preliminary data indicated a decrease in migration speed upon β1 blockage at E13.5. We attempted to confirm the involvement of Cxcl12 by CxcR4 blockage in brain slices, however CxcR4 blockage did not affect migration speed. Possibly, CxcR4 is involved in recruitment of microglial cells towards the brain, which we cannot observe in brain slices or, this receptor might play a role in regulating directed migration. It should be tested if microglial cells lose their direction upon CxcR4 blockage. Blockage of PI3K and MEK1/2 resulted in a decreased migration speed, indicating the importance of this pathway in β1 dependent migration. Although other effects, such as an effect on the cytoskeleton, can not be excluded. Surprisingly, at E17.5 β1 blockage resulted in an increased migration speed. This suggests a shift in integrin function during embryonic development towards the more adhesive

aspect of integrins, resulting in stimulation of adhesion and not migration. However, data with the PI3K and MEK1/2 blockers did not confirm this result. PI3K blockage resulted in a decreased migration speed whereas MEK1/2 blockage did not have an effect. Again, effect on other mechanisms involved in migration or on cytoskeleton dynamics cannot be excluded. More profound research in this shift from migration towards adhesion is needed. It would be clarifying to investigate the effect of β1 blockage and the intracellular pathway on E15.5 and eventually later in embryonic development since at postnatal age again an upregulation in migration speed is described.

All together, we showed that microglial cells migrate *in vitro* towards Cxcl12, dependent on integrin β1 by activation of the PI3K/MEK1/2 pathway. Our results suggest an increase in the active β1 conformation upon Cxcl12 stimulation. However, the exact functional effect of Cxcl12 on integrin β1 should be elucidated. We showed *ex vivo* a decreased migration speed upon β1, PI3K and MEK1/2 blockage at E13.5. At E17.5, an increased migration speed was present upon β1 blockage, while PI3K resulted in a decreased migration speed. We could not confirm the Cxcl12 dependency in our brain slices. Deeper research in this shift in migration speed and integrin functioning is needed.

This project will contribute to a better understanding of microglial behavior and migration during embryonic development. It is widely accepted by now that microglial cells have important functions during development, including by involvement of synaptic pruning. For that reason, it is important to expand our knowledge regarding microglia in development. Revealing the exact migration pattern and the accompanying mechanisms will result in a better understanding of this process. It is essential to expand the fundamental principles before further steps regarding dysfunctioning of microglia during development can be attempted.

REFERENCES

1. Prinz M, Tay TL, Wolf Y, Jung S. Microglia: unique and common features with other tissue macrophages. Acta Neuropathol. 2014;128(3):319-31.
2. Tay TL, Savage J, Hui CW, Bisht K, Tremblay ME. Microglia across the lifespan: from origin to function in brain development, plasticity and cognition. J Physiol. 2016.
3. Frost JL, Schafer DP. Microglia: Architects of the Developing Nervous System. Trends Cell Biol. 2016.
4. Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, et al. Synaptic pruning by microglia is necessary for normal brain development. Science. 2011;333(6048):1456-8.
5. Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R, et al. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron. 2012;74(4):691-705.
6. Sierra A, Beccari S, Diaz-Aparicio I, Encinas JM, Comeau S, Tremblay ME. Surveillance, phagocytosis, and inflammation: how never-resting microglia influence adult hippocampal neurogenesis. Neural Plast. 2014;2014:610343.
7. Bechade C, Cantaut-Belarif Y, Bessis A. Microglial control of neuronal activity. Front Cell Neurosci. 2013;7:32.
8. Bessis A, Bechade C, Bernard D, Roumier A. Microglial control of neuronal death and synaptic properties. Glia. 2007;55(3):233-8.
9. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science. 2010;330(6005):841-5.
10. Ginhoux F, Lim S, Hoeffel G, Low D, Huber T. Origin and differentiation of microglia. Front Cell Neurosci. 2013;7:45.
11. Elmore MR, Najafi AR, Koike MA, Dagher NN, Spangenberg EE, Rice RA, et al. Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. Neuron. 2014;82(2):380-97.
12. van Furth R, Cohn ZA, Hirsch JG, Humphrey JH, Spector WG, Langevoort HL. The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. Bull World Health Organ. 1972;46(6):845-52.
13. Lichanska AM, Hume DA. Origins and functions of phagocytes in the embryo. Exp Hematol. 2000;28(6):601-11.
14. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell. 2008;132(4):631-44.
15. Arno B, Grassivaro F, Rossi C, Bergamaschi A, Castiglioni V, Furlan R, et al. Neural progenitor cells orchestrate microglia migration and positioning into the developing cortex. Nat Commun. 2014;5:5611.
16. Swinnen N, Smolders S, Avila A, Notelaers K, Paesen R, Ameloot M, et al. Complex invasion pattern of the cerebral cortex bymicroglial cells during development of the mouse embryo. Glia. 2013;61(2):150-63.
17. Arnold T, Betsholtz C. The importance of microglia in the development of the vasculature in the central nervous system. Vasc Cell. 2013;5(1):4.
18. Pixley FJ, Stanley ER. CSF-1 regulation of the wandering macrophage: complexity in action. Trends Cell Biol. 2004;14(11):628-38.
19. Stanley ER, Guilbert LJ, Tushinski RJ, Bartelmez SH. CSF-1--a mononuclear phagocyte lineage-specific hemopoietic growth factor. J Cell Biochem. 1983;21(2):151-9.
20. Guilbert LJ, Stanley ER. Specific interaction of murine colony-stimulating factor with mononuclear phagocytic cells. J Cell Biol. 1980;85(1):153-9.
21. Nandi S, Gokhan S, Dai XM, Wei S, Enikolopov G, Lin H, et al. The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate neural progenitor cell maintenance and maturation. Dev Biol. 2012;367(2):100-13.
22. Wei S, Nandi S, Chitu V, Yeung YG, Yu W, Huang M, et al. Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. J Leukoc Biol. 2010;88(3):495-505.
23. Ginhoux F, Prinz M. Origin of Microglia: Current Concepts and Past Controversies. Cold Spring Harb Perspect Biol. 2015;7(8).
24. Martynoga B, Drechsel D, Guillemot F. Molecular control of neurogenesis: a view from the mammalian cerebral cortex. Cold Spring Harb Perspect Biol. 2012;4(10).
25. Gotz M, Huttner WB. The cell biology of neurogenesis. Nat Rev Mol Cell Biol. 2005;6(10):777-88.
26. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. Immunity. 2000;12(2):121-7.
27. NCBI. Cxcl12 chemokine (C-X-C motif) ligand 12 [Mus musculus (house mouse)].
28. Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol

Pharmacol. 2003;63(6):1256-72.
29. Li M, Ransohoff RM. Multiple roles of chemokine CXCL12 in the central nervous system: a migration from immunology to neurobiology. Prog Neurobiol. 2008;84(2):116-31.
30. Bernhagen J, Krohn R, Lue H, Gregory JL, Zernecke A, Koenen RR, et al. MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. Nat Med. 2007;13(5):587-96.
31. Yoshida D, Nomura R, Teramoto A. Signalling pathway mediated by CXCR7, an alternative chemokine receptor for stromal-cell derived factor-1alpha, in AtT20 mouse adrenocorticotrophic hormone-secreting pituitary adenoma cells. J Neuroendocrinol. 2009;21(5):481-8.
32. Schonemeier B, Kolodziej A, Schulz S, Jacobs S, Hoellt V, Stumm R. Regional and cellular localization of the CXCl12/SDF-1 chemokine receptor CXCR7 in the developing and adult rat brain. J Comp Neurol. 2008;510(2):207-20.
33. Lavi E, Strizki JM, Ulrich AM, Zhang W, Fu L, Wang Q, et al. CXCR-4 (Fusin), a co-receptor for the type 1 human immunodeficiency virus (HIV-1), is expressed in the human brain in a variety of cell types, including microglia and neurons. Am J Pathol. 1997;151(4):1035-42.
34. Nagasawa T, Tachibana K, Kishimoto T. A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. Semin Immunol. 1998;10(3):179-85.
35. McGrath KE, Koniski AD, Maltby KM, McGann JK, Palis J. Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. Dev Biol. 1999;213(2):442-56.
36. Katsumoto K, Kume S. The role of CXCL12-CXCR4 signaling pathway in pancreatic development. Theranostics. 2013;3(1):11-7.
37. Zhang M, Song A, Lai S, Qiu L, Huang Y, Chen Q, et al. Applications of stripe assay in the study of CXCL12-mediated neural progenitor cell migration and polarization. Biomaterials. 2015;72:163-71.
38. Teicher BA, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clin Cancer Res. 2010;16(11):2927-31.
39. Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. Annu Rev Immunol. 2004;22:891-928.
40. Lu DY, Tang CH, Yeh WL, Wong KL, Lin CP, Chen YH, et al. SDF-1alpha up-regulates interleukin-6 through CXCR4, PI3K/Akt, ERK, and NF-kappaB-dependent pathway in microglia. Eur J Pharmacol. 2009;613(1-3):146-54.
41. Dehghani M, Kianpour S, Zangeneh A, Mostafavi-Pour Z. CXCL12 Modulates Prostate Cancer Cell Adhesion by Altering the Levels or Activities of β1-Containing Integrins. Int J Cell Biol. 2014;2014:981750.
42. Izumi D, Ishimoto T, Miyake K, Sugihara H, Eto K, Sawayama H, et al. CXCL12/CXCR4 Activation by Cancer-Associated Fibroblasts Promotes Integrin ß1 Clustering and Invasiveness in Gastric Cancer. Int J Cancer. 2015.
43. Takada Y, Ye X, Simon S. The integrins. Genome Biol. 2007;8(5):215.
44. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell. 2002;110(6):673-87.
45. Luo BH, Carman CV, Springer TA. Structural basis of integrin regulation and signaling. Annu Rev Immunol. 2007;25:619-47.
46. Takagi J, Petre BM, Walz T, Springer TA. Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. Cell. 2002;110(5):599-11.
47. Takagi J, Strokovich K, Springer TA, Walz T. Structure of integrin alpha5beta1 in complex with fibronectin. EMBO J. 2003;22(18):4607-15.
48. Stephens LE, Sutherland AE, Klimanskaya IV, Andrieux A, Meneses J, Pedersen RA, et al. Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. Genes Dev. 1995;9(15):1883-95.
49. Fassler R, Meyer M. Consequences of lack of beta 1 integrin gene expression in mice. Genes Dev. 1995;9(15):1896-908.
50. Anderson R, Fassler R, Georges-Labouesse E, Hynes RO, Bader BL, Kreidberg JA, et al. Mouse primordial germ cells lacking beta1 integrins enter the germline but fail to migrate normally to the gonads. Development. 1999;126(8):1655-64.
51. Milner R. Microglial expression of alphavbeta3 and alphavbeta5 integrins is regulated by cytokines and the extracellular matrix: beta5 integrin null microglia show no defects in adhesion or MMP-9 expression on vitronectin. Glia. 2009;57(7):714-23.
52. Milner R, Campbell IL. The extracellular matrix and cytokines regulate microglial integrin expression and activation. J Immunol. 2003;170(7):3850-8.
53. Zheng W, Yuan X. Guidance of cortical radial migration by gradient of diffusible factors. Cell Adh Migr. 2008;2(1):48-50.
54. Stettler EM, Galileo DS. Radial glia produce and align the ligand fibronectin during neuronal migration in the developing chick brain. J Comp Neurol. 2004;468(3):441-51.
55. Chun JJ, Shatz CJ. A fibronectin-like molecule is present in the developing cat cerebral cortex and is

correlated with subplate neurons. J Cell Biol. 1988;106(3):857-72.
56. De Gasperi R, Gama Sosa MA, Elder GA. Presenilin-1 regulates the constitutive turnover of the fibronectin matrix in endothelial cells. BMC Biochem. 2012;13:28.
57. Kavinsky CJ, Garber BB. Fibronectin associated with the glial component of embryonic brain cell cultures. J Supramol Struct. 1979;11(2):269-81.
58. Shen W, Bendall LJ, Gottlieb DJ, Bradstock KF. The chemokine receptor CXCR4 enhances integrin-mediated in vitro adhesion and facilitates engraftment of leukemic precursor-B cells in the bone marrow. Exp Hematol. 2001;29(12):1439-47.
59. Yang Z, Zhou X, Liu Y, Gong C, Wei X, Zhang T, et al. Activation of integrin beta1 mediates the increased malignant potential of ovarian cancer cells exerted by inflammatory cytokines. Anticancer Agents Med Chem. 2014;14(7):955-62.
60. Yu Y, Shi X, Shu Z, Xie T, Huang K, Wei L, et al. Stromal cell-derived factor-1 (SDF-1)/CXCR4 axis enhances cellular invasion in ovarian carcinoma cells via integrin beta1 and beta3 expressions. Oncol Res. 2013;21(4):217-25.
61. Kiss DL, Windus LC, Avery VM. Chemokine receptor expression on integrin-mediated stellate projections of prostate cancer cells in 3D culture. Cytokine. 2013;64(1):122-30.
62. Huang YC, Hsiao YC, Chen YJ, Wei YY, Lai TH, Tang CH. Stromal cell-derived factor-1 enhances motility and integrin up-regulation through CXCR4, ERK and NF-kappaB-dependent pathway in human lung cancer cells. Biochem Pharmacol. 2007;74(12):1702-12.
63. Koyama R, Ikegaya Y. Microglia in the pathogenesis of autism spectrum disorders. Neurosci Res. 2015;100:1-5.
64. Bilimoria PM, Stevens B. Microglia function during brain development: New insights from animal models. Brain Res. 2015;1617:7-17.
65. Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. Mol Cell Biol. 2000;20(11):4106-14.
66. Meijering E, Dzyubachyk O, Smal I. Methods for cell and particle tracking. Methods Enzymol. 2012;504:183-200.
67. Swinnen N, Smolders S, Avila A, Notelaers K, Paesen R, Ameloot M, et al. Complex invasion pattern of the cerebral cortex bymicroglial cells during development of the mouse embryo. Glia. 2013;61(2):150-63.
68. Hartmann TN, Burger JA, Glodek A, Fujii N, Burger M. CXCR4 chemokine receptor and integrin signaling co-operate in mediating adhesion and chemoresistance in small cell lung cancer (SCLC) cells. Oncogene. 2005;24(27):4462-71.
69. Bazzoni G, Shih DT, Buck CA, Hemler ME. Monoclonal antibody 9EG7 defines a novel beta 1 integrin epitope induced by soluble ligand and manganese, but inhibited by calcium. J Biol Chem. 1995;270(43):25570-7.
70. Lipfert J, Odemis V, Wagner DC, Boltze J, Engele J. CXCR4 and CXCR7 form a functional receptor unit for SDF-1/CXCL12 in primary rodent microglia. Neuropathol Appl Neurobiol. 2013;39(6):667-80.
71. Tissir F, Wang CE, Goffinet AM. Expression of the chemokine receptor Cxcr4 mRNA during mouse brain development. Brain Res Dev Brain Res. 2004;149(1):63-71.
72. Xavier AL, Menezes JR, Goldman SA, Nedergaard M. Fine-tuning the central nervous system: microglial modelling of cells and synapses. Philos Trans R Soc Lond B Biol Sci. 2014;369(1654):20130593.
73. Itakura A, Aslan JE, Kusanto BT, Phillips KG, Porter JE, Newton PK, et al. p21-Activated kinase (PAK) regulates cytoskeletal reorganization and directional migration in human neutrophils. PLoS One. 2013;8(9):e73063.
74. Jimenez C, Portela RA, Mellado M, Rodriguez-Frade JM, Collard J, Serrano A, et al. Role of the PI3K regulatory subunit in the control of actin organization and cell migration. J Cell Biol. 2000;151(2):249-62.
75. Xue G, Hemmings BA. PKB/Akt-dependent regulation of cell motility. J Natl Cancer Inst. 2013;105(6):393-404.
76. Eyo UB, Miner SA, Weiner JA, Dailey ME. Developmental changes in microglial mobilization are independent of apoptosis in the neonatal mouse hippocampus. Brain Behav Immun. 2015.
77. Huttenlocher A, Horwitz AR. Integrins in cell migration. Cold Spring Harb Perspect Biol. 2011;3(9):a005074.
78. Fraley SI, Feng Y, Krishnamurthy R, Kim DH, Celedon A, Longmore GD, et al. A distinctive role for focal adhesion proteins in three-dimensional cell motility. Nat Cell Biol. 2010;12(6):598-604.
79. Gallant ND, Michael KE, Garcia AJ. Cell adhesion strengthening: contributions of adhesive area, integrin binding, and focal adhesion assembly. Mol Biol Cell. 2005;16(9):4329-40.
80. Cavalcanti-Adam EA, Volberg T, Micoulet A, Kessler H, Geiger B, Spatz JP. Cell spreading and focal adhesion dynamics are regulated by spacing of integrin ligands. Biophys J. 2007;92(8):2964-74.
81. Roca-Cusachs P, Gauthier NC, Del Rio A, Sheetz MP. Clustering of alpha(5)beta(1) integrins

determines adhesion strength whereas alpha(v)beta(3) and talin enable mechanotransduction. Proc Natl Acad Sci U S A. 2009;106(38):16245-50.
82. Selhuber-Unkel C, Lopez-Garcia M, Kessler H, Spatz JP. Cooperativity in adhesion cluster formation during initial cell adhesion. Biophys J. 2008;95(11):5424-31.
83. Truong H, Danen EH. Integrin switching modulates adhesion dynamics and cell migration. Cell Adh Migr. 2009;3(2):179-81.
84. Dumstrei K, Mennecke R, Raz E. Signaling pathways controlling primordial germ cell migration in zebrafish. J Cell Sci. 2004;117(Pt 20):4787-95.
85. Heit B, Liu L, Colarusso P, Puri KD, Kubes P. PI3K accelerates, but is not required for, neutrophil chemotaxis to fMLP. J Cell Sci. 2008;121(Pt 2):205-14.

SUPPLEMENTAL INFORMATION

1 Material and methods supplement

1.1 Adhesion assay

Cells were maintained overnight on DMEM 5796 with 1 % P/S, from now on referred to as serumfree medium. The next morning cells were harvested by trypsinisation, centrifugated for 10' at 400G, washed with PBS, centrifugated and resuspended in serumfree medium. A 24-well plate was uncoated or precoated during 60' at 37 °C with fibronectin (10 µg/ml, Sigma), ICAM-1 (10 µg/ml, Sino Biological, Beijing, China) or 1 % BSA. Wells were washed twice with PBS after coating. Cells were seeded at a concentration of 2.10^5 cells in 200 µl serumfree medium. Cells were allowed to attach for 60'. Non-adherent cells were washed away with PBS, adherent cells were fixed for 5' with 4 % PFA. After 2' staining with 0.05 % crystal violet, pictures were taken at 10x magnification with the Zeiss Primovert microscope and Axiocam camera. Pictures were tresholded using the Default threshold in Fiji and the mean grey value of the total picture was calculated using the measure function in Fiji. Grey values were calculated relative to adhesion to the fibronectin coating condition, set as 1.0 adhesion. The assay was performed in triplo (n=1).

1.2 Flow cytometry

BV-2 cells were seeded overnight in a 24 well plate precoated with 1 % BSA, fibronectin (10 µl/ml), ICAM-1 (10 µl/ml) or uncoated at a density of 2.10^5 cells/well in serumfree medium. Cells were fixed for 20' with 4 % PFA. After fixation, cells were detached by scraping the bottom of the plate with a 200 µl yellow tip and transferred to a 96 well plate. Cells were washed with PBS and centrifugated for 5' at 400 G, 4°C. The cell pellet was stained for 25' at room temperature (RT) with the primary 9EG7 antibody 50 µl/well (1:1000, 553715, BD) or isotype control (Rat IgG2a, 553922, BD) in FACS buffer (PBS, 2% FCS, sodium azide). After 25' incubation cells were centrifugated, washed with FACS buffer, centrifugated and incubated for 15' with the secondary antibody (1:600, Goat-anti rat Alexa 488, 11006, Life Technologies) at RT in FACS buffer (50 µl/well). Finally, a wash step was performed and the pellet was resolved in FACS buffer. Cells were analyzed with the FACS Aria II and the FACS Diva 6.1.3 software (BD Biosciences). N=number of experiments performed in mono.

2 Results supplement

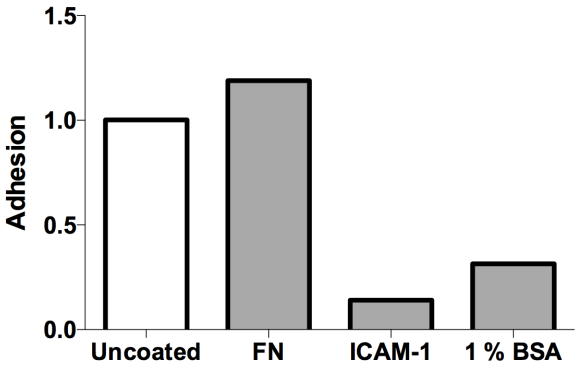

Figure S1: BV-2 cells were seeded on different coatings. Adhesion on the uncoated surface was set as control level. Adhesion increased on fibronectin coating and decreased on ICAM-1 and 1 % BSA coating (n=1).

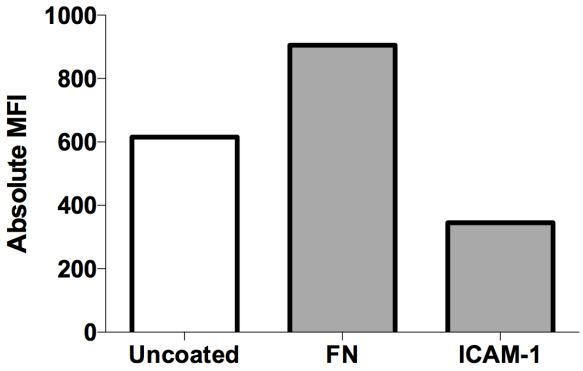

Figure S2: The active β1 conformation was analyzed by means of flow cytometry. Absolute expression of integrin β1 in active conformation increases upon FN coating and decreases upon ICAM-1 coating (n=1).

Auteursrechtelijke overeenkomst

Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
The link between Cxcl12/CxcR4 and integrin □ ß1 in microglia migration during brain development in mice

Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
Jaar: **2016**

in alle mogelijke mediaformaten, - bestaande en in de toekomst te ontwikkelen - , aan de Universiteit Hasselt.

Niet tegenstaand deze toekenning van het auteursrecht aan de Universiteit Hasselt behoud ik als auteur het recht om de eindverhandeling, - in zijn geheel of gedeeltelijk -, vrij te reproduceren, (her)publiceren of distribueren zonder de toelating te moeten verkrijgen van de Universiteit Hasselt.

Ik bevestig dat de eindverhandeling mijn origineel werk is, en dat ik het recht heb om de rechten te verlenen die in deze overeenkomst worden beschreven. Ik verklaar tevens dat de eindverhandeling, naar mijn weten, het auteursrecht van anderen niet overtreedt.

Ik verklaar tevens dat ik voor het materiaal in de eindverhandeling dat beschermd wordt door het auteursrecht, de nodige toelatingen heb verkregen zodat ik deze ook aan de Universiteit Hasselt kan overdragen en dat dit duidelijk in de tekst en inhoud van de eindverhandeling werd genotificeerd.

Universiteit Hasselt zal mij als auteur(s) van de eindverhandeling identificeren en zal geen wijzigingen aanbrengen aan de eindverhandeling, uitgezonderd deze toegelaten door deze overeenkomst.

Voor akkoord,

Arnauts, Kaline

Datum: **8/06/2016**