Die Rolle und Funktionsweise der Chemokinrezeptoren CXCR4 und CXCR7 in Mikroglia und Astrozyten

Dissertation

zur Erlangung des akademisches Grades
Dr. rer. med.

an der Medizinischen Fakultät
der Universität Leipzig

eingereicht von:
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geboren am 10.10.1983 in Sonneberg

angefertigt am:
Institut für Anatomie
AG Molekulare Neuroanatomie

Betreuer:
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Leipzig, im Februar 2013

Beschluss über die Verleihung des Doktorgrades vom: 16. Juli 2013

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Abkürzungsverzeichnis

DARC	engl.: *Duffy antigen receptor for chemokines*
EGF	engl.:*epidermal growth factor*
GABA	γ-Aminobuttersäure
GCSF	engl.: *granulocyte colony-stimulating factor*
GDP	Guanosindiphosphat
GFAP	engl.: *glial fibrillary acidic protein*
GPCR	G-Protein-gekoppelter Rezeptor
Grk	G-Protein-gekoppelte Rezeptorkinase
GSH	Glutathion
GTP	Guanosintriphosphat
HIV	humanes Immundefizienz-Virus
HPV	humanes Papillomavirus
IP-10	engl.: *interferon γ-inducible protein* 10
I-TAC	engl.: *interferon-inducible T cell alpha chemoattractant*
$InsP_3$	Inositol-1,4,5-triphosphat
LPS	Lipopolysaccharide
MCAO	engl.: *middle cerebral artery occlusion*
MIG	engl.: *monokine induced by γ-interferon*
MIF	engl.: *macrophage migration inhibitory factor*
MS	Multiple Sklerose
PTX	Pertussistoxin
SDF	engl.: *stromal derived factor*
SIV	simianes Immundefizienz-Virus
WHIM	Warzen-Hypogammaglobulinämie-Immundefizienz-Myelokathexis
ZNS	zentrales Nervensystem

1. Bibliographische Beschreibung

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Die Rolle und Funktionsweise der Chemokinrezeptoren CXCR4 und CXCR7 in Mikroglia und Astrozyten

Universität Leipzig, Dissertation

86 Seiten, 108 Literaturangaben, 4 Anlagen

Referat:

Das Chemokin *stromal derived factor* (SDF)-1 spielt eine wichtige Rolle bei der Hämatopoese, bei Immunreaktionen sowie bei der Entwicklung des Herzens, der Extremitätenmuskulatur und des zentralen und peripheren Nervensystems. Lange Zeit galt CXCR4 als der einzige Chemokinrezeptor für SDF-1, bis vor wenigen Jahren CXCR7 als ein alternativer Rezeptor für SDF-1 identifiziert wurde. Da alle Zelltypen des zentralen Nervensystems (ZNS) sensitiv für SDF-1 sind, sollte in dieser Arbeit die Funktion der beiden Rezeptoren in primärer Mikroglia und primären Astrozyten untersucht werden. Bisher konnte CXCR7 nur als *Scavenger*-Rezeptor für SDF-1 oder als atypischer Chemokinrezeptor nachgewiesen werden.

Die Untersuchungen ergaben einen mitogenen und chemotaktischen Effekt von SDF-1 auf primäre Mikroglia, wobei sowohl CXCR4 als auch CXCR7 für das SDF-1-Signalverhalten essentiell sind. Nach Aktivierung von Mikroglia in vitro und in vivo wurden beide Rezeptoren verstärkt expremiert. In primären Astrozyten ergab sich ein ligandenabhängiges Signalverhalten von CXCR7. So führte die Bindung von SDF-1 an CXCR7 zu einer Aktivierung von G-Proteinen, während die Kopplung von *interferon-inducible T cell alpha chemoattractant* (I-TAC), als zweiten Liganden von CXCR7, eine Signalweiterleitung über β-Arrestin2 zur Folge hatte. Zudem konnte die G-Protein-gekoppelte Rezeptorkinase (Grk)2 als ein positiver Regulator des SDF-1-CXCR7-Signalverhaltens in Astrozyten identifiziert werden.

2. Einführung

2.1. Chemokine

2.1.1. Klassifizierung und Funktion

Zur Familie der Chemokine gehören über 45 verschiedene Moleküle, die aus 70-100 Aminosäuren bestehen. Der Name Chemokine leitet sich von ihrer chemotaktischen Wirkung auf Leukozyten und ihren Zytokin-ähnlichen Eigenschaften ab [1]. Chemokine haben an ihrem N-Terminus konservierte Cysteinreste, deren Anordnung eine Einteilung der Moleküle in vier verschiedene Chemokinklassen ermöglicht. CXC Chemokine haben dabei zwischen ihren beiden ersten Cysteinresten eine Aminosäure, während bei den CC Chemokinen die Cysteinreste direkt nebeneinander liegen. Fractaline (CX3CL1), als einziges Chemokin der CX3C Familie, hat drei Aminosäuren zwischen den zwei Cysteinen. Bei den Mitgliedern der (X)C Familie fehlt das erste und dritte Cystein [2].

Neben ihrer strukturellen Klassifizierung können Chemokine auch in funktionell unterschiedliche Gruppen eingeteilt werden. Dabei ist die Unterteilung in homöostatische und inflammatorische Chemokine nicht gänzlich starr, da einige Chemokine beiden Gruppen zugeordnet werden können [2].

Homöostatische Chemokine werden konstitutiv in verschiedenen Organen wie Thymus, Lymphknoten und Knochenmark gebildet, wo sie essentiell für die Leukozytenproduktion und -migration sind [1]. Beispielsweise weisen Mäuse, die durch eine Mutation das Chemokin CCL21 nicht produzieren können, eine gestörte T-Zell Reifung in ihren Lymphknoten auf [3]. Ein defektes Gen für das Chemokin CXCL13 beziehungsweise für seinen Rezeptor CXCR5 in der Maus führt dazu, dass keine peripheren Lymphknoten und Peyerschen Plaques ausgebildet werden [4]. Chemokine spielen weiterhin eine entscheidende Rolle während der embryonalen Entwicklung (siehe Abschnitt 2.2.3 und 2.2.4). Neuere Untersuchungen haben gezeigt, dass

Chemokine unter physiologischen Bedingungen im ZNS Neurotransmitter-ähnliche Funktion haben können, also für die Signalweiterleitung von Neuronen wichtig sind [5].

Inflammatorische Chemokine werden bei Entzündungsreaktionen vermehrt produziert und dienen in erster Linie der Rekrutierung von Leukozyten an den Entzündungsherd. CXC Chemokine dieser Gruppe mit einem ELR-Motiv (Glutaminsäure-Leucin-Arginin) direkt vor den Cysteinresten haben angiogenetische Eigenschaften, während solche ohne ELR-Motiv angiostatisch wirken [2]. Auch für die Wundheilung sind Chemokine von essentieller Bedeutung. Beispielsweise führt die Inaktivierung des Chemokinrezeptors CXCR2 in Mäusen zu einer verringerten Einwanderung von Neutrophilen in die verletzte Region sowie zu einer beeinträchtigten Migration und Proliferation von Keratinozyten und damit zu einer verlangsamten Wundheilung [6].

Darüber hinaus spielen Chemokine bei verschiedenen Krankheiten wie Arthritis, Multipler Sklerose (MS), Asthma und Arteriosklerose eine Rolle [7]. Zum Beispiel ist das Risiko für Arteriosklerose bei Menschen, die eine Mutation im Gen für den Chemokinrezeptor CX3CR1 aufweisen, geringer als bei Menschen ohne diese Mutation [8, 9]. Chemokine sind zudem wichtige Faktoren bei der Tumorgenese sowie Invasion und Metastasierung von Tumorzellen (siehe Abschnitt 2.2.3 und 2.2.4).

2.1.2. SDF-1

SDF-1 gehört zur Gruppe der CXC Chemokine und wird auch als CXCL12 bezeichnet. Das Chemokin wurde zuerst aus Stromazellen des Knochenmarkes isoliert und als wachstumsfördernder Faktor von B-Vorläuferzellen beschrieben [10].

Bis jetzt sind sechs verschiedene Spliceformen von SDF-1 bekannt. Alle Isoformen haben die ersten 89 Aminosäuren gemein und unterscheiden sich nur am C-Terminus. SDF-1α als die häufigste und am besten untersuchte Splicevariante besteht aus 89 Aminosäuren, während zum Beispiel SDF-1β aus 93 Aminosäuren aufgebaut ist [11].

SDF-1α wird ubiquitär in allen Organen von verschiedenen Zelltypen expremiert. Im Gehirn können Astrozyten, Mikroglia und Neurone SDF-1α produzieren [12, 13]. Im Blut erfolgt ein schneller proteolytischer Abbau des SDF-1α durch die Caboxypeptidase N. SDF-1β dagegen wird im Blut wesentlich langsamer abgebaut als SDF-1α. Dies könnte erklären, weshalb SDF-1β am stärksten in sehr gut durchbluteten Organen wie Leber, Niere, Milz und Knochemmark expremiert wird. SDF-1γ findet

sich vor allem im Herzen. Die Expression der weiteren Splicevarianten SDF-1δ, SDF-1ϵ und SDF-1ϕ konnte in verschiedenen Organen wie Leber, Niere, Pankreas und Darm nachgewiesen werden [11, 14].

Die Rezeptorbindung des SDF-1 wird durch den N-Terminus des Peptides realisiert. Zusätzlich kommt es zu einer Stabilisierung der Bindung durch Wechselwirkungen zwischen negativ geladenen Glucoseaminoglukanen auf der Zellmembran und dem positiv geladenen N-Terminus von SDF-1 [15].

Das Gen für SDF-1 liegt beim Menschen als einziges der CXC Chemokine auf Chromosom 10. Die meisten anderen Gene dieser Chemokinklasse liegen auf Chromosom 4. Dieser Fakt und eine über 90%ige Homologie von SDF-1 zwischen Mensch und Maus, lassen auf essentielle biologische Funktionen des Chemokins schließen [11]. Bei welchen physiologischen und pathologischen Prozessen SDF-1 eine Rolle spielt, wird in Zusammenhang mit seinen Rezeptoren in den Abschnitten 2.2.3 und 2.2.4 näher beschrieben.

2.1.3. I-TAC

Das 73 Aminosäuren lange Peptid I-TAC gehört wie SDF-1 zur Familie der CXC Chemokine (CXCL11). Identifiziert wurde I-TAC in primären, humanen Astrozyten, die zuvor mit dem Zytokin Interferon-γ stimuliert wurden [16]. Eine Expression von I-TAC konnte neben Astrozyten in weiteren Zellen bzw. Zelllinien und Organen wie Mikroglia [17], peripheren Leukozyten, diversen Krebszelllinien [18], Pankreas, Leber und Thymus nachgewiesen werden [16].

Das Gen für I-TAC ist beim Menschen auf Chromosom 4 lokalisiert. Die größte Homologie in seiner Aminosäuresequenz weist I-TAC mit den CXC Chemokinen *monokine induced by γ-interferon* (MIG/CXCL9) und *interferon γ-inducible protein* 10 (IP-10/CXCL10) auf. Alle drei Chemokine binden an den Rezeptor CXCR3, wobei I-TAC die höchste Rezeptoraffinität zeigt [16]. Sowohl I-TAC als auch MIG und IP-10 sind durch Zytokine induzierbar und werden daher vermehrt bei Infektionen, Verletzungen oder Entzündungsreaktionen gebildet [19]. Mäuse, die kein I-TAC expremieren, zeigten eine verlangsamte und gestörte Neubildung der Epidermis und Dermis [20].

Die Annahme, dass I-TAC einzig als Ligand für den Chemokinrezeptor CXCR3 fungiert, wurde von Burns et al. [21] widerlegt. Die Arbeitsgruppe konnte zeigen, dass

I-TAC ebenfalls ein Ligand für den Chemokinrezeptor CXCR7 (Abschnitt 2.2.4) ist.

2.2. Chemokinrezeptoren

2.2.1. Struktur und Wirkungsweise

Chemokinrezeptoren gehören zur Gruppe der G-Protein-gekoppelten 7-transmembran-Rezeptoren. Sieben Helices durchspannen die Zellmembran, mit drei extrazellulären und vier intrazellulären Schleifen. Für die Ligandenbindung ist der extrazelluläre N-Terminus verantwortlich. Der intrazelluläre C-Terminus enthält viele Serin- und Threoninreste, die phosphoryliert werden und damit wichtig für die Rezeptordesensitivierung sind [22]. Chemokinrezeptoren können Di- und Oligomere bilden, wobei sich gleiche, aber auch unterschiedliche Rezeptoren zusammenschließen können [23]. Die Rezeptoren inflammatorischer Chemokine haben oftmals viele Liganden, während Rezeptoren homöostatischer Chemokine meist nur einen oder zwei Liganden besitzen. Die Chemokinrezeptoren werden analog zu ihren wichtigsten Chemokinliganden in vier Gruppen eingeteilt. Bisher sind 23 Chemokinrezeptoren identifiziert worden, wobei fünf von ihnen als atypische Rezeptoren gelten [2].

G-Protein gekoppelte Rezeptoren (GPCRs) haben an ihren intrazellulären Schleifen G-Proteine gebunden. Diese bestehen aus einer Gα-Untereinheit, welche direkt an den Rezeptor gekoppelt ist, und einer daran gebundenen G$\beta\gamma$-Untereinheit. Zusätzlich bindet die Gα-Untereinheit im inaktiven Zustand Guanosindiphosphat (GDP). Kommt es zur Ligandenbindung, wird das heterotrimere G-Protein aktiviert und GDP durch Guanosintriphosphat (GTP) ersetzt. Gα-GTP löst sich daraufhin vom Rezeptor und dissoziiert von der G$\beta\gamma$-Untereinheit [22]. Sowohl die Gα- als auch die G$\beta\gamma$-Untereinheit führen nun zur Aktivierung zahlreicher Signalkaskaden. Beispielsweise kommt es nach Ligandenbindung zu einer Steigerung der intrazellulären Calciumkonzentration durch Inositol-1,4,5-triphosphat ($InsP_3$). $InsP_3$ wiederum wird durch eine Phospholipase Cβ_2 gebildet, welche von der G$\beta\gamma$-Untereinheit aktiviert wird [24]. Es gibt unterschiedliche Klassen von G-Proteinen, wobei gezeigt wurde, dass nur die Kopplung von G_i-Proteinen an den Rezeptor zu einer Zellmigration führt [25].

GPCRs besitzen in ihrer zweiten intrazellulären Schleife ein sogenanntes DRYLAIV-Motiv, welches für die Kopplung der Gα_i-Untereinheit an den Rezeptor wichtig ist.

Atypische Chemokinrezeptoren besitzen kein oder ein verändertes DRYLAIV-Motiv. Dadurch sind sie nicht in der Lage, typische Signalkaskaden nach Ligandenbindung zu aktivieren [26]. Dennoch binden sie Chemokine, internalisieren sie und können so einen extrazellulären Chemokingradienten erzeugen. CCRL1 und CCRL2 beispielsweise binden homöostatische Chemokine des Chemokinrezeptors CCR7 und spielen dabei eine regulatorische Rolle bei der Migration von Lymphozyten und dendritischen Zellen [2]. *Duffy antigen receptor for chemokines* (DARC) als ein weiterer atypischer Chemokinrezeptor ist wichtig für die Transzytose von Chemokinen durch Endothelzellen [26]. Auch CXCR7 zählt bisher zu den atypischen Chemokinrezeptoren. Auf seine Eigenschaften und Funktionen wird in Abschnitt 2.2.4 speziell eingegangen.

2.2.2. Grks

Grks erkennen aktivierte GPCRs, was zu einer Phosphorylierung der Serin- und Threoninreste des Rezeptor-C-Terminus durch die Kinaseaktivität der Grks führt. Der phosphorylierte Rezeptor wird dann in einem nächsten Schritt durch Arrestine erkannt, welche an den Rezeptor binden und dadurch die G-Protein-Kopplung an diesen blockieren. Damit wird die G-Protein-vermittelte Aktivierung von Signalkaskaden gestoppt [27]. Der an Arrestin gekoppelte Rezeptor wird anschließend über sogenannte *clathrin coated pits* internalisiert und in Endosomen abgebaut oder recycelt und zur Zellmembran zurückgeführt [28]. Arrestine können darüber hinaus selbst mit Signalmolekülen wie Src, p38, RhoA und JNK interagieren und Signalkaskaden aktivieren [27].

Zur Familie der Grks gehören sieben verschiedene Proteine (Grk1-Grk7). Grk1 und Grk7 werden als visuelle Grks bezeichnet, da sie nur in den Zapfen und Stäbchen der Retina expremiert werden. Ein Verlust von funktionellem Grk1 führt bei Mäusen zur Erblindung. Das Oguchi-Syndrom beim Menschen wird durch eine Mutation im Grk1-Gen verursacht, was zu einer verminderten katalytischen Aktivität des Enzyms führt und mit Nachtblindheit einhergeht [29].

Die Expression von Grk4 ist hauptsächlich auf die Hoden und Nieren beschränkt, wohingegen Grk2, Grk3, Grk5 und Grk6 in fast allen Organen gebildet werden [27]. Diese fünf nicht-visuellen Grks stehen in Säugetieren über 800 verschiedenen GPCRs gegenüber, was schlussfolgern lässt, dass ein Grk viele unterschiedliche Rezeptoren phosphorylieren kann. Über die Spezifität der Grks in Bezug auf bestimmte GPCRs

ist bisher kaum etwas bekannt. Neben GPCRs können Grks auch andere Substrate phosphorylieren und damit Signalkaskaden regulieren. Zu diesen Substraten zählen zum Beispiel nicht G-Protein-gekoppelte Rezeptoren wie LRP6, Transkriptionsfaktoren wie NFκB1 p105, Signalmoleküle wie p38 und Proteine des Zytoskelettes wie Tubulin. Weiterhin können Grks Moleküle binden und beeinflussen ohne diese zu phosphorylieren. Besonders Grk2 und Grk3 sind große Proteine mit vielen unterschiedlichen Domänen, über die sie mit anderen Proteinen auf vielfältige Weise interagieren können. Zu den von Grks beeinflussten Signalmolekülen zählen unter anderem PI3K, Akt, PKA, PKC und Erk1/2 [29].

2.2.3. CXCR4

Der Chemokinrezeptor CXCR4 ist beim Menschen auf Chromosom 2 lokalisiert und wurde erst 1996 von Oberlin [30] und Bleul [31] als funktioneller Rezeptor für SDF-1 charakterisiert, obwohl er schon länger als LESTR beschrieben worden war [32]. CXCR4 wird ubiquitär auf verschiedenen Zelltypen expremiert. Hämatopoetische Stamm- und Vorläuferzellen, aber auch Vorläuferzellen anderer Gewebe und Organe wie Leber, Skelettmuskulatur, Herz, Endothel, Nieren und Nervensystem tragen CXCR4 auf ihrer Zellmembran [33]. Im Hirn wird CXCR4 sowohl von Neuronen als auch von Mikroglia und Astrozyten expremiert [34].

Die elementaren Funktionen von CXCR4 im Organismus wurden durch Ausschalten des Rezeptors in Mäusen deutlich. CXCR4-defiziente ($CXCR4^{-/-}$) Tiere sterben bereits im Uterus oder kurz nach der Geburt. Sie weisen eine fehlerhafte Entwicklung des Herzens, der Großhirnrinde und des Hippocampus auf. Zudem kommt es zu einer verminderten Bildung von B-Vorläuferzellen in der Leber und im Knochenmark. Auch die Anzahl myeloider Vorläuferzellen im Knochenmark ist bei $CXCR4^{-/-}$ Tieren stark reduziert [35]. Darüber hinaus ist die Vaskularisierung des Gastrointestinaltraktes [36] und die Innervation der Extremitäten durch Verlust von Spinalganglienneuronen gestört. Auch die Entwicklung der Extremitätenmuskulatur läuft bei $CXCR4^{-/-}$ Tieren fehlerhaft ab [37]. Die Beobachtung, dass vergleichbare Defekte bei Mäusen auftreten, die kein SDF-1 expremieren, führte zu der Schlußfolgerung, dass SDF-1 der einzige Chemokinligand für CXCR4 sei [38, 39].

Viele Untersuchungen haben sich mit der Rolle von CXCR4 und SDF-1 während der Hämatopoese beschäftigt. Hämatopoetische Vorläuferzellen migrieren in Rich-

tung eines SDF-1 Gradienten, was ein entscheidender Mechanismus beim sogenannten *Homing* von Vorläuferzellen ins Knochenmark ist. Nach Knochenmarkstransplantationen wird das Zytokin *granulocyte colony-stimulating factor* (GCSF) eingesetzt, um diese Vorläuferzellen wieder aus dem Knochenmark zu mobilisieren. GCSF führt zur verstärkten Bildung verschiedener Proteasen im Knochenmark wie Cathepsin G, die neben Adhäsionsmolekülen auch SDF-1 und den N-Terminus von CXCR4 spalten. Erst dadurch kann es zum Auswandern von hämatopoetischen Vorläuferzellen ins periphere Blut kommen [40]. Zudem scheinen CXCR4 und SDF-1 auch an der Mobilisierung von nicht-hämatopoetischen Vorläuferzellen nach Verletzungen von beispielsweise Leber, Lunge, Pankreas oder nach Herzinfarkt und Schlaganfall beteiligt zu sein [33, 41].

Neben seinen physiologischen Funktionen ist CXCR4 auch an pathologischen Prozessen beteiligt. CXCR4 ist ein Corezeptor für den Eintritt von Viren der humanen Immundefizienz (HIV) in CD4-positive Lymphozyten. SDF-1 kann durch Rezeptorblockierung den Eintritt der HI-Viren verhindern [30]. Beim sogenannten Warzen-Hypogammaglobulinämie-Immundefizienz-Myelokathexis (WHIM)-Syndrom kommt es zu einer Mutation des C-Terminus von CXCR4, was zu einer gesteigerten Aktivität des Rezeptors führt. Patienten mit WHIM leiden unter einer erhöhten Anfälligkeit für das humane Papillomavirus (HPV). Infolgedessen weisen diese Patienten Warzen am ganzen Körper auf und erkranken häufiger an Gebärmutterhalskrebs. Zudem ist die Anzahl von neutrophilen Granulozyten und B-Lymphozyten im Blut vermindert. Dadurch kommt es bei diesen Patienten zu einem gehäuften Auftreten von bakteriellen Infektionen [42]. Die Expression von CXCR4 konnte beim Menschen in über 23 verschiedenen Krebsarten nachgewiesen werden [43]. Entscheidend ist die CXCR4-SDF-1-Achse bei der Metastasierung von Tumoren. Organe, die eine hohe SDF-1-Expression aufweisen, wie zum Beispiel Lymphknoten, Lunge oder Knochen, rekrutieren verstärkt CXCR4-positive Krebszellen [33]. Nicht nur die Metastasierung, sondern auch das Wachstum von Tumoren wird durch CXCR4 beeinflusst. In vitro konnte das Wachstum von humanen Glioblastomlinien durch Inhibierung von CXCR4 oder SDF-1 deutlich vermindert werden [44]. In vivo führte das Ausschalten von CXCR4 bei Mäusen zu einem geringeren Wachstum von subcutanen Lymphomen und einer gesteigerten Überlebensrate [45].

Das Zytokin *macrophage migration inhibitory factor* (MIF) und Ubiquitin kön-

nen als Nicht-Chemokine an CXCR4 binden. MIF, welches neben CXCR4 auch an den Chemokinrezeptor CXCR2 bindet, wirkt proinflammatorisch und spielt bei der Atherogenese eine Rolle [46]. Ubiqutin dagegen wirkt antiinflammatorisch, immunsuppressiv und neuroprotektiv. Es bindet und aktiviert CXCR4 über eine andere extrazelluläre Bindungsstelle als SDF-1 [47, 48].

2.2.4. CXCR7

Das Gen für den Chemokinrezeptor wurde vor über 20 Jahren zum ersten Mal aus der cDNA-Sequenz des Hundes kloniert und zunächst als RDC1 (*Receptor Dog* cDNA) bezeichnet [49]. Erste postulierte Liganden für RDC1 wie VIP und Adrenomedullin erwiesen sich als falsch und der Rezeptor wurde als sogenannter *orphan*-Rezeptor eingestuft. Hinweise, dass RDC1 ein Chemokinrezeptor sein könnte, ergaben sich aus seiner Lage auf dem humanen Chromosom 2. Auf diesem Chromosom sind auch die Chemokinrezeptoren CXCR1, CXCR2 und CXCR4 lokalisiert. Zudem weist RDC1 eine 43%ige Sequenzhomologie zu CXCR2 auf [50]. Wie andere Chemokinrezeptoren ist auch RDC1 ein Corezeptor für Viren der humanen und simianen Immundefizienz (SIV) [51]. Balabanian et al. [52] konnten schließlich zeigen, dass SDF-1 neben CXCR4 auch an RDC1 bindet und forderten die Umbenennung des Rezeptors in CXCR7. Neben SDF-1 wurde auch das Chemokin I-TAC als ein Ligand für CXCR7 identifiziert, wobei SDF-1 eine höhere Bindungsaffinität für CXCR7 als I-TAC aufweist [21].

Wie CXCR4 wird auch CXCR7 von vielen verschiedenen Geweben und Zellen expremiert. Im Gehirn konnte CXCR7 in Neuronen, Astrozyten und Endothelzellen nachgewiesen werden [53]. Auch B-Zellen, Monozyten und Neutrophile expremieren CXCR7 [52, 54, 55]. Die CXCR7 mRNA-Expression korreliert dabei aber nicht zwangsläufig mit der Menge an Protein in beziehungsweise auf der Zelle [21, 56].

Um die Funktion von CXCR7 zu untersuchen, wurden $CXCR7^{-/-}$ Mäuse generiert. Diese kommen im Gegensatz zu $CXCR4^{-/-}$ Mäusen lebend zur Welt, sterben aber kurz nach der Geburt. Ursache ist eine fehlerhafte Herzentwicklung [55, 57]. Daneben konnten noch weitere Funktionen von CXCR7 nachgewiesen werden. Zum Beispiel werden renale Vorläuferzellen nach Nierenverletzungen durch SDF-1 sowie durch CXCR4 und CXCR7 zum Ort der Verletzung geleitet, wobei CXCR7 für das Überleben der Vorläuferzellen und deren Adhäsion an Endothelzellen verantwort-

lich ist [58]. Auch während der Angiogenese spielt CXCR7 eine Rolle, indem es das Überleben von endothelialen Vorläuferzellen sichert [59]. Aber nicht nur normale Zellen, sondern auch viele verschiedene Krebszellen expremieren CXCR7. Bei Prostatakrebs steigt die Tumoraggressivität mit dem Expressionslevel von CXCR7 [60]. CXCR7 führt zu einem verstärkten Wachstum von Brust- und Lungentumoren [61] und korreliert mit der Metastasierung von Blasentumoren [62]. In verschiedenen Gliomzellen mediiert CXCR7 antiapoptotische Effekte [63]. Auch Tumor-assoziierte Blutgefäße expremieren viel CXCR7, was für eine wichtige Funktion des Rezeptors bei der Neovaskularisierung in Tumoren spricht [43, 61]. Liberman et al. [64] konnten im Kontrast dazu zeigen, dass CXCR7 das Wachstum von Neuroblastomen in vitro und in vivo reduziert.

Bisherige Untersuchungen zur Wirkungsweise von CXCR7 zeigen, dass der Rezeptor auf verschiedene Art und Weise agieren kann. Beim Zebrafisch bindet und internalisiert CXCR7 SDF-1 und erzeugt so einen extrazellulären SDF-1-Gradienten, der für die gerichtete Migration von CXCR4-expremierenden primordialen Stammzellen entscheidend ist [65]. In Muskelzellen hat CXCR7 ebenfalls eine *Scavenger*-Funktion. Die Expression von CXCR7 nimmt während der Differenzierung der Muskelzellen zu. CXCR7 bindet SDF-1 und verhindert so die inhibitorischen Effekte des CXCR4 auf die Muskelzelldifferenzierung [66]. Auch in humanen Endothelzellen konnte gezeigt werden, dass CXCR7 als *Scavenger* für SDF-1 und I-TAC fungiert [67]. Darüber hinaus kann CXCR7 die Funktion von CXCR4 durch Bildung von Heterodimeren modulieren [55, 68, 69]. Beispielsweise führt in HEK-293T-Zellen die Heterodimerisierung der beiden Rezeptoren zu einem verringerten G-Protein-abhängigen, intrazellulären Calciumanstieg nach Stimulation mit SDF-1 [68]. CXCR7 scheint auch mit dem Rezeptor für *epidemal growth factor* (EGF) Komplexe zu bilden und ihn dabei zu phosphorylieren und damit zu aktivieren [20, 70]. Eine alleinige SDF-1-Signaltransduktion durch CXCR7 wurde ebenfalls schon in verschiedenen Zelltypen wie Astrozyten [71], Oligodentrozyten [72], T-Zellen [73], HEK293-Zellen [74] und diversen Tumorzellen [60, 62, 63] beschrieben. Allerdings konnte bisher noch keine G-Protein-abhängige Aktivierung von Signalmolekülen nach SDF-1 Bindung an CXCR7 nachgewiesen werden [21, 55, 68, 74]. Als Ursache dafür wird das modifizierte DRYLAIV-Motiv in der zweiten intrazellulären Schleife von CXCR7 vermutet [50]. Stattdessen wird davon ausgegangen, dass es nach Ligandenbindung an CXCR7

zu einer β-Arrestin-Rekrutierung an den Rezeptor und infolgedessen zu einer Signaltransduktion kommt [69, 74–77].

2.3. Mikroglia und Astrozyten

Mikroglia

Mikroglia sind die immunkompetenten Zellen des ZNS. Während der Embryogenese differenzieren Mikroglia aus Makrophagen, die im Dottersack, dem ersten Ort der Hämatopoese, entstehen und in die Anlagen des Gehirns migrieren [78]. Bei der Maus lassen sich ab dem achten Tag und bei der Ratte ab dem elften Tag der embryonalen Entwicklung Mikroglia im Gehirn nachweisen. Die Zahl der Mikroglia steigt danach stetig an und erreicht etwa zwei Wochen nach der Geburt ihren Höhepunkt [79]. Mikroglia finden sich in allen Hirnregionen, wobei die Mikrogliadichte zwischen den einzelnen Regionen variiert. Insgesamt sind im adulten Gehirn etwa 15-20% aller Zellen Mikroglia [80]. Während ihrer Migration ins Gehirn weisen Mikroglia eine runde, amöboide Form auf. Im Hirnparenchym angekommen, verändern sich die Zellen und gehen in einen sogenannten ramifizierten oder ruhenden Zustand über. Morphologisch zeichnen sich ramifizierte Mikroglia durch lange und vielfach verzweigte Fortsätze aus, wobei auch hier Unterschiede zwischen unterschiedlichen Hirnbereichen bestehen. In der weißen Substanz haben Mikroglia meist einen langgezogenen Zellkörper und ihre Fortsätze sind parallel oder im rechten Winkel zu den Nervensträngen angeordnet, während in der grauen Substanz die Fortsätze in alle Richtungen zeigen [81].

Die Funktion der Mikroglia im Gehirn ist vielfältig. Während der Hirnentwicklung phagozytieren Mikroglia überflüssige und abgestorbene Neurone oder können sogar deren Apoptose induzieren. Auch im adulten Hirn sind Mikroglia für die Phagozytose von beispielsweise abgestorbenen Zellen oder Amyloidablagerungen verantwortlich [82]. Zudem sind Mikroglia während der Entwicklung des ZNS immer mit dem Gefäßsystem assoziiert, was eine Rolle der Mikroglia bei der Angiogenese vermuten lässt. Diese Annahme wird durch Untersuchungen untermauert, die zeigen, dass Mäuse mit weniger oder keiner Mikroglia ein geringer ausgebildetes Gefäßsystem aufweisen [79]. Mikroglia können die synaptische Plastizität durch Kontakt ihrer Fortsätze mit den Synapsen und ihrer Phagozytosekompetenz beeinflussen [80, 82]. Zudem wirken

Mikroglia durch die Produktion sogenannter Neurotrophine und neurotrophischer Zytokinen protektiv auf Nervenzellen und sind essentiell für deren Regeneration nach Läsionen [81, 82].

Die Fortsätze der Mikroglia sind nicht steif, sondern bewegen sich ständig, um ihre Umgebung zu überwachen [80]. Kommt es zum Eindringen von Pathogenen wie Bakterien oder Viren ins Hirn, aber auch nach Verletzungen wie Traumata oder Ischämie, wird die Mikroglia aktiviert. Aktivierte Mikrogliazellen phagozyzieren potentielle Pathogene und können daraufhin Antigene für T-Zellen auf ihrer Oberfläche präsentieren. Darüber hinaus sezernieren sie immunmodulatorische Moleküle wie Zytokine, Chemokine und reaktive Sauerstoffspezies, die zu einer Verstärkung der Immunreaktion führen. Durch die Sektretion von Chemokinen werden weitere aktivierte Mikrogliazellen zum Entzündungsherd gelockt [83]. Bei Beeinträchtigung der Blut-Hirn-Schranke können auch Makrophagen aus der Peripherie ins Gehirn gelangen und sind dann morphologisch kaum von aktivierten Mikrogliazellen zu unterscheiden [81]. Die Immunkompetenz der Mikroglia bedingt ihre Beteiligung an allen Erkrankungen des ZNS wie zum Beispiel MS, Morbus Alzheimer und Morbus Parkinson. Allerdings wird ihre Rolle dabei oftmals kontrovers diskutiert [84–88]. Beispielsweise führt ein Ausschalten von Mikroglia zu weniger Myelin- und Axonschäden im Mausmodell für MS. Gleichzeitig produziert Mikroglia aber auch antiinflammatorische Moleküle und trägt zur Remyelisierung bei [86]. Bei Morbus Alzheimer und Morbus Parkinson ist nicht vollständig geklärt, ob aktivierte Mikroglia die Ursache für einen Neuronenverlust ist oder eher als deren Folge auftritt [85]. Eine weitere Hypothese nimmt an, dass auch die Mikroglia Alterungsprozessen unterliegt und dadurch ihre neuroprotektiven Wirkungen geschwächt sind, was die Entstehung neurodegenerativer Erkrankungen fördert [89].

Astrozyten

Astrozyten entstehen während der embryonalen Hirnentwicklung aus radialen Gliazellen, aus denen sich zuvor Neurone differenzieren. Im adulten Gehirn sind Astrozyten die am häufigsten vorkommende Zellart [90]. Man unterscheidet zwischen protoplasmatischen Astrozyten der grauen Substanz und sogenannter Faserglia der weißen Substanz. Zudem gibt es im Kleinhirn die Bergmannglia und in der Retina die Müllerglia, welche morphologisch radialen Gliazellen ähneln [91]. Vom Soma der Astrozy-

ten zweigen viele Fortsätze ab, die sich wiederum noch feiner aufgliedern. Mit diesen Fortsätzen stehen Astrozyten in Kontakt mit den Fortsätzen, dem Soma und den Synapsen von Neuronen, wobei beim Menschen ein einzelner Astrozyt mit bis zu zwei Millionen verschiedenen Synapsen assoziiert sein kann [90].

Astrozyten sind im Hirn aufgrund ihrer verschiedenen Funktionen essentiell für die Integrität von Neuronen. Sie puffern beispielsweise den extrazellulären Gehalt an Kalium- und Wasserstoffionen, indem sie diese Ionen aufnehmen. Astrozyten besitzen auch Transporter für die Neurotransmitter Glutamat und γ-Aminobuttersäure (GABA). Diese werden von Synapsen ausgeschüttet und im Anschluss von Astrozyten aufgenommen. Durch die Astrozyten-spezifische Glutaminsynthase wird Glutamat in Glutamin umgewandelt. Dieses wird durch die Astrozyten abgegeben und kann wieder von Neuronen aufgenommen und in Glutamat umgewandelt werden. Zu hohe extrazälluläre Glutamatkonzentrationen wirken toxisch auf Synapsen. Astrozyten schützen Neurone weiterhin, indem sie Glutathion (GSH) als Antioxidans produzieren und Neuronen Cystein für die GSH-Synthese zur Verfügung stellen. Zudem wird angenommen, dass sie Nervenzellen mit Laktat als Energiequelle versorgen. Während der Hirnentwicklung sind Astrozyten maßgeblich an der Synapsenbildung beteiligt und produzieren im adulten Hirn Neurotrophine [91, 92]. Mit ihren Fortsätzen bilden Astrozyten ein Netzwerk um alle Blutgefäße im Gehirn und können über die Produktion von vasokonstriktorischen oder -dilatorischen Substanzen modulatorisch auf die glatten Muskelzellen der Arteriolen und damit auf den Blutfluss wirken [93].

Neben ihren vielfältigen Funktionen im gesunden Gehirn spielen Astrozyten auch bei diversen pathologischen Zuständen eine Rolle. Nach Hirnverletzungen bilden Astrozyten eine sogenannte Glia-Narbe, die eine Regeneration der Axone beeinträchtigt [94]. Bei der Alexander-Krankheit kommt es zu einer Mutation im *glial fibrillary acidic protein* (GFAP), einem Astrozyten-spezifischen Protein. Die Folgen sind eine Degeneration der weißen Substanz im Gehirn und Rückenmark, Entwicklungsverzögerung und eine Zunahme der Schädelgröße [91]. Bei der hepatischen Enzephalopathie kommt es durch Leberversagen zu einer Zunahme von Ammoniak im ZNS. Dieser wird von den Astrozyten aufgenommen und mit Glutamat zu Glutamin kondensiert. Eine Überproduktion von Glutamin hat aber toxische Effekte auf Astrozyten [92]. Ebenfalls wird eine astrozytäre Dysfunktion im Zusammenhang mit Epilepsie diskutiert. Hierbei kommt es zu vielfältigen Veränderungen in den Astrozyten wie

zum Beispiel einer verminderten Aufnahme von Kaliumionen und Glutamat [95]. Bei Morbus Alzheimer können Astrozyten Amyloid-β-haltige Plaques internalisieren und degradieren. Ist deren Kapazität überschritten, kommt es zur Ausschüttung von inflammatorischen Mediatoren und Glutamat durch Astrozyten, was zur Schädigung von Neuronen führt [92].

2.4. Zielsetzung

Mikroglia als immunkompetente Zellen des ZNS sind essentiell für dessen Integrität. Verschiedene Faktoren wie das Eindringen von Pathogenen oder auch Verletzungen führen zur Aktivierung von Mikroglia. Es konnte bereits gezeigt werden, dass Mikroglia sensitiv für das Chemokin SDF-1 ist und dass SDF-1 vermehrt bei Ischämie [96] oder Erkrankungen wie HIV-Enzephalopathie [97] und MS [98] im Gerhirn expremiert wird. Allerdings gibt es bisher noch keine Studien zur Expression und Funktion von CXCR7 in Mikroglia. In dieser Arbeit sollte deshalb untersucht werden, inwieweit der Chemokinrezeptor CXCR7 von Mikroglia expremiert wird und welche Rolle CXCR4 und CXCR7 beim SDF-1-Signalverhalten spielen.

Der zweite Teil dieser Arbeit beschäftigt sich mit der Funktionsweise von CXCR7 in Astrozyten. In einer vorangegangenen Untersuchung von Ödemis et al. [71] wurde CXCR7 als funktioneller SDF-1 Rezeptor in primären Astrozyten nachgewiesen. Da CXCR7 offensichtlich auf verschiedene Art und Weise arbeiten kann, sollten hier die molekularen Mechanismen des SDF-1-CXCR7-*Signallings* in Astrozyten genauer erforscht werden.

3. Publikationen

3.1. CXCR4 and CXCR7 form a functional receptor unit for SDF-1/CXCL12 in primary rodent microglia

Titel:	CXCR4 and CXCR7 form a functional receptor unit for SDF-1/CXCL12 in primary rodent microglia
Autoren:	Jana Lipfert, Veysel Ödemis, Daniel-Christoph Wagner, Johannes Boltze, Jürgen Engele
eingereicht am:	01.08.2012
akzeptiert am:	30.11.2012
Veröffentlichung:	Neuropathology and Applied Neurobiology online seit 07.01.2013 doi: 10.1111/nan.12015

Neuropathology and Applied Neurobiology (2013) doi: 10.1111/nan.12015

CXCR4 and CXCR7 form a functional receptor unit for SDF-1/CXCL12 in primary rodent microglia

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J. Lipfert, V. Ödemis, D.-C. Wagner, J. Boltze and J. Engele (2013) *Neuropathology and Applied Neurobiology*
CXCR4 and CXCR7 form a functional receptor unit for SDF-1/CXCL12 in primary rodent microglia

Aims: Microglial cells have been originally identified as a target for the CXC chemokine, SDF-1, by their expression of CXCR4. More recently, it has been recognized that SDF-1 additionally binds to CXCR7, which depending on the cell type acts as either a nonclassical, a classical or a scavenger chemokine receptor. Here, we asked whether primary microglial cells additionally express CXCR7 and if so how this chemokine receptor functions in this cell type. **Methods:** CXCR4 and CXCR7 expression was analysed in cultured rat microglia and in the brain of animals with permanent occlusion of the middle cerebral artery (MCAO) by either Western blotting, RT-PCR, flow cytometry and/or immunocytochemistry. The function of CXCR4 and CXCR7 was assessed in the presence of selective antagonists. **Results:** Cultured primary rat microglia expressed CXCR4 and CXCR7 to similar levels. Treatment with SDF-1 resulted in the activation of Erk1/2 and Akt signalling. Erk1/2 and Akt signalling were required for subsequent SDF-1-dependent promotion of microglial proliferation. In contrast, Erk1/2 signalling was sufficient for SDF-1-induced migration of microglial cells. Both SDF-1-dependent signalling and the resulting effects on microglial proliferation and migration were abrogated following pharmacological inactivation of either CXCR4 or CXCR7. Moreover, treatment of cultured microglia with lipopolysaccharide resulted in the co-ordinated up-regulation of CXCR4 and CXCR7 expression. Likewise, reactive microglia accumulating in the area adjacent to the lesion core in MCAO rats expressed both CXCR4 and CXCR7. **Conclusions:** CXCR4 and CXCR7 form a functional receptor unit in microglial cells, which is up-regulated during activation of microglia both *in vitro* and *in vivo*.

Keywords: brain injury, cell proliferation, cell signalling, chemokines, chemotaxis, SDF-1/CXCL12

Introduction

Microglia are the resident immunocompetent and phagocytic cells of the central nervous system and represent the prime mediator of innate immune response to infection, injury and neurodegeneration in the brain [1–3]. They direct the immune response by the release of multiple factors with pro-inflammatory or immunoregulatory effects. They clear cell and tissue debris as well as pathogens by phagocytosis. By the release of chemoattractive factors they further direct immune cells to the site of injury and allow for the activation of T cells by antigen presentation. While it is well established that microglia respond to pathological conditions by the release of potentially neurotoxic substances that induce or promote the progression of neurological disorders, evidence exists that microglia also release neuroprotective substances [1–3].

In addition to cytokines, microglia are a recognized source and/or target for various chemokines, including macrophage inflammatory protein 1α (MIP-1α), MIP-1β, monocyte chemoattractant protein-1 (MCP-1) and

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stromal cell-derived factor-1 (SDF-1) [4–6]. The CXC chemokine, SDF-1, was initially identified as a regulator of haematopoiesis and immune cell function and was subsequently shown to exert pleiotropic effects during the development of the cardiovascular system, nervous system and limb musculature [7–11]. While it was originally assumed that SDF-1 would signal exclusively through the chemokine receptor, CXCR4, a more recent study identified CXCR7 as a second SDF-1 receptor [12], which in addition binds I-TAC as an alternative ligand [13]. The data currently available imply that depending on the cell type and/or the biological condition, CXCR7 might exert diverse functions. During development, CXCR7 seems to act as a SDF-1 scavenger, which directs CXCR4-induced migration of primordial cells by shaping the extracellular SDF-1 gradient [14]. CXCR7 further modulates CXCR4-dependent cell signalling by forming heterodimers with CXCR4 [15,16]. In addition, CXCR7 acts as an atypical G protein-coupled receptor, which signals through β-arrestin in some tumour cell lines and cell lines transiently transfected with CXCR7 [17–20]. Finally, we recently obtained evidence that CXCR7 actively mediates SDF-1 signalling through Gi/o proteins in primary astrocytes and astrocytoma cell lines [21,22].

Early work demonstrated that cultured rodent microglia express CXCR4 and in addition revealed that SDF-1 promotes microglial migration [23,24]. Low levels of CXCR4 are further present in microglia of the adult rat brain [25]. Moreover, CXCR4 is up-regulated in microglia and astrocytes in various brain diseases, such as HIV encephalitis and experimental allergic encephalomyelitis [26–28]. Whether microglia would also express CXCR7 and if so, how this chemokine receptor functions in microglial cells is presently unknown. We now demonstrate that a major subpopulation of rodent microglia co-express CXCR4 and CXCR7. In addition, we provide evidence that CXCR4 and CXCR7 form a functional receptor unit in microglial cells.

Materials and methods

Cell cultures

Enriched microglial cultures were established according to a previously established protocol [29] with some minor modifications. In brief, cortical hemispheres were removed from postnatal day 2–3 Sprague–Dawley rats and trypsinized (0.2%) for 10 min. Following mechanical dissociation in plastic pipettes, obtained cells were seeded into 75-cm^2 culture flasks (Sarstedt, Nümbrecht, Germany) and grown in Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% foetal calf serum (FCS; Invitrogen, Darmstadt, Germany). Under these culture conditions a confluent layer of astrocytes formed within the following days on top of which round cells, representing microglial cells accumulated. For subsequent isolation of microglial cells, bottles were gently shaken and detached cells were collected and placed with DMEM and 10% FCS in 12-well culture plates (TPP, Trasadingen, Switzerland) or on glass coverslips (immunohistochemistry). For experiments, cells were further maintained with serum-free N_2 medium additionally supplemented with SDF-1 (Almac, Edinburgh, UK), AMD3100 (10–100 ng/ml; dissolved in water; Calbiochem, Gaitherburg, MD, USA), T140 (50–100 ng/ml; dissolved in water; Bachem, Bubendorf, Switzerland), CCX771 (100 nM; dissolved in DMSO; ChemoCentryx, Mountain View, CA, USA), CCX704 (100 nM; dissolved in DMSO; ChemoCentryx), CCX733 (50–100 nM; dissolved in DMSO; ChemoCentryx), UO126, Ly294002 (both 250 nM; dissolved in DMSO; Calbiochem), pertussis toxin (PTX, 50 ng/ml; Calbiochem) or lipopolysaccharide (LPS; 1–20 ng/ml; Sigma, Saint Louis, MO, USA) as specified in the text. C2C12^{CXCR7} cells, a subclone of C2C12 myoblasts only expressing CXCR7 [30], were propagated in DMEM containing 10% FCS.

Western blot analysis

Cells were lysed by ultrasonication in 62.5 mM Tris-HCl, containing 2% sodium dodecyl sulphate (SDS) and 10% sucrose. Proteins were denatured at 95°C for 5 min and further diluted in sample buffer (250 mM Tris-HCl, pH 6.8 containing 4% SDS, 10% glycerol and 2% β-mercaptoethanol). For detection of phosphorylated proteins, sample buffer was additionally supplemented with sodium orthovanadate (10 mM). Protein content of cell lysates was determined using the BCA protein estimation kit (Pierce, Rockford, IL, USA) and bovine serum albumin (BSA) as a standard. Proteins (15 μg/lane) were separated by SDS-(10%) polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. Upon blocking nonspecific binding sites with 3% BSA for 60 min, blots were incubated overnight at 4°C with one of the following antibodies: anti-CXCR7 (1:1000; Acris, Herford, Germany), anti-CXCR4 (1:1000; Abcam, Cambridge, UK),

phosphospecific anti-Erk1/2 (1:3000) or phosphospecific anti-Akt (1:3000; both Cell Signaling Technology Danvers, MA, USA). Antibody labelling was detected by incubating cultures for 2 h at room temperature with appropriate horseradish peroxidase-labelled secondary antibodies (Dianova, Hamburg, Germany) and visualized with the enhanced chemiluminescence kit (Serva, Heidelberg, Germany). To control for protein loading, blots were additionally stained with either anti-GAPDH (1:10 000; BD Transduction Laboratories, San Jose, CA) antibodies, or antibodies against nonphosphorylated Erk1/2 (1:2000) or Akt (1:2000, both Cell Signaling Technology). Integrated optical densities of immunoreactive protein bands were measured using the MF CheminBis 1.6 imager (Biostep, Jahnsdorf, Germany).

Proliferation assay

Proliferating microglial cells were identified by Ki67 expression and quantified by flow cytometric analysis. Following permeabilization with 0.05% saponin, cells were incubated for 1 h at 4°C with anti-Ki67 antibodies (1:500; Abcam) and for further 30 min with Alexa Fluor 488-labelled secondary antibodies (1:700; Invitrogen). Cells were fixed with paraformaldehyde (1%, w/v) in PBS. Flow cytometric analysis was performed with a FACScan (Becton Dickinson, Heidelberg, Germany). The forward narrow angle light scatter was used to exclude dead and aggregated cells.

Cell counts

For determining total number of microglial cells present in culture, cells were trypsinized (0.2%) for 10 min and counted with a Neubauer chamber.

Chemotaxis

The chemotactic response of primary microglial cells to SDF-1 was evaluated using a modified 48-well Boyden chamber (Neuro Probe, Cabin John, MD, USA). After cell harvest, microglia was resuspended in fresh DMEM containing 1% BSA. Fifty microlitres of the cell suspension, containing 10 000 cells and the according receptor antagonists, were added to the upper well of the chamber. The lower well received 28 µl of N_2 medium supplemented with the indicated concentrations of SDF-1. The upper and lower wells were separated by polyornithin-coated Nucleopore® PVP-free polycarbonate filters (Corning, Acton, MA, USA) with 5 µm pore size, and the chamber was incubated for 4 h at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. After incubation, nonmigrating cells were scraped off from the upper surface of the filter. Migrated cells, attached to the lower surface, were fixed in ice-cold methanol for 10 min, stained with DAPI (1:1000; AAT Bioquest, Sunnyval, CA, USA) and counted under a microscope at 400× magnification in six high-power fields. Data were normalized by calculating the migration index, which was defined to be the ratio of cells migrating in the presence and absence of chemokines

Experimental stroke

All animal experiments were performed in accordance with the guidelines defined by the governmental authorities for care and use of experimental animals (reference number TVV18/07). Spontaneously hypertensive rats (SHR, 250 g; Charles River Laboratories) were housed at constant temperature (+23°C) under a 12-h light/dark cycle and *ad libitum* access to food and water. Experimental brain ischemia was induced by unilateral permanent occlusion of the middle cerebral artery (MCAO) as previously described [31]. Briefly, animals were anaesthetized with ketamine hydrochloride (100 mg/kg; Merial, Hallbergmoos, Germany), Xylacin (10 mg/kg; Bayer, Monheim, Germany) and Atropin (0.1 mg/kg; Ratiopharm, Ulm, Germany) given as intraperitoneal injection. Body core temperature was maintained at 37.5°C using a feedback controlled heating pad connected to a rectal probe. Pulse rate, systolic arterial blood pressure and oxygen saturation were non-invasively monitored at the beginning of the surgical intervention and before and after MCAO. After subtemporal craniotomy, the middle cerebral artery was permanently occluded by electrocoagulation. For preparing brain sections, subjects were deeply anaesthetized after 25 h with carbon dioxide and transcardially perfused with 200 ml phosphate-buffered solution followed by perfusion of 250 ml ice-cold 4% formalin solution. Subsequently, the removed brains were fixed in 4% formalin solution for another 24 h, drained in ascending concentrations of sucrose for 3 days and cryoconserved at −80°C. Frozen brains were cut into 20 µm thick sections and mounted on coated microscope slides.

RNA isolation and RT-PCR analysis

RNA was isolated from microglia using the PeqGold (PeqLab, Schwalbach, Germany) isolation kit according to the manufacturer's instructions. RNA concentrations were measured by spectrophotometric absorbance at 260 nm. Five micrograms of RNA was reversed transcribed using 200 units/µl of moloney leukaemia virus reverse transcriptase (Sigma), random hexamere primers (Thermo Hybaid, Ulm, Germany) and dNTPs (Fermentas, St. Leon-Rot, Germany). For real-time PCR Biotag™ DNA Polymerase Kit (Bioline GmbH, Luckenwalde, Germany), dNTPs and the following primers (Thermo Scientific, Ulm, Germany) were used: CXCR4 sense 5′-AGTGGCTGA CCTCCTCTTTGT-3′ and antisense 5′-GCCCACATAGACTG CCTTTCC-3′, product size, 235 bp; CXCR7 sense 5′-TCA CCTACTTCACCAGCACC-3′ and antisense 5′-ACATGGC TCTGGCGAGCAGG-3′, product size, 282 bp; GAPDH sense 5′-CCGTGTTCCTACCCCCAATG-3′ and antisense 5′-GTCCACCACCCTGTTGCTGTA-3′, product size, 280 bp. PCR conditions were 93°C for 5 min, 95°C for 1 min, 40 cycles of 52°C for 1 min (CXCR4, GAPDH) or 40 cycles of 60°C for 1 min (CXCR7). In all cases, reaction was terminated by heating samples to 72°C for 2 min. Reaction products were separated on 1.5% agarose gel and visualized with ethidium bromide.

Immunohistochemistry

For immunohistochemistry, paraformaldehyde-fixed tissue sections or cell cultures (coverslips; 4%, 20 min) were permeabilized with 0.05% saponin (in PBS) for 20 min and nonspecific binding sites were blocked with 2% (v/v) normal goat or donkey serum (Invitrogen) in PBS for 30 min. Tissue sections or cell cultures were subsequently incubated overnight in a humid chamber at 4°C with one the following primary antibodies: anti-rabbit Iba-1 (1:2000; Wako Chemikals, Neuss, Germany), fluorescein-labelled anti-lectin (1:50; Vector Laboratories, Bulingame, CA, USA); anti-rat CXCR4 (1:250; Abcam) or anti-goat CXCR4 (1:250; Abcam) and anti-mouse CXCR7 (1:250, ChemoCentryx; Mountain View, CA, USA). With exception of lectin staining, antibody labelling was detected by incubating tissue slices or cell cultures for 2 h at room temperature with either appropriate Alexa Fluor488-labelled (1:400, Invitrogen) and/or Cy™ 3-labelled secondary antibodies (1:400; Jackson ImmunoResearch, Newmarket, UK). Cell nuclei were visualized by staining with DAPI (AAT Bioquest, Sunnyval, CA, USA). Tissue sections or coverslips were mounted with DAPCO/glycerol (DAKO, Carpinteria, CA, USA) and staining was examined under an Olympus BX-50 microscope with narrow-band filters.

Immunoprecipitation

For immunoprecipitation, cells were lysed in ice-cold NP-40 buffer (0.15 M NaCl, 0.01 M Na_2PO_4, 1% NP-40, pH 7.2). After preclearing, lysates were incubated with either anti-rat CXCR4 (1:100, Abcam) or anti-mouse CXCR7 antibodies (1:100, ChemoCentryx) at 4°C for 1 h, followed by another 1-h incubation period in the additional presence of protein A/G Sepharose beads (Calbiochem). Bound proteins were resuspended in sample buffer and separated (30 µg protein/lane) by 10%-SDS-PAGE.

CXCR4 and CXCR7 cell surface expression

Cell surface co-expression of CXCR4 and CXCR7 was analysed by flow cytometry. Cultured microglial cells were suspended in PBS supplemented with anti-rat CXCR4 (1:400; Abcam) and anti-rabbit CXCR7 antibodies (1:400; Acris), and for another 30 min with FITC-labelled secondary antibodies (1:400; Jackson ImmunoResearch) and PE-labelled secondary antibodies (1:400; Santa Cruz Biotechnology, Heidelberg, Germany). Flow cytometric analysis was performed with a FACScan (Becton Dickinson). The forward narrow-angle light scatter was used to exclude dead and aggregated cells. Double- and single-labelled cells were analysed by gating on forward and side scatter.

Statistics

Data, obtained from at least three independent experiments, are given as mean ± SD. One-way analysis of variance (ANOVA) followed by pairwise multiple comparison procedures (Student–Newman–Keuls method) was used for statistical analysis. Differences with $P \leq 0.05$ were considered significant.

Results

Analysis of cultured microglia for CXCR7 and CXCR4 expression

Highly enriched cultures of primary microglia were established according to the previous protocol by Guilian and

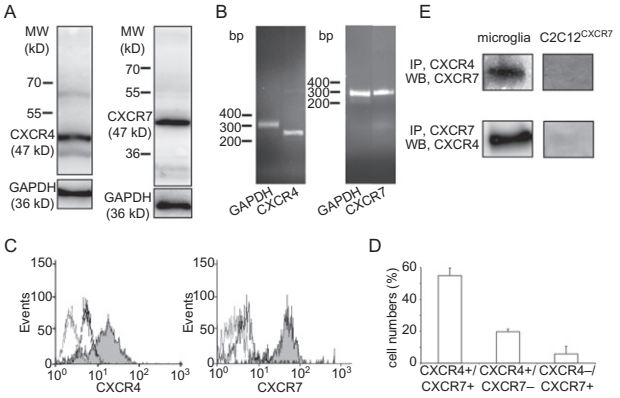

Figure 1. Primary microglia co-express CXCR4 and CXCR7 at their cell surface. (**A**) Western blot analysis and (**B**) RT-PCR analysis demonstrated that cultured rat microglia express CXCR4 and CXCR7 to similar levels. GADPH served as loading control (Western blotting) or internal control (RT-PCR). (**C**) Flow cytometry further revealed that cultured primary microglia express both CXCR4 and CXCR7 at their cell surface. Grey area/black line, primary and secondary antibody; open area/black line, only secondary antibody; open area/grey line, only primary antibody. Note that staining is reduced in cultures incubated with either the secondary or the primary antibody alone, thus, confirming the specificity of the CXCR4 and CXCR7 antibodies used. (**D**) Analysis of CXCR4 and CXCR7 co-expression in cultured primary microglia. Co-expression of CXCR4 and CXCR7 was determined by flow cytometry as described under Materials and Methods. Bars represent mean ± SD ($n = 3$). A major portion of cultured microglia co-expressed CXCR4 and CXCR7 ($CXCR4^+/CXCR7^+$). A clearly smaller portion of the cultured cells only expressed CXCR4 ($CXCR4^+/CXCR7^-$) whereas a minor portion only expressed CXCR7 ($CXCR4^-/CXCR7^+$). (**E**) Evidence for the existence of preformed CXCR4-CXCR7 heterodimers in primary microglial cells. CXCR4 or CXCR7 was immunoprecipitated (IP) from cultured microglia and analysed for co-precipitated CXCR7 or CXCR4, respectively, by Western blotting (WB). A subclone of C2C12 cells only expressing CXCR7 ($C2C12^{CXCR7}$) served as a control.

Baker [29]. This protocol involved the shake-off of microglial cells from primary cell cultures of the postnatal day (P)2 to 3 rat cortical hemispheres and the subsequent replating of the collected cells. In accordance with previous reports [e.g. 2,3] almost all cells (>99%) in the resulting cultures showed immunoreactivity for the microglia/macrophage markers, Iba-1 and lectin, and only very few cells (<1%) exhibited immunolabelling for the astrocytic marker, GFAP (supplementary Figure S1). Further corroborating previous studies [24,26], RT-PCR analysis in combination with Western blot analysis demonstrated that cultured primary microglia express CXCR4 (Figure 1**A**,**B**). These analyses further revealed that cultured microglia additionally express CXCR7 at levels similar to those of CXCR4 (Figure 1**A**,**B**). The specificity of the antibodies used was confirmed in a previous study [22]. Flow cytometric analysis finally demonstrated that 55% of the cells in culture co-express CXCR4 and CXCR7 at their cell surface (Figure 1**C**,**D**). Almost 20% of the cells only expressed CXCR4 at their surface, whereas a small population of about 6% of the cells showed surface expression of only CXCR7 (Figure 1**D**). The remaining cells were either dead or formed aggregates and subsequently escaped analysis. Collectively these findings establish that in culture a major subpopulation of microglial cells co-express CXCR4 and CXCR7.

Previous studies using FRET and BRET provided evidence that CXCR4 and CXCR7 not only exist as preformed homodimers, but also as preformed heterodimers [15,34,35]. To additionally determine whether CXCR4 and CXCR7 form heterodimers in microglial cells, we immunoprecipitated CXCR4 or CXCR7 from microglial cells and analysed the isolated proteins for coprecipitated CXCR7 or CXCR4, respectively, by Western blotting. For control purposes, immunoprecipitation experiments were performed with a previously established subclone of C2C12 myoblasts only expressing CXCR7 ($C2C12^{CXCR7}$) [30]. Analysis of CXCR4 immunoprecipitated from microglial cells allowed the detection of large amounts of coprecipitated CXCR7 and vice versa analysis of immunoprecipitated CXCR7 allowed the detection of coprecipitated CXCR4 (Figure 1**E**). As expected, CXCR7 remained undetectable in CXCR4 immunoprecipitates from cells of $C2C12^{CXCR7}$ subclone (Figure 1**E**). These findings further reveal that at least in part CXCR4 and CXCR7 form heterodimers in microglial cells.

SDF-1-dependent signalling in primary microglia requires both CXCR4 and CXCR7

Signalling molecules reportedly activated by SDF-1 in its target cells include p38, extracellular-signal-regulated kinases 1 and 2 (Erk1/2), Akt, the conventional protein kinase C (PKC) isoforms α and β (PKCα/β), and the atypical PKC isoforms ζ and λ (PKCζ/λ) [9,21,36–38]. Western blot analysis and the use of phosphospecific antibodies revealed that treatment of cultured microglia with SDF-1 resulted in the phosphorylation (activation) of Erk1/2 and Akt (Figure 2), but not in the phosphorylation (activation) of PKC isoforms (data not shown). Activation of Erk1/2 and Akt was maximal after 3 min of SDF-1 treatment and returned to control levels after 6–10 min (supplementary Figure S2). Moreover, the effects of SDF-1 on Erk1/2 and

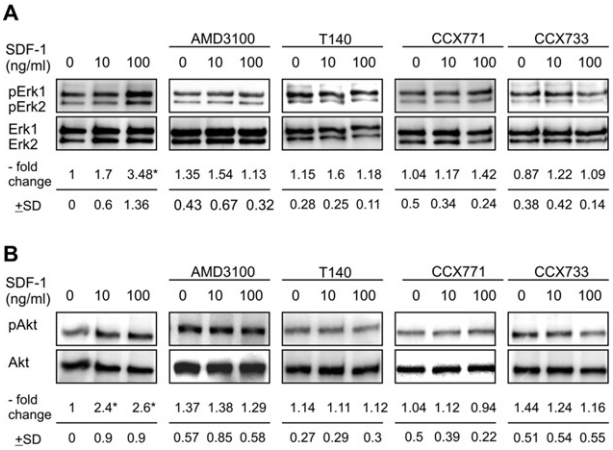

Figure 2. SDF-1-dependent signalling in primary microglia requires the presence of CXCR4 and CXCR7. Cultures of primary rat microglia were maintained for 1 h in the presence or absence of AMD3100 (100 ng/ml), T140 (100 ng/ml), CCX771 (100 nM) or CCX733 (100 nM) followed by a 3-min treatment with SDF-1 at the indicated concentrations. Effects of the various treatments on activation (phosphorylation) of (**A**) Erk1/2 and (**B**) Akt were analysed by Western blotting and the use of phosphospecific antibodies. To control for protein loading, blots were additionally stained with antibodies recognizing Erk1/2 and Akt independent of their phosphorylation status, Numbers show average levels ± SD of phosphorylated (p)Erk1/2 and phosphorylated (p)Akt corrected for protein loading as determined in four to nine independent experiments. Levels of pErk1/2 and pAkt present in untreated controls were set to 1. SDF-1 allowed for a dose-dependent increase in the activity of both Erk1/2 and Akt. SDF-1 failed to activate Erk1/2 and Akt in the presence of either CXCR4 antagonists or CXCR7 antagonists. $^{*}P \leq 0.002$.

Akt activation were dose-dependent with maximal activation of both signalling molecules in the presence of 100 ng/ml of the chemokine (Figure 2). To subsequently determine whether SDF-1 activates cell signalling in microglial cells through CXCR4 and/or CXCR7, we analysed SDF-1-dependent cell signalling in the presence of either the CXCR4 receptor antagonists, AMD3100 and T140 [39,40], or the CXCR7 antagonists, CCX771 and CCX733 [18,20]. Both AMD3100 and T140 prevented SDF-1-dependent activation of Akt and Erk1/2 in microglial cells (Figure 2). In addition, SDF-1-dependent cell signalling was abrogated in the presence of either CCX771 or CCX733 (Figure 2). In contrast, SDF-1 (100 ng/ml) signalling persisted in the presence of the control peptide CCX704 (100 ng/ml) resulting in a 2.0 ± 0.2-fold ($P < 0.001$; $n = 5$) and 2.3 ± 0.5-fold increase ($P < 0.001$; $n = 6$) in Erk1/2 and Akt activity respectively. Together, these findings establish that in microglial cells both CXCR7 and CXCR4 are required for mediating SDF-1 cell signalling.

CXCR4- and CXCR7-dependent signalling controls proliferation and migration of primary microglia

A prime function of SDF-1 is the control of cell proliferation and cell migration [9,21,41]. To assess whether SDF-1 acts as a mitogen for microglia, we maintained cultured microglial cells for 48 h with SDF-1 and subsequently analysed the cells for expression of the proliferation marker, Ki67. We found that treatment with SDF-1 (100 ng/ml) approximately doubled the number of Ki67-expressing cells (Figure 3**A**). To further assess whether SDF-1 would also represent a chemoattractant for microglial cells, we analysed the migratory response of microglia to the chemokine in a modified Boyden chamber. We found that cultured microglial cells migrated towards SDF-1 (Figure 3**B**). The migratory response was absent when we applied SDF-1 to both the lower and the upper well of the Boyden chamber (data not shown). This further suggests that the observed migratory response results from chemotaxis, and not from chemokinesis. We also found that the chemotactic response induced by SDF-1 was similar or even slightly higher than that elicited by MCP-1 or MIP1α (supplementary Figure S3). Both chemokines represent established chemoattractants for microglial cells [42,43]. Only ATP, another established chemoattractant for microglia [44,45], elicited a more pronounced migratory response than SDF-1 (supplementary Figure S3).

To determine whether SDF-1 controls microglial proliferation and migration through Erk1/2 and/or Akt, we studied the SDF-1-dependent effects in the presence of either the Mek1/2 inhibitor, UO126, which subsequently prevents activation of Erk1/2 or the phospho-inositol-3-kinase/Akt inhibitor, Ly294002. The proliferative response of microglial cells to SDF-1 (100 ng/ml; 48 h) was completely abrogated in the presence of either UO126 or Ly294002 (Figure 3**A**). In contrast, microglial cells failed to migrate towards SDF-1 following treatment with UO126, whereas the migratory response fully persisted following treatment with Ly294002 (Figure 3**B**). These findings establish that SDF-1 controls microglial proliferation through Erk1/2 and Akt signalling and further demonstrate that SDF-1-dependent chemotaxis of microglial cells solely depends on Erk1/2 signalling.

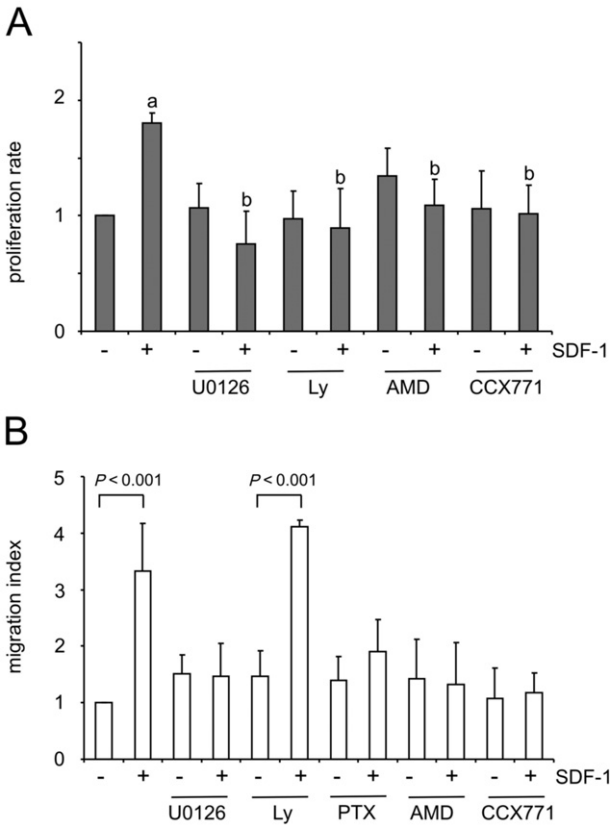

Figure 3. SDF-1 controls proliferation and migration of cultured primary microglia. (**A**) Analysis of mitogenic effects of SDF-1 on microglia. Cultures of primary rat microglia were treated for 48 h with SDF-1 in the absence or presence of UO126 (250 nM), Ly294002 (250 nM), AMD3100 (50 ng/ml) or CCX771 (100 nM) and subsequently analysed for expression of the proliferation marker, Ki67, as described in Materials and Methods. The number of Ki67-expressing cells present in untreated controls was set to 1. SDF-1 promoted proliferation of cultured primary microglia. Proliferative effects were absent in the presence of either the Mek/Erk and Akt inhibitors, UO126 and Ly294002 respectively. Microglial cells further showed no proliferative response to SDF-1 in the presence of either the CXCR4 antagonist, AMD3100, or the CXCR7 antagonist, CCCX771. Bars represent average mitogenic response ± SD as determined in four to six independent experiments. [a]$P < 0.001$, SDF-1 treated cultures *vs.* untreated cultures; [b]$P < 0.001$, cotreatment *vs.* SDF-1 alone. (**B**) Analysis of the migratory response of microglia to SDF-1. SDF-1-dependent chemotaxis of primary microglia was assessed in a modified Boyden chamber as described in Materials and Methods. The number of cells migrating in the absence of SDF-1 was set to 1. Bars represent relative numbers of migrated cells (mean ± SD) as determined in 4–10 independent experiments. SDF-1 promoted migration of primary microglia. The migratory response was sensitive to the Mek/Erk inhibitor, UO126, but not to the Akt inhibitor Ly294002. Microglial cells failed to respond to SDF-1 with chemotaxis in the presence of either AMD3100 or CCX771. SDF-1-dependent chemotaxis was further undetectable in the presence of PTX (50 ng/ml).

To additionally verify that SDF-1-dependent control of microglial proliferation and migration requires both CXCR7 and CXCR4, we tested the responses of cultured microglia to SDF-1 in the presence of CXCR4 and CXCR7 antagonists. We found that SDF-1 failed to increase the number of Ki67-expressing cells in the presence of either the CXCR4 antagonist, AMD3100, or the CXCR7 antagonists, CCX771 (Figure 3**A**). Likewise, microglial cells showed no SDF-1-dependent chemotactic response in the presence of either AMD3100 or CCX771 (Figure 3**B**). However, SDF-1-dependent chemotaxis occurred with the control compound CCX704 (data not shown). These findings confirm that in microglia CXCR4 and CXCR7 form a functional receptor unit, which is essential for the SDF-1-dependent control of microglial proliferation and migration.

A previous study demonstrated that in several cell lines the formation of CXCR4/CXCR7 complexes impairs G protein-mediated cell signalling and potentiates β-arrestin-dependent signalling [46]. To assess whether or not SDF-1 signals through G proteins in microglia, we tested primary microglia for SDF-1-dependent chemotaxis in the presence of PTX. We found that PTX treatment completely abolished the chemotactic response of primary microglia to SDF-1 (Figure 3**B**). These findings further establish that the function of CXCR4 and CXCR7 as a receptor unit in microglial cells is not associated with a switch from G protein- to β-arrestin-mediated cell signalling.

Microglial activation both *in vitro* and *in vivo* is associated with the increased expression of CXCR4 and CXCR7

It is generally agreed that during cultivation microglia do not remain in a resting state, but rather become partially activated [1,47]. Moreover, previous studies obtained evidence that in the normal brain microglial cells exhibit low expression levels of CXCR4 and are devoid of CXCR7 [25,48]. To assess whether activation of microglial cells is associated with the increased expression of CXCR4 and/or CXCR7, we maintained cultured microglial cells for 24 h in the presence or absence of LPS and double-stained them with antibodies for Iba-1 and either CXCR4 or CXCR7. In the absence of LPS, few Iba-1-positive cells exhibited faint immunolabelling for CXCR4 or CXCR7. Following LPS treatment, the number of CXCR4- and CXCR7-positive cells clearly increased (Figure 4**A**). Double

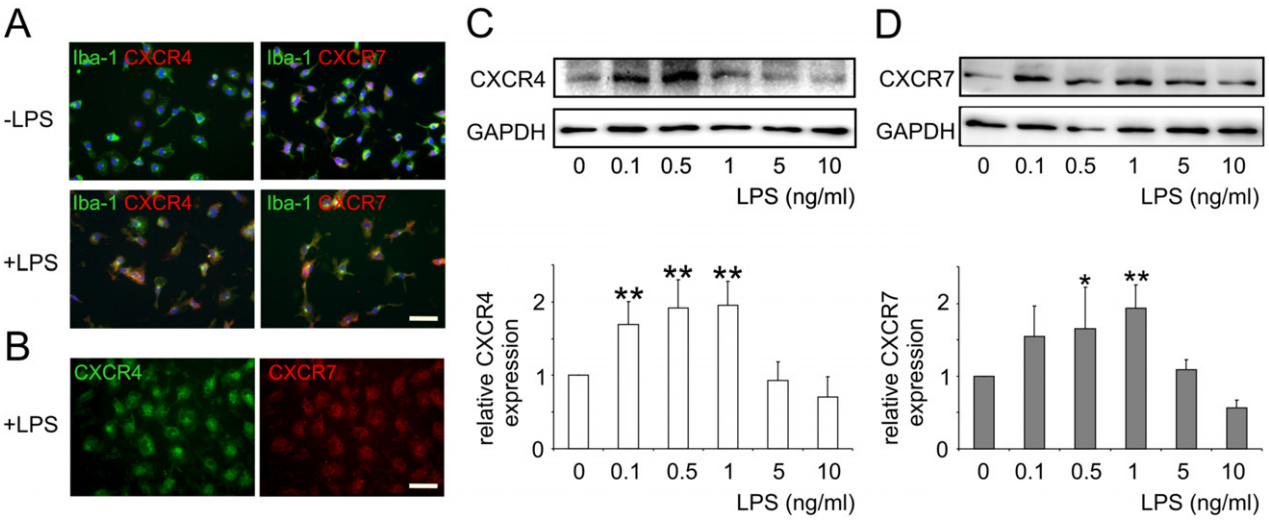

Figure 4. Activation of cultured primary microglia with lipopolysaccharide (LPS) results in the increased expression of CXCR4 and CXCR7. (**A**) Microglial cultures were maintained for 24 h in the presence or absence of LPS (1 ng/ml) and subsequently double-labelled with antibodies against Iba-1 (green) and CXCR4 or CXCR7 (both red). The overlay of the stainings (orange to yellow) demonstrates that LPS increases the number of microglial cells expressing CXCR4 and CXCR7. Cell nuclei were visualized by DAPI (blue). Scale bar, 25 µm. (**B**) Double immunostaining revealing that CXCR4 and CXCR7 are co-expressed by LPS (1 ng/ml, 24 h)-activated microglia. Scale bar, 25 µm. (**C**, **D**) Microglial cultures were treated for 24 h with the indicated concentrations of LPS and CXCR4 and CXCR7 protein levels were determined by Western blotting. GAPDH served as loading control. Numbers represent relative changes (mean ± SD, $n = 5–7$) in CXCR4 or CXCR7 protein levels under the various treatments. CXCR4 and CXCR7 protein levels present in untreated controls were set to 1. LPS at 1 ng/ml, but not at higher concentrations increased both CXCR4 and CXCR7 expression. $^{*}P < 0.02$, $^{**}P < 0.001$.

immunolabelling additionally revealed that LPS results in the co-ordinated up-regulation of both CXCR4 and CXCR7 in cultured microglia (Figure 4**B**). When we quantified expression levels of both chemokine receptors by Western blotting, we found that treatment with LPS (1 ng/ml) for 24 h resulted in a similar 1.5-fold to 1.6-fold increase in CXCR4 and CXCR7 protein levels (Figure 4**C**,**D**). Increases in CXCR4 and CXCR7 protein levels were absent with LPS at higher concentrations (>1 ng/ml). We actually noticed that at these high concentrations the cultured cells started to deteriorate, which is consistent with the previous demonstration that high concentrations of LPS induce microglial apoptosis [49–51]. In fact, whereas microglial cell numbers remained virtually unchanged in cultures maintained with 1 ng/ml of LPS (104 ± 7% of controls, $n = 6$), microglial cell numbers decreased by 47 ± 11% ($P < 0.001$, $n = 5$) and 66 ± 2% ($P < 0.001$, $n = 6$) in the presence of 5 ng/ml and 10 ng/ml of LPS respectively. Flow cytometry further revealed that the increase in CXCR4 and CXCR7 protein levels induced by LPS treatment (1 ng/ml, 24 h) is associated with a 2.5-fold and 2.0-fold increase in cell surface expression levels of CXCR4 and CXCR7 respectively (supplementary Figure S4). To assess whether a similar co-ordinated up-regulation of CXCR4 and CXCR7 would occur during activation of microglial cells *in vivo*, we studied microglial responses in the brain of rats at different time points after unilateral permanent MCAO. Labelling of coronal brain sections prepared from MCAO animals with antibodies against the microglial marker, Iba-1, confirmed that up to 24 h following permanent MCAO, microglial numbers increase in the tissue surrounding the infarct core (Figure 5**A**) [52,53]. Microglial numbers further increased up to 96 h post infarction and remained high up to 168 h (Figure 5**A**). The Iba-1-immunoreactive cells present in the area adjacent to the infarct core initially exhibited a process-bearing morphology. However, with time most Iba-1-immunoreactive cells showed a round morphology with either no or stout processes and, hence, resembled activated microglial cells (Figure 5**A**,**B**). Double labelling of brain sections with antibodies against Iba-1 and either CXCR4 or CXCR7 demonstrated that expression of CXCR4, but not of CXCR7 increases in Iba-1-positive microglia 72 h following MCAO (supplementary Figure S5). Starting 96 h following MCAO, Iba-1-positive cells

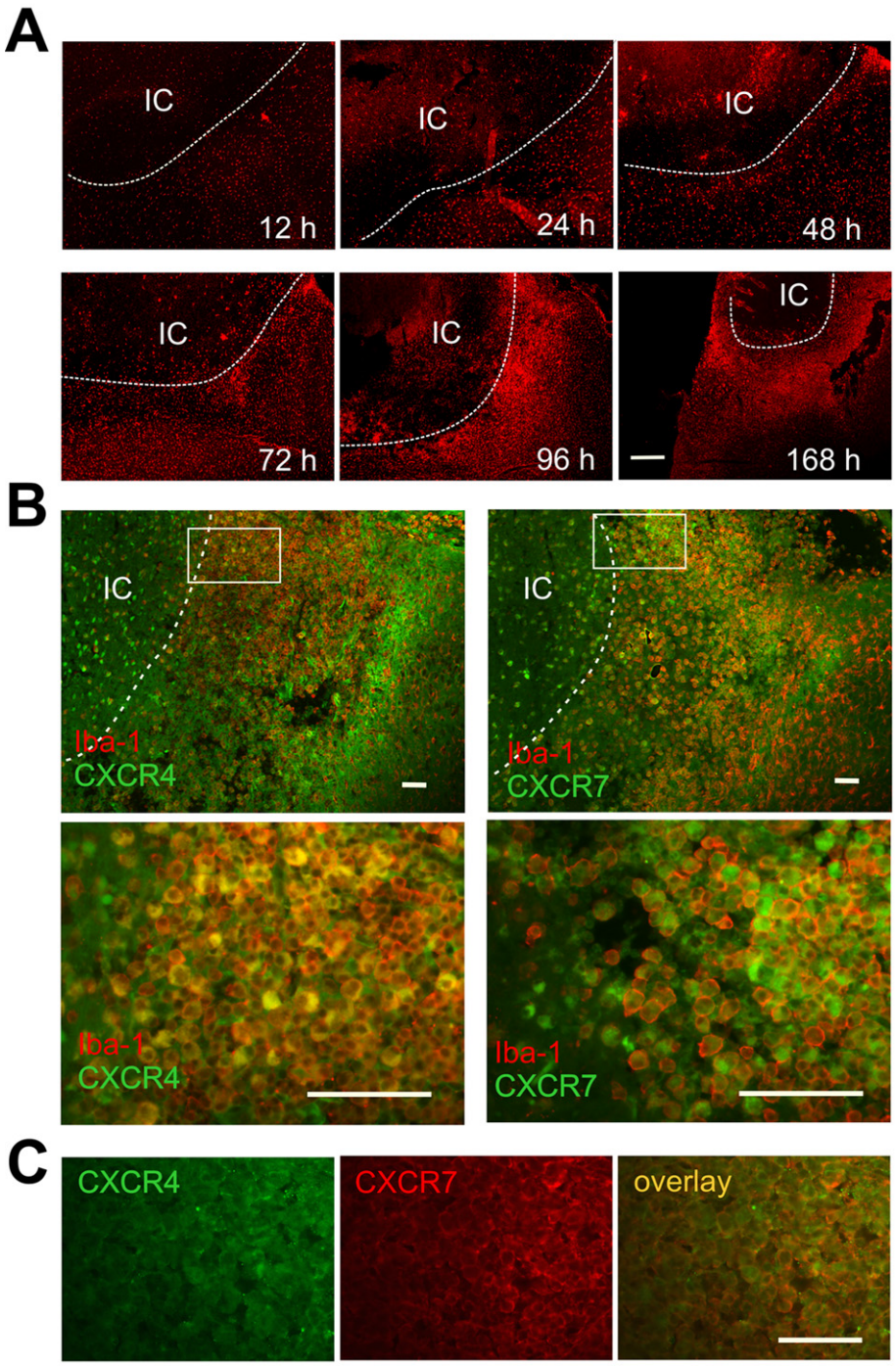

Figure 5. Activated microglia in the brain of animals with experimental stroke express CXCR4 and CXCR7. (**A**) Time-course of microglial accumulation within the area surrounding the infarct core (IC). Rats were subjected to unilateral permanent occlusion of the middle cerebral artery (MCAO) and sacrificed after the indicated time. Brains were removed, cryosectioned and stained with antibodies against the microglial marker, Iba-1. Within the area adjacent to the infarct core, numbers of Iba-1-positive microglial cells increased up to 96 h after MCAO. Iba-1-positive cells were initially process-bearing and attained a roundish morphology with time. Scale bar, 200 μm. (**B**) Double staining of coronal brain sections from animals 96 h after MCAO with Iba-1 (red) and CXCR4 or CXCR7 (green). Note that many Iba-1-positive cells locate adjacent to the infarct core express CXCR4 and CXCR7. IC, infarct core. Scale bar, 25 μm. (**C**) Double immunostaining of coronal brain sections from MCAO animals demonstrating that microglia accumulating adjacent to the infarct core co-express CXCR4 (green) and CXCR7 (red). Scale bar, 25 μm.

surrounding the infarct core exhibited increased expression of both CXCR4 and CXCR7 (Figure 5**B**). Double immunolabelling further revealed that similar to the situation *in vitro*, a major portion of microglia *in vivo* co-express CXCR4 and CXCR7 (Figure 5**C**). The analysis of brain sections obtained from animals after longer survival times further demonstrated that expression levels of CXCR4 and CXCR7 remain high for at least up to 240 h following MCAO (supplementary Figure S5). Notably, even after long survival times (>96 h), the immunohistochemical analyses allowed the detection of only small numbers of Iba-1-positive microglial cells in the contralateral (non-injured) hemisphere of MCAO animals, which showed faint immunostaining for CXCR4, and were virtually devoid of immunostaining for CXCR7 (supplementary Figure S5). Collectively, these findings establish that the CXCR4/CXCR7 receptor unit is up-regulated during activation of microglial cells.

Discussion

In contrast to the SDF-1 receptor, CXCR4, which represents a classical G protein-coupled receptor, the alternative SDF-1 receptor, CXCR7, seems to exert a rather diverse set of functions. Indeed, depending on the cell type and/or the biological condition, CXCR7 was reported to modulate the function of CXCR4 either by scavenging SDF-1 and thus controlling the extracellular availability of the chemokine or by forming heterodimers with CXCR4 [14–16]. Moreover, CXCR7 was found to act as a nonclassical G protein-coupled receptor signalling through β-arrestin in several nonneural cells/cell lines [17–20], and was further shown to function as a classical G protein-coupled receptor in primary astrocytes and astroglioma cell lines [22]. We now unravel that *in vitro* and *in vivo*, activated microglia express both CXCR4 and CXCR7. In addition, we provide evidence that CXCR4 and CXCR7 form a functional receptor unit in microglial cells.

In accordance with previous work [23,24], we found that cultured rodent microglia express CXCR4 at the mRNA and protein levels. We further observed that cultured rodent microglial cells also express CXCR7 at levels similar to those of CXCR4. FACS analysis finally revealed that a major subpopulation of cultured rodent microglia co-express CXCR4 and CXCR7 at their cell surface. Treatment of cultured microglia with SDF-1 resulted in the activation of Erk1/2 and Akt and the subsequent induction of microglial proliferation and migration. The use of the selective Mek (Erk) and Akt inhibitors, UO126 and Ly294002, respectively, further demonstrated that SDF-1 promotes microglial proliferation by signalling through Akt and Erk1/2 [54]. Moreover, in line with previous findings [55,56], these experiments revealed that SDF-1-dependent chemotaxis of microglial cells solely requires activation of Erk signalling.

The subsequent analysis of SDF-1-dependent cell signalling and resulting effects on cell function provided evidence that CXCR4 and CXCR7 form a functional receptor unit in microglial cells. Specifically, we observed that SDF-1-dependent activation of Erk1/2 and Akt is likewise abolished following pharmacological inactivation of either CXCR4 or CXCR7. Similarly, SDF-1 failed to promote proliferation and migration of cultured microglia in the presence of either CXCR4 or CXCR7 antagonists. It is of note that we previously obtained evidence that CXCR4 and CXCR7 would form a functional receptor unit in primary Schwann cells [21]. Indeed, similar to our present findings with microglial cells, SDF-1-dependent cell signalling in Schwann cells only occurred in the presence of both CXCR4 and CXCR7 and was abolished when either CXCR4 or CXCR7 was impaired [21]. As noted above SDF-1 controls microglial migration solely through Erk signalling, which in turn is only activated in the presence of both CXCR4 and CXCR7, we currently favour the assumption that at least in microglial cells, CXCR4 and CXCR7 co-operate by forming receptor heterodimers [15]. In line with this view, co-immunoprecipitation studies provided evidence for the physical interference of CXCR4 and CXCR7 in microglial cells. However, contrasting the previous demonstration that the formation of CXCR4/CXCR7 complexes is associated with a switch from G protein-dependent to β-arrestin-dependent cell signalling [46], we found that SDF-1 still signals through G proteins in microglial cells. Consequently, we can at present not dismiss the possibility that in microglial cells both chemokine receptors interact by a distinctly different mechanism.

Previous studies demonstrated that CXCR4 expression increases in microglia during HIV encephalitis and experimental allergic encephalomyelitis [26–28]. Underscoring the view that CXCR4 and CXCR7 function as a receptor unit in microglia of the diseased brain, we obtained evidence that CXCR4 and CXCR7 are up-regulated in a co-ordinated manner in activated microglia both *in vitro* and *in vivo*. In fact, treatment of cultured microglia with LPS resulted in similar increases in CXCR4 and CXCR7

expression. In addition, we found that a major portion of cultured microglia co-express CXCR4 and CXCR7. Likewise, numbers of microglia cells co-expressing CXCR4 and CXCR7 increased in the brain of rats with experimental cerebral ischaemia (unilateral permanent MCAO occlusion). In accordance with previous studies [52], we found that CXCR4- and CXCR7-expressing microglia accumulated in the area adjacent to the lesion core within the first week following MCAO and were basically undetectable in non-affected brain areas. The absence of both chemokine receptors in microglia from non-injured brain areas within the initial days after MCAO reflects the previous demonstration that in the normal brain, CXCR4 is expressed only at low levels by microglia [25] as well as with the finding that CXCR7 expression is absent from microglia of the developing and adult rat brain [48]. Notably, the accumulation of Iba-1-positive cells within the area surrounding the infarct core was first detectable 12–24 h after MCAO with a further increase in cell numbers between 48 h and 72 h after MCAO. Moreover, increases in microglial CXCR4 expression first became evident 72 h after MCAO whereas CXCR7 expression first increased 96 h following MCAO. Based on the data available, we are unable to discern whether the initial increase in Iba-1-positive cells within the affected brain area followed by an increase in CXCR4- and CXCR7-expressing microglial cells results from the ongoing recruitment of activated, amoeboid microglial or from infiltrating blood-borne macrophages [53]. Depending on the experimental design, infiltration of blood-borne macrophages into the infarct area has been demonstrated to occur 2–7 days after MCAO [53]. Further consistent with the view that CXCR4 and CXCR7 are functional in microglia of the injured brain, a previous work demonstrated that 24 h following transient MCAO SDF-1 expression increases in the infarct area and remains elevated for at least 30 days [57]. Whether beyond microglial growth and migration, the ligand-activated CXCR4/CXCR7 receptor unit controls additional injury-related processes remains to be established. In this respect, it is interesting to note that a previous study using the microglial cell line, BV-2, provided evidence that SDF-1 by signalling through Akt and Erk1/2 regulates IL-6 production in microglia [58].

In conclusion, our findings provide evidence that CXCR4 and CXCR7 form a functional receptor unit in microglial cells, which is up-regulated during microglial activation and subsequently controls microglial proliferation and migration in the injured brain.

Acknowledgements

We thank Dr Mark E. Penfold (ChemoCentryx) for providing us with CCX771, CCX704, CCX733 and CXCR7 antibodies. This work was supported by the Deutsche Forschungsgemeinschaft (En187/9-1). J.L., V.Ö., D.-C.W. and J.B. planned and performed experiments and analysed data. J.E. designed the study and wrote the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Characterization of microglial cultures. Microglial cells were shaken off from dissociated cell cultures of P2–3 rat cortex and replated. Staining of the replated cells with antibodies against the microglial markers, (**A**) Iba-1 and (**B**) lectin, demonstrated that the established cultures predominantly (≥99%) consist of microglial cells. Cell nuclei were visualized by DAPI. Scale bar, 25 μm.

Figure S2. Time-course of SDF-1-dependent activation of Erk1/2 and Akt signalling in primary microglial cells. Cultured primary rat microglia were treated for the indicated time with SDF-1 (100 ng/ml) and subsequently analysed for activation (phosphorylation) of (**A**) Erk1/2 and (**B**) Akt by Western blotting and the use of phosphospecific antibodies. Protein loading was controlled by additionally staining blots with antibodies recognizing Erk1/2 and Akt independent of their phosphorylation status. Immunoreactive protein bands were quantified by densitometry and corrected for protein loading. Levels of phosphorylated

(p)Erk1/2 and pAkt present in untreated controls were set to 1. Bars represent mean ± SD ($n = 3$). Levels of both pErk1/2 and pAkt were maximal after 3 min of SDF-1 treatment and returned to control levels after 6 min and 10 min respectively. $^{**}P < 0.005$; $^{*}P < 0.02$, treatment *vs.* untreated control.

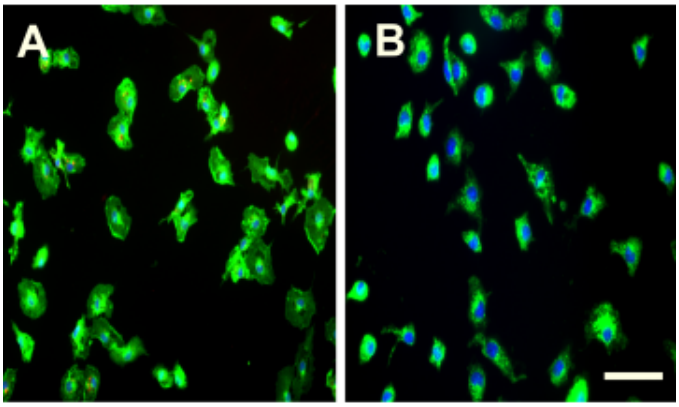
Figure S3. Comparative analysis of the chemotactic response of microglial cells to various chemokines and to ATP. The chemotactic response of primary rat microglial cells was tested using a modified Boyden chamber. Cell migration was determined after 4 h. The number of cells that migrated in the absence of any compound was set to 1. Bars represent the mean number of migrated cells ± SD relative to controls (migration index) as determined in three to four independent experiments. The chemokines MCP-1, MIP1α and SDF-1 induced microglial cell migration with similar potencies. Compared with chemokines, ATP more potently stimulated microglial migration. $^{*}P < 0.05$; $^{**}P < 0.001$.

Figure S4. Effects of LPS on CXCR4 and CXCR7 cell surface expression. Cultured primary microglia were treated with the indicated concentrations of LPS for 24 h and subsequently analysed for (**A**) CXCR4 and (**B**) CXCR7 cell surface expression levels by flow cytometry. Expression levels of CXCR4 and CXCR7 determined in untreated controls were set to 1. Bars represent mean expression levels ± SD as determined in five to six independent experiments. Cell surface expression of both chemokine receptors increased following treatment with LPS at 1 ng/ml, but not following treatment with higher concentrations. $^{*}P < 0.001$, treatment vs. untreated control.

Figure S5. Time-course of CXCR4 and CXCR7 expression in microglia cells following experimental stroke. Rats were subjected to unilateral permanent MCAO and sacrificed after the indicated times. Brains were removed, cyrosectioned, and double stained with antibodies against Iba-1 (red) and CXCR4 or CXCR7 (both green). On the lesioned (ipsilateral) side, microglia located adjacent to the infarct core showed increased expression of CXCR4 72 h after MCAO whereas increases in CXCR7 expression became first detectable after 96 h (see Figure 5). Expression levels of CXCR4 and CXCR7 remained high for at least up to 240 h following MCAO. Further note that Iba-1-positive microglial cells, present in the contralateral (non-injured) hemisphere, express only extremely low levels of CXCR4, and are virtually devoid of CXCR7. Scale bar, 50 μm.

Received 1 August 2012
Accepted after revision 30 November 2012
Published online Article Accepted on 7 January 2013

Supplementary Material



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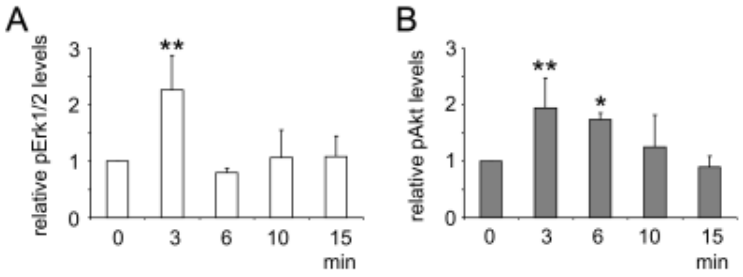

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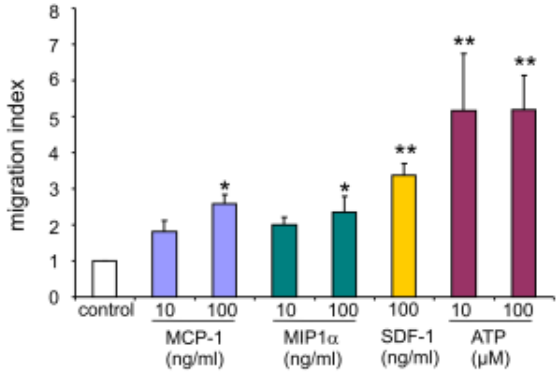

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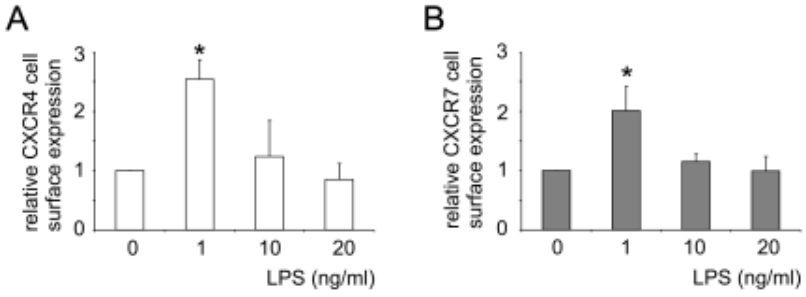

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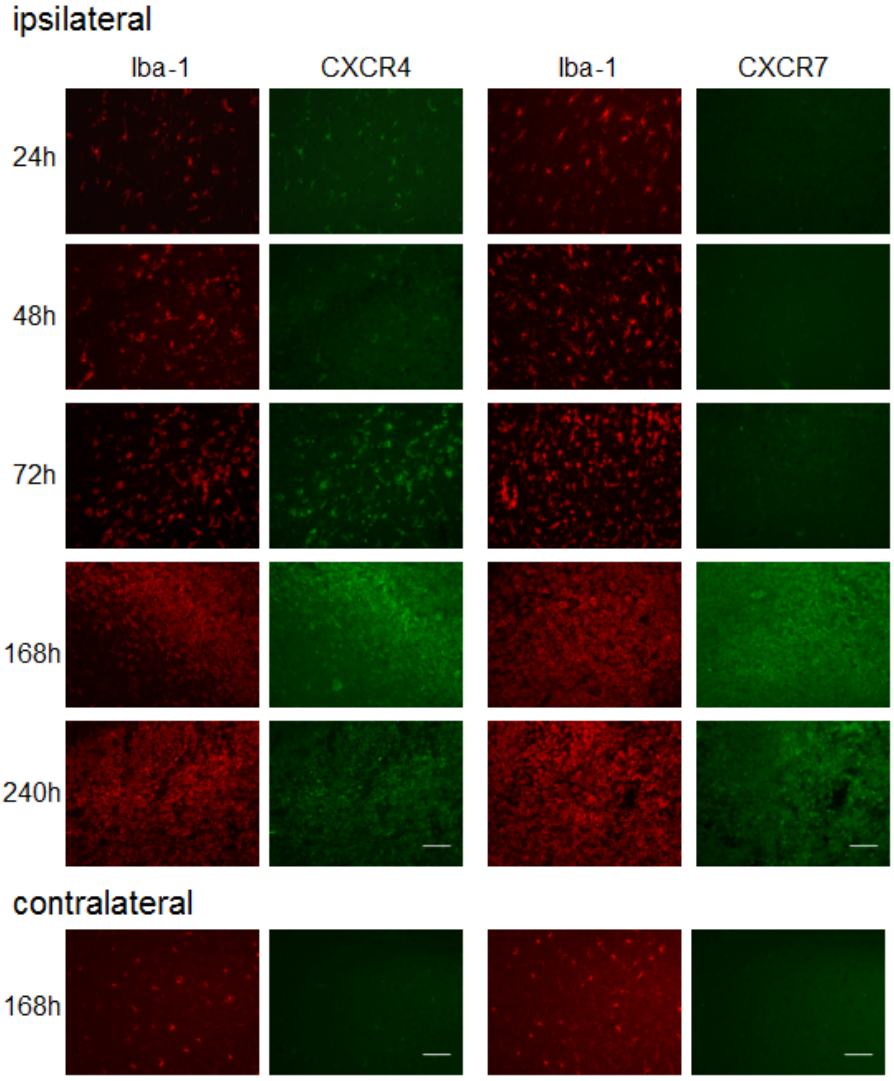

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eingereicht am: 12.07.2011
akzeptiert am: 26.10.2011

Veröffentlichung: Glia 60:372-381 (2012)

GLIA

GLIA 60:372–381 (2012)

The Presumed Atypical Chemokine Receptor CXCR7 Signals Through $G_{i/o}$ Proteins in Primary Rodent Astrocytes and Human Glioma Cells

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KEY WORDS
CXCR4; G proteins; SDF-1; I-TAC

ABSTRACT
SDF-1/CXCL12 binds to the chemokine receptors, CXCR4 and CXCR7, and controls cell proliferation and migration during development, tumorigenesis, and inflammatory processes. It is currently assumed that CXCR7 would represent an atypical or scavenger chemokine receptor which modulates the function of CXCR4. Contrasting this view, we demonstrated recently that CXCR7 actively mediates SDF-1 signaling in primary astrocytes. Here, we provide evidence that CXCR7 affects astrocytic cell signaling and function through pertussis toxin-sensitive $G_{i/o}$ proteins. SDF-1-dependent activation of $G_{i/o}$ proteins and subsequent increases in intracellular Ca^{2+} concentration persisted in primary rodent astrocytes with depleted expression of CXCR4, but were abolished in astrocytes with depleted expression of CXCR7. Moreover, CXCR7-mediated effects of SDF-1 on Erk and Akt signaling as well as on astrocytic proliferation and migration were all sensitive to pertussis toxin. Likewise, pertussis toxin abolished SDF-1-induced activation of Erk and Akt in CXCR7-only expressing human glioma cell lines. Finally, consistent with a ligand-biased function of CXCR7 in astrocytes, the alternate CXCR7 ligand, I-TAC/CXCL11, activated Erk and Akt through β-arrestin. The demonstration that SDF-1-bound CXCR7 activates $G_{i/o}$ proteins in astrocytes could help to explain some discrepancies previously observed for the function of CXCR4 and CXCR7 in other cell types.

INTRODUCTION

The CXC chemokine, SDF-1/CXCL12, was originally identified as a regulator of hematopoiesis and immune cell function (Broxmeyer, 2008), and was subsequently shown to additionally control the development of cardiovascular system, limb musculature, as well as peripheral and central nervous system (Buckingham, 2006; Ödemis et al., 2005; 2007; Tachibana et al., 1998; Zou et al., 1998). In the developing and mature brain, virtually all types of CNS cells such as neural progenitors, neurons, microglial cells, and astrocytes as well as endothelial cells of brain blood vessels are targets for SDF-1 (Li and Ransohoff, 2008). Developmental aspects controlled by SDF-1 in the CNS include cell proliferation, migration, and (neuronal) cell survival (Li and Ransohoff, 2008). A recognized function of SDF-1 in the mature CNS is the control of intercellular communication between brain cells (Bezzi et al., 2001; Rostene et al., 2007). In the injured CNS, SDF-1 regulates inflammatory processes (Cartier et al., 2005; Li and Ransohoff, 2008) and promotes tissue regeneration by recruiting neural progenitor cells to the site of injury (Tran et al., 2007).

While it has been originally held that SDF-1 would exclusively signal through the chemokine receptor, CXCR4, more recently the previous orphan seven transmembrane receptor, RDC-1/CXCR7, has been identified as an alternative SDF-1 receptor (Balabanian et al., 2005). CXCR7 shows a roughly 10 times higher binding affinity for SDF-1 as compared with CXCR4 (Balabanian et al., 2005; Crump et al., 1997) and additionally binds interferon-inducible T-cell α chemoattractant (I-TAC, CXCL11) as a second ligand (Burns et al., 2006). The currently available data imply that in contrast to CXCR4, CXCR7 would represent an atypical or scavenger chemokine receptor. Similar to the atypical chemokine receptors, D6 and CCX-CKR (Graham, 2009), CXCR7 contains a modified DRYLAIV motif; this motif is assumed to be essential for G protein binding (Thelen and Thelen, 2008). Moreover, several studies failed to demonstrate CXCR7-dependent activation of G proteins and subsequent Ca^{2+}-induced chemotaxis (Burns et al., 2006; Levoye et al., 2009; Rajagopal et al., 2010b; Sierro et al., 2007). Studies on the migration of primordial germ cells further revealed that CXCR7 controls CXCR4-mediated cell migration by sequestering extracellular SDF-1 and, hence, shaping the extracellular chemokine gradient (Boldajipour et al., 2008). More recently, this scavenger function was additionally highlighted by the demonstration of a constant cycling of

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: DFG; Grant number: KR 3408/2-1 (to R.K.).

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Received 12 July 2011; Accepted 26 October 2011

DOI 10.1002/glia.22271

Published online 14 November 2011 in Wiley Online Library (wileyonlinelibrary.com).

CXCR7 between the plasma membrane and intracellular compartments as well as a nonsaturating binding of SDF-1 to CXCR7 (Naumann et al., 2010). Intriguingly, despite this atypical function, CXCR7 induces cell signaling in various types of tumor cells and controls their growth, adhesion, and transendothelial migration (Burns et al., 2006; Grymula et al., 2010; Miao et al., 2007; Wang et al., 2008; Zabel et al., 2009). In addition, CXCR7-mediated cell signaling occurs in primary interneurons (Wang et al., 2011). Along the same line, we recently demonstrated that in primary astrocytes, SDF-1-dependent activation of several signaling molecules/pathways including Erk, Akt, and PKCζ/λ is entirely mediated by CXCR7 (Ödemis et al., 2010). Since CXCR7 associates β-arrestin, it has been suggested that CXCR7 would affect cell function through a β-arrestin-dependent mechanism (Luker et al., 2009; Kalatskaya et al., 2009; Rajagopal et al., 2010a; Zabel et al., 2009). We now provide evidence that in primary rodent astrocytes and human astrocytoma cell lines, CXCR7 induces cell signaling and controls cell proliferation and migration through pertussis toxin-sensitive $G_{i/o}$ proteins.

MATERIALS AND METHODS

Cell Cultures

Astroglial cultures, containing >90% GFAP-expressing cells, were established from the cerebral hemispheres of postnatal day (P) 2 to 3 Sprague Dawley rats or embryonic day (E)18 $CXCR4^{-/-}$ mice (Zou et al., 1998) as described previously (Ödemis et al., 2010). The human glioma cell lines, U343 and A764, were propagated in 90% DMEM (Lonzan) and 10% fetal calf serum (FCS; Hattermann et al., 2010). Primary astrocytic cultures and glioma cell cultures were switched to serum-free N2-medium 24 h and 6 h before experiments, respectively, and additionally treated with the following substances as specified in the text: SDF-1α (Millipore, Billerica, MA), I-TAC (Biozol, Eching, Germany); FGF-2 (Peprotech, Rocky Hill, NJ); AMD3100 (dissolved in water; Sigma-Aldrich, St. Louis, MO), CCX771 (dissolved in DMSO; ChemoCentryx, Mountain View, CA), U73122, U73343 (both Merck, Darmstadt, Germany), PTX (Merck).

Western Blot Analysis

Cells were lysed by ultrasonication in 62.5 mM Tris-HCl, containing 2% SDS and 10% sucrose. Proteins were denatured at 95°C for 5 min and further diluted in sample buffer (250 mM Tris-HCl, pH 6.8 containing 4% SDS, 10% glycerol, and 2% β-mercaptoethanol). For detection of phosphorylated proteins, sample buffer was additionally supplemented with sodium orthovanadate (10 mM). Protein content of cell lysates was determined using the BCA protein estimation kit (Pierce; Rockford, IL) and bovine serum albumin (BSA) as a standard. Proteins (15 μg/lane) were separated by SDS-(10%) polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. Upon blocking nonspecific binding sites with 3% BSA for 60 min, blots were incubated overnight at 4°C with one of the following antibodies: anti-CXCR3 (1:1,000; sc-133121, Santa Cruz Biotechnology, Santa Cruz, CA); anti-CXCR4 (1:1,000; ab2074, Abcam, Cambridge, MA), anti-CXCR7 (1:1,000; AP17961PU-N, Acris, Herford, Germany), anti-β-arrestin1 (1:1,000; sc-9182, Santa Cruz Biotechnology), anti-β-arrestin2 (1:1,000; sc-13140, Santa Cruz Biotechnology), phosphospecific anti-Erk1/2 (1:3,000; ab #4377, Cell Signaling Technology Danvers, MA) or phosphospecific anti-Akt (1:3,000; ab #9271, Cell Signaling Technology). Antibody labeling was detected by incubating cultures for 2 h at room temperature with appropriate horseradish peroxidase-labeled secondary antibodies (Dianova, Hamburg, Germany) and visualized with the enhanced chemiluminescence kit (Amersham Pharmacia, Freiburg, Germany). To control for protein loading, blots were additionally stained with either anti-GAPDH (1:6,000; 10R-G109a, Fitzgerald Industries International, North Acton, MA) antibodies, or antibodies against nonphosphorylated Erk1/2 (1:2,000; ab #9107) or Akt (1:2,000; ab #2920, both Cell Signaling Technology). Blots were recorded using the MF-Chemi-BIS 1.6 gel imaging system (Biostep, Jahnsdorf, Germany) and integrated optical densities of immunoreactive protein bands were measured using the Gel-Pro Analyzer (Media Cybernetics, Bethesda, MD). Data were normalized to the respective loading controls.

Calcium Imaging

Measurements of $[Ca^{2+}]i$ in single astrocytes were carried out using the fluorescent indicator fura-2 in combination with a monochromator-based imaging system (T.I.L.L. Photonics). For experiments, cells were seeded on poly-L-lysine coated glass coverslips in 12-well plates. Cells were loaded with 5 μM fura-2-AM (Molecular Probes) supplemented with 0.01% Pluronic F127 for 60 min at 20°C to 22°C in a standard bath solution containing (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 Hepes, adjusted to pH 7.4 with NaOH. For measurements of $[Ca^{2+}]i$, cells were kept in standard bath solution and fluorescence was excited at 340 and 380 nm. Emitted fluorescence from single cells exhibiting glial morphology was acquired at intervals of 2 s and the fluorescence ratio R = F340/F380 was calculated. Quantities for $[Ca^{2+}]i$ were calculated with the equation $[Ca^{2+}]i = K_D\beta(R - R_{min})/(R_{max} - R)$, where K_D = 224 nM, β = 4.076, R_{min} = 0.300, and R_{max} = 2.753, obtained from single dye-loaded cells (n = 47) in the presence of 10 μM ionomycin added to standard bath solution or to a solution containing 10 mM EGTA instead of 2 mM $CaCl_2$. In each experiment, signals from 9 to 11 cells were averaged.

[^{35}S]-GTPγS Binding Assay

Astrocytes were lysed in ice-cold assay buffer (50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 10 mM $MgCl_2$, 200 mM

NaCl, 0.2 mM DTT) and centrifuged at 40,000*g* at 4°C for 30 min. After washing, membrane fractions were resuspended in ice-cold assay buffer (1–2 mg protein/mL) and processed for [^{35}S]-GTPγS binding assay the same day. Assay was carried out in duplicate in assay buffer, containing guanosine 5′-diphosphate (10 μM), [^{35}S]GTPγS (0.5 nM), SDF-1 or I-TAC, and 3 μg membrane protein for 120 min at 30°C. The assay was terminated by adding 6 mL ice-cold assay buffer and rapid filtration through presoaked GF/B glass fibre filters (Whatman, Clifton, NJ). Filters were washed, dried overnight, and bound radioactivity was measured by liquid scintillation counting. [^{35}S]-GTPγS binding was expressed as per cent increase in radioactivity in the presence of drugs relative to basal levels of binding. Nonspecific [^{35}S]-GTPγS binding was determined in the presence of unlabelled GTPγS.

Proliferation Assay

Proliferating cells were identified by Ki67 expression and quantified by flow cytometric analysis. Cells were fixed with paraformaldehyde (2%, w/v) in PBS for 15 min. Following permeabilization with 0.05% saponin, cells were incubated for 45 min at 4°C with anti-Ki67 antibodies (1:500; ab15580, Abcam) and for further 30 min with fluorescein isothiocyanate-labeled secondary antibodies (1:500; Jackson Laboratories, West Grove, PA). Flow cytometric analysis was performed with a FACScan (Becton Dickinson, Heidelberg, Germany). The forward narrow angle light scatter was used to exclude dead and aggregated cells.

RNA Interference

Predesigned rat CXCR3, CXCR4, CXCR7, β-arrestin1 and β-arrestin2, and respective nonhomologous (nh) siRNAs were purchased from Qiagen (Hilden, Germany). One hour before transfection, culture medium was replaced by 400 μL MEM containing 2% FCS. Transfection was performed with the siPORT™ Amine Transfection Agent (Ambion, Huntingdon, UK) according to the manufacturer's recommendations. For transfection, cultures additionally received overnight 100 μL of MEM supplemented with 2% FCS, the indicated concentrations of siRNA or nh siRNAs and 2.5 μL of transfection reagent. Transfected cultures were subsequently maintained with MEM containing 10% FCS and serum-free N2-medium for 24 h and submitted to experiments.

Chemotaxis

The chemotactic response of astrocytes to SDF-1 was evaluated using a modified 48-well Boyden chamber (Neuro Probe, Cabin John, MD), as previously described (Ödemis et al., 2002). For cell harvest, cultures were rinsed three times with N2-medium and cells were resuspended in fresh N2-medium. Fifty microliters of the cell suspension, containing 25,000 cells, were added to the upper well of the chamber. The lower well received 28 μL of N2 medium supplemented with the indicated concentrations of SDF-1. The upper and lower wells were separated by polyornithin-coated Nucleopore® PVP-free polycarbonate filters (Corning, Acton, MA) with 5 μm pore size, and the chamber was incubated for 4 h at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. After incubation, nonmigrating cells were scraped off from the upper surface of the filter. Migrated cells, attached to the lower surface, were fixed in methanol for 10 min, stained with hematoxylin/eosin and counted under a microscope at 100× magnification within a total area of 2 mm^2. Data were normalized by calculating the migration index, which was defined to be the ratio of cells migrating in the presence and absence of chemokines.

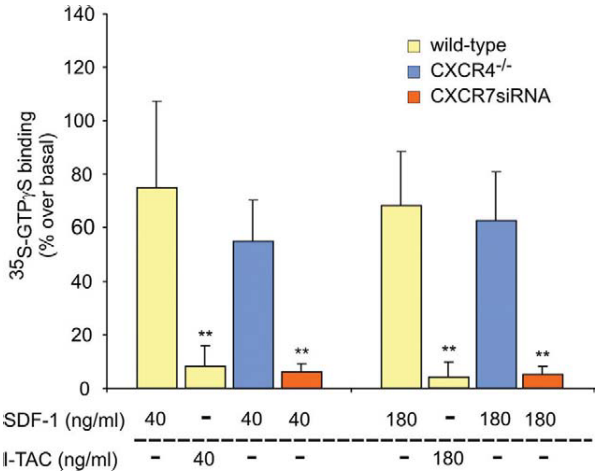

Fig. 1. SDF-1-bound CXCR7, but not SDF-1-bound CXCR4, activates G proteins in cortical rodent astrocytes. [^{35}S]GTPγS-binding assay demonstrated that SDF-1, but not I-TAC, activates G proteins in rat (wild-type) astrocytes. SDF-1-dependent activation of G proteins still occurred in astrocytes obtained from CXCR4 knockout mice (CXCR4$^{-/-}$). By contrast, SDF-1 failed to activate G proteins in wild-type astrocytes previously transfected with selective CXCR7 siRNA. Data represent mean ± s.d. from six to eight independent experiments. [^{35}S]GTPγS-binding is expressed relative to binding in untreated controls. $^{**}P < 0.001$; ANOVA with Student-Newman-Keuls. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS

SDF-1-Bound CXCR7 Activates G Proteins in Astrocytes

Incubation of crude cell membranes prepared from cultured rat (wild-type) cortical astrocytes with SDF-1 resulted in a 170% increase in [^{35}S]-GTPγS binding when compared with nontreated membranes (Fig. 1). Similar increases in [^{35}S]-GTPγS binding were previously seen following activation of other G protein-coupled receptors (GPCR) present on glial or neural cells (e.g. Fumagalli et al., 2004; Tonazzini et al., 2007; Trincavelli et al., 2008). By contrast, I-TAC did not increase [^{35}S]-GTPγS binding to membrane fractions (Fig. 1); I-TAC represents a second ligand for CXCR7 which in

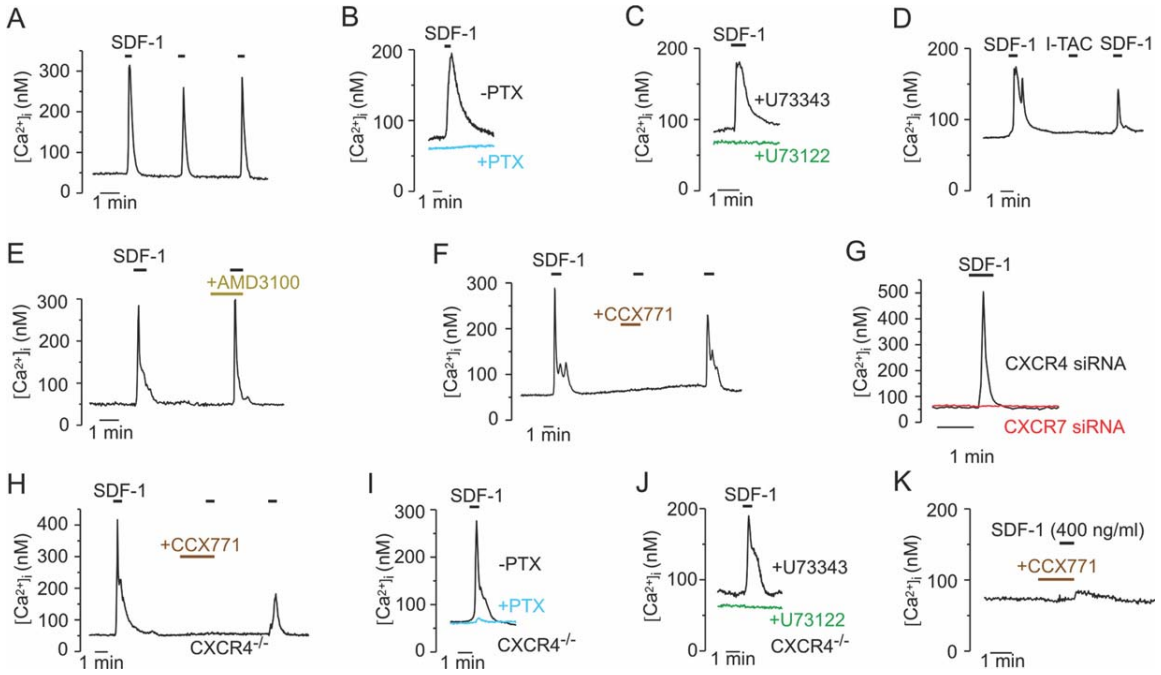

Fig. 2. SDF-1 induces calcium signaling in cultured rodent astrocytes through CXCR7. (**A**) Analysis of calcium signaling by fura-2 imaging demonstrated that application of SDF-1 (100 ng/mL) to cultured rat astrocytes evokes repetitive calcium transients. (**B**, **C**) SDF-1 (100 ng/mL)-induced calcium responses were completely abolished following treatment of cultured rat astrocytes with PTX (100 ng/mL) for 24 h or after incubation with U73122 (10 μM) for 10 min. Incubation with U73343 (10 μM) for 30 min was ineffective. (**D**) In contrast to SDF-1 (100 ng/mL), the alternative CXCR7 ligand, I-TAC (100 ng/mL), failed to evoke calcium responses in rat astrocytes. (**E**, **F**) SDF-1 (100 ng/mL)-evoked calcium transients in rat astrocytes were abolished by the CXCR7-inhibitor, CCX771 (1 μM), but not by the CXCR4-inhibitor, AMD3100 (1 μM). (**G**) RNAi-mediated depletion of CXCR7, but not of CXCR4, prevented SDF-1 (100 ng/mL)-dependent calcium responses in rat astrocytes. (**H**) SDF-1 (100 ng/mL)-induced calcium signals persisted in astrocytes from $CXCR4^{-/-}$ mice and were prevented by CCX771 (1 μM). (**I**, **J**) SDF-1 (100 ng/mL)-induced calcium responses in astrocytes derived from $CXCR4^{-/-}$ mice were suppressed following treatment with either PTX (100 ng/mL) for 24 h or with U73122 (10 μM) for 10 min. (**K**) Demonstration that even increased concentrations of SDF-1 (400 ng/mL) failed to induce Ca^{2+} responses in rat astrocytes in which CXCR7 is pharmacologically silenced with CXC771 (1 μM). Each trace represents the average signal from 9 to 11 cells from representative experiments.

addition binds to CXCR3 (Burns et al., 2006). Surprisingly, when we performed [^{35}S]-GTPγS binding assays with cell membranes of cortical astrocytes obtained from a CXCR4-deficient mouse line, we could still observe SDF-1-dependent activation of G proteins (Fig. 1). Moreover, the level to which SDF-1 activated G proteins in $CXCR4^{-/-}$ astrocytes was similar to that seen in wild-type astrocytes. Since $CXCR7^{-/-}$ animals are not at our disposal, we additionally measured [^{35}S]-GTPγS binding in rat astrocytes in which CXCR7 expression was inhibited by RNAi. As shown previously (Ödemis et al., 2010), transfection of primary astrocytes with CXCR7 siRNA allowed for a 73 ± 10% (n = 4) decline in CXCR7 protein levels after 72 h (Supp. Info. Fig. 1). RNAi-mediated depletion of CXCR7 resulted in an almost complete loss of SDF-1-dependent activation of G proteins (Fig. 1). Collectively, these findings establish that in astrocytes SDF-1-bound CXCR7, but not I-TAC-bound CXCR7, allows for activation of G proteins.

SDF-1-Bound CXCR7 Evokes Ca^{2+} Signals in Astrocytes

The finding that CXCR7 activates G proteins in astrocytes prompted us to determine next whether CXCR7 would also mediate SDF-1-dependent Ca^{2+} responses in astrocytes. By using fura-2 imaging, we observed that in accordance with previous studies (Bajetto et al., 1999; Tanabe et al., 1997), SDF-1 induced transient increases in intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) (Fig. 2A). These increases were completely abolished by pertussis toxin (PTX; Fig. 2B), implying that the effects of SDF-1 are mediated via $G_{i/o}$ signaling pathways. In addition, SDF-1-dependent Ca^{2+} signals were prevented by the selective phospholipase C (PLC) inhibitor, U73122, but not by its inactive analogue, U73343 (Fig. 2C). This confirms that in astrocytes, SDF-1 evokes a rise in $[Ca^{2+}]i$ through a PLC-sensitive, inositol-1,4,5-triphosphate-dependent mechanism. In contrast to SDF-1, I-TAC failed to activate $[Ca^{2+}]i$ in cultured astrocytes (Fig. 2D). To define the role of CXCR4 and CXCR7 in astrocytic Ca^{2+} responses, we measured SDF-1-induced increases in $[Ca^{2+}]i$ in the presence of the CXCR4 antagonists, AMD3100, and the CXCR7 antagonist, CCX771. SDF-1-induced increases in $[Ca^{2+}]i$ remained detectable in the presence of AMD3100 (Fig. 2E), but were abrogated by CCX771 (Fig. 2F). Notably, in these experiments AMD3100 and CCX771 by their own had no obvious effects on $[Ca^{2+}]i$. To additionally verify the findings obtained by this pharmacological approach, we determined the effects of SDF-1 on $[Ca^{2+}]i$ in astrocytes with

siRNA-mediated inhibition of CXCR4 or CXCR7 expression (Ödemis et al., 2010). Transfection of astrocytes with CXCR4 siRNA allowed for a 61 ± 10% (n = 4) decline in CXCR4 levels (Supp. Info. Fig. 1). We found that CXCR4-depleted astrocytes still responded to SDF-1 with increases in $[Ca^{2+}]i$, whereas such increases were undetectable in CXCR7-depleted astrocytes (Fig. 2G). Similarly, SDF-1-induced increases in $[Ca^{2+}]i$ still occurred in astrocytes cultured from the cortex of $CXCR4^{-/-}$ mice and were abolished by CCX771 (Fig. 2H). Moreover, SDF-1-evoked Ca^{2+} responses in $CXCR4^{-/-}$ astrocytes were sensitive to PTX (Fig. 2I) and U73122 (Fig. 2J). Since CXCR7 reportedly exhibits a higher affinity for SDF-1 than CXCR4 (Balabanian et al., 2005; Crump et al., 1997), we further reasoned that CXCR4 might only induce astrocytic Ca^{2+} responses at distinctly higher concentrations of the chemokine. Exposure of astrocytes in which CXCR7 was pharmacologically silenced by CCX771 to SDF-1 at 400 ng/mL (instead of 100 ng/mL) did not again increase $[Ca^{2+}]i$ (Fig. 2K). Together, these findings identify CXCR7 as the main mediator of SDF-1-induced Ca^{2+} responses in astrocytes.

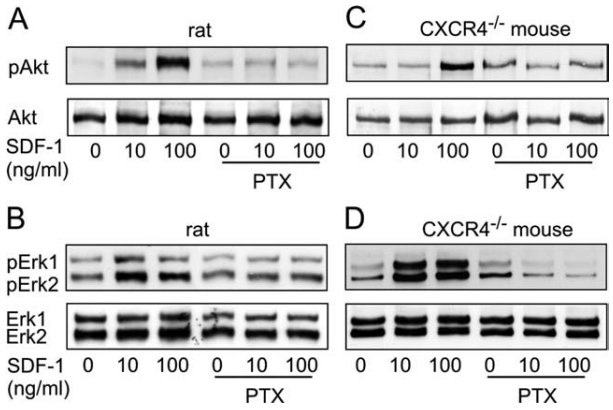

Fig. 3. SDF-1-bound CXCR7 activates astrocytic Akt and Erk signaling through PTX-sensitive $G_{i/o}$ proteins. (**A**, **B**) Western blotting demonstrated that PTX prevents the SDF-1 (10 min)-induced increase in phosphorylated (activated) Akt (pAkt) and phosphorylated Erk (pErk) in rat astrocytes. (**C**, **D**) SDF-1 still allowed for the activation of both signaling molecules in astrocytes from CXCR4-deficient mice ($CXCR4^{-/-}$). The CXCR7-mediated activation of Akt and Erk in $CXCR4^{-/-}$ astrocytes was again sensitive to PTX. Antibodies recognizing Akt and Erk independent of their phosphorylation status served as loading controls. Experiments were replicated three to four times with similar results.

SDF-1-Bound CXCR7 Activates Akt and Erk Through $G_{i/o}$ Protein

We have previously demonstrated that in astrocytes SDF-1-dependent activation of Akt and Erk1/2 exclusively occurs through CXCR7 (Ödemis et al., 2010). In order to distinguish whether CXCR7 induces these signaling responses via a G protein-dependent or -independent mechanism, we analyzed the activation of the signaling molecules following a 24-h pretreatment with PTX. Western blotting and the use of phosphospecific antibodies confirmed that treatment of cultured astrocytes with SDF-1 results in the activation (phosphorylation) of Akt and Erk1/2 (Fig. 3A,B; Supp. Info. Table 1). Activation of both signaling molecules was undetectable in PTX-pretreated cells (Fig. 3A,B). In another set of experiments, we further analyzed the effects of PTX on SDF-1-dependent cell signaling in astrocytes cultured from a CXCR4 knockout mouse line ($CXCR4^{-/-}$; Fig. 3C,D). As shown previously (Ödemis et al., 2010), SDF-1 still activated Akt and Erk1/2 in CXCR4-deficient astrocytes (Fig. 3C,D; Supp. Info. Table 1). However, SDF-1 again failed to activate both signaling molecules following PTX-treatment (Fig. 3C,D). Together, these observations imply that in astrocytes SDF-1-bound CXCR7 activates Akt and Erk through $G_{i/o}$ proteins.

I-TAC-Bound CXCR7 Activates Astrocytic Signaling Through β-Arrestin

The findings that I-TAC neither resulted in activation of $[^{35}S]$-GTPγS binding nor in increases in $[Ca^{2+}]i$ in cultured astrocytes, prompted us to examine further the signaling response of astrocytes to this chemokine. We found that similar to SDF-1, I-TAC leads to the activation of Erk1/2 and Akt (Fig. 4A,B; Supp. Info. Table 1). The signaling responses persisted in PTX-pretreated astrocytes (Fig. 4A,B), implying that both signaling molecules are activated by G protein-independent mechanism. I-TAC reportedly signals through CXCR7 and CXCR3 (Burns et al., 2006). Moreover, CXCR3 was previously shown to be expressed by astrocytes *in vitro* and *in vivo* (Biber et al., 2002; Goldberg et al., 2001; Subileau et al., 2009; Tanuma et al., 2006). This prompted us to additionally examine I-TAC-dependent signaling in astrocytes with RNAi-mediated inhibition of CXCR3 expression. Transfection of cultured astrocytes with selective CXCR3 siRNA resulted in a 78 ± 4% (n = 3) decline in CXCR3 protein levels after 72 h (Fig. 4C). Subsequent analysis of these cells showed that I-TAC-dependent activation of Erk1/2 and Akt is virtually unaffected by CXCR3 depletion (Fig. 4D,E; Supp. Info. Table 1), hence, indicating that both signaling molecules are predominantly activated through CXCR7. Since many GPCRs also signal through β-arrestin (DeWire et al., 2007), we asked next whether astrocytic I-TAC signaling depends on β-arrestin. Western blotting demonstrated that cultured cortical astrocytes express β-arrestin1 and β-arrestin2 (Fig. 5A,B). Transfection of cultured astrocytes with siRNA selective for either one of the β-arrestin isoforms resulted in a 81 ± 4% and 82 ± 7% (n = 3) decrease in β-arrestin1 and β-arrestin2 expression levels within 72 h, respectively (Fig. 5A,B). When we analyzed the signaling response of β-arrestin1-depleted astrocytes to I-TAC, we found persisting increases in Akt and Erk activity (Fig. 5C,D; Supp. Info. Table 1). However, I-TAC failed to activate Akt and Erk in β-arrestin2-depleted astrocytes (Fig. 5E,F; Supp. Info. Table 1). By contrast, astrocytes with siRNA-medi-

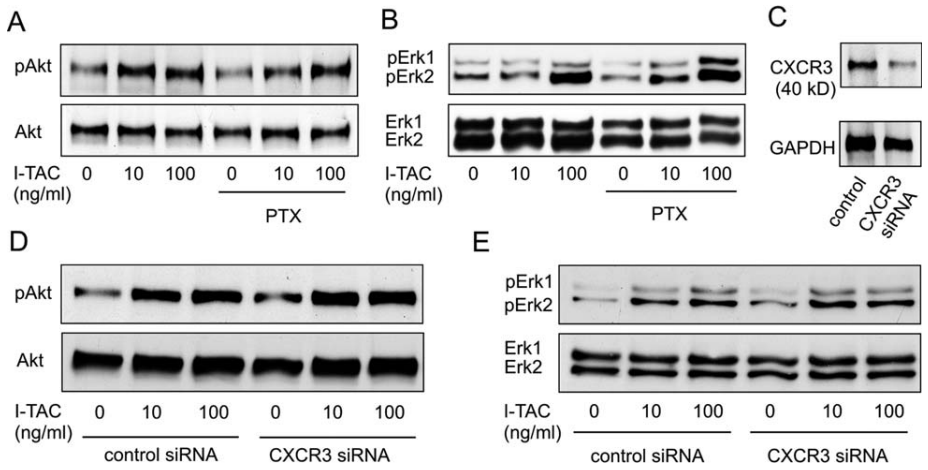

Fig. 4. Characterization of I-TAC-induced signaling in cultured astrocytes. (**A**, **B**) Western blot analysis demonstrated that I-TAC (10 min) leads to the activation (phosphorylation) of Akt and Erk in rat astrocytes and that activation of both signaling molecules persists following 24 h treatment with PTX (100 ng/mL). (**C**) Cortical astrocytes expressed detectable protein levels of the alternative I-TAC receptor, CXCR3, which are depleted 3 days after transfection with selective CXCR3 siRNA (50 nM). (**D**, **E**) I-TAC (10 min)-induced activation of Akt and Erk remained detectable in astrocytes with RNAi-mediated inhibition of CXCR3, indicating that activation of both signaling molecules predominantly occurs through CXCR7. For each analysis, one representative blot out of three to four independent experiments is shown.

ated inhibition of either β-arrestin1 or β-arrestin2 fully responded to SDF-1 with increases in Akt and Erk activity (Fig. 5G–J; Supp. Info. Table 1). These findings suggest that in contrast to SDF-1-bound CXCR7, I-TAC-bound CXCR7 signals through β-arrestin.

SDF-1-Bound CXCR7 Controls Astrocytic Proliferation and Migration Through $G_{i/o}$ Proteins

We recently demonstrated that the mitogenic response of astrocytes to SDF-1 is entirely mediated by CXCR7 (Ödemis et al., 2010). To determine whether this mitogenic effect involves CXCR7-dependend activation of $G_{i/o}$ proteins, we analyzed the proliferative response of PTX-pretreated rat astrocytes. As shown before (Ödemis et al., 2010), SDF-1 increased the number of cells expressing the proliferation marker, Ki-67, within 24 h (Fig. 6A). This increase was absent in cultures co-treated with SDF-1 and PTX for 24 h (Fig. 6A). To additionally determine whether the absence of a proliferative response under this experimental paradigm is due to putative toxic effects of PTX, we compared the mitogenic effects of SDF-1 with those of FGF-2. FGF-2 represents another potent mitogen for astrocytes (astrocytic precursors) which acts through tyrosine kinase receptors (Reuss and von Bohlen und Halbach, 2003). In the absence of PTX, FGF-2 produced a distinctly higher increase in the number of Ki-67 expressing cells than SDF-1 (Fig. 6B). This increase was only slightly reduced by the additional presence of PTX, hence, implying that PTX does not interfere with astrocytic proliferation *per se*.

We were previously unable to observe SDF-1-mediated chemotaxis in replated astrocytes unless the cells had been pretreated with dibutyryl cAMP and/or Il-6 (Ödemis et al., 2002). We subsequently found that omitting the replating step allowed for SDF-1-mediated chemotaxis without pretreatment (see also Fig. 6). To additionally determine whether this chemotactic response again involves CXCR7-dependent activation of $G_{i/o}$ proteins, we initially verified that SDF-1-dependent chemotaxis of astrocytes is in fact mediated by CXCR7. To this end, we studied the migratory response of astrocytes cultured from $CXCR4^{-/-}$ mice and age-matched wild-type littermates to SDF-1 in a modified Boyden chamber. We found that nonreplated astrocytes derived from wild-type mice migrated toward SDF-1 (Fig. 6C). The migratory response toward SDF-1 was preserved in nonreplated astrocytes derived from $CXCR4^{-/-}$ animals (Fig. 6C). Likewise, SDF-1 stimulated the migration of nonreplated rat astrocytes with siRNA-mediated inhibition of CXCR4 expression whereas SDF-1 failed to induce migration of nonreplated astrocytes with siRNA-mediated inhibition of CXCR7 expression (Supp. Info. Fig. 1). Finally, we found that the migratory response of wild-type (rat) astrocytes toward SDF-1 is completely prevented following treatment with PTX (Fig. 6D). Collectively, these findings unravel that CXCR7 controls astrocytic proliferation and migration through a $G_{i/o}$-dependent mechanism.

SDF-1-Bound CXCR7 Activates $G_{i/o}$ Proteins in Human Astrocytomas

CXCR7 expression increases in human astrocytoma tissue and is largely confined to GFAP-expressing cells (Hattermann et al., 2010). Likewise, several human astroglioma cell lines express detectable levels of CXCR7, but not of CXCR4 (Hattermann et al., 2010). To assess whether CXCR7 is also coupled to $G_{i/o}$ proteins in malignant astroglial cells, we examined the effects of PTX on SDF-1-induced cell signaling in the CXCR7-posi-

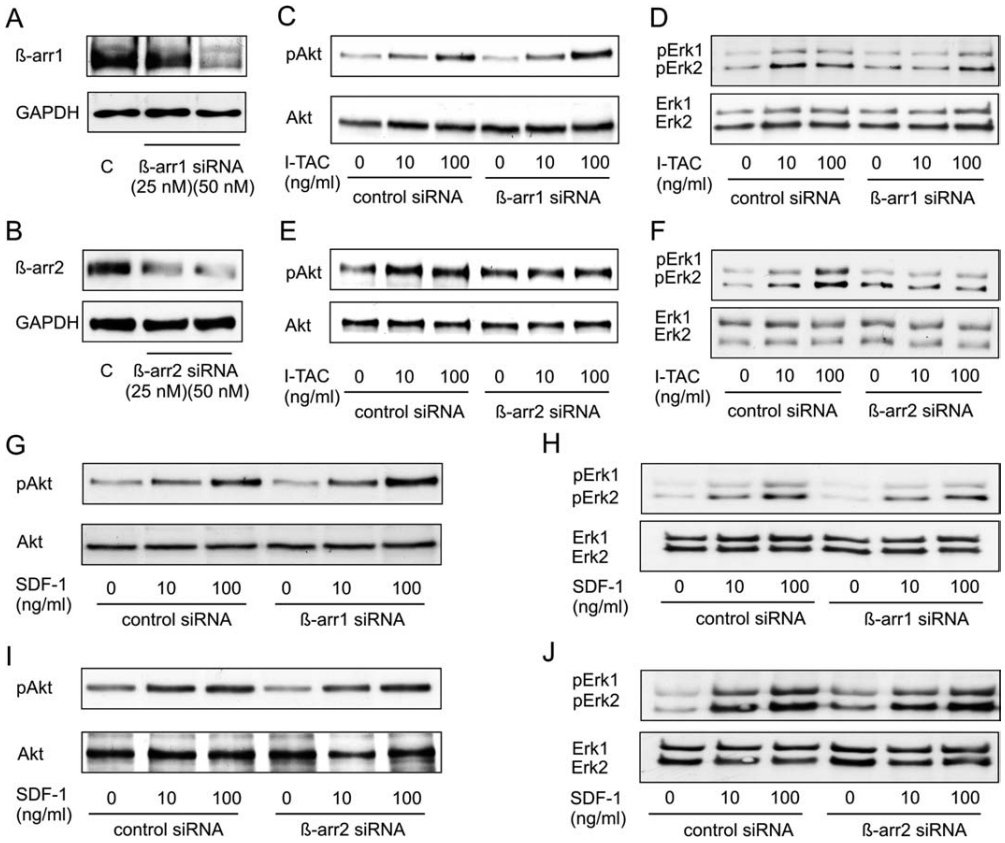

Fig. 5. I-TAC-bound CXCR7 signals through β-arrestin in astrocytes. (**A**, **B**) Western blot analysis demonstrated that rat astrocytes express similar levels of β-arestin1 (β-arr1) and β-arrestin2 (β-arr2). Expression levels of both β-arrestin isoforms were reduced 72 h following transfection with selective siRNA. (**C**–**F**) Demonstration that the stimulatory influences of I-TAC-bound CXCR7 (10 min) on Akt and Erk activity were abolished in cortical astrocytes with depleted expression of β-arrestin2 (50 nM siRNA; E, F), but not β-arrestin1 (50 nM siRNA; C, D). (**G**–**J**) SDF-1-bound CXCR7 (10 min) still allowed for activation of Akt and Erk in β-arrestin1 (G, H) or β-arrestin2-depleted astrocytes (I, J). All results were reproduced in at least three independent experiments.

tive, CXCR4-negative human glioma cell lines, A764 and U343 (Hattermann et al., 2010). Corroborating previous findings (Hattermann et al., 2010), SDF-1 activated Erk in both cell lines (Fig. 7; Supp. Info. Table 1). In addition, SDF-1 resulted in the activation of Akt in A764 and U343 cells (Fig. 7; Supp. Info. Table 1). As expected, SDF-1-induced activation of Erk and Akt was abolished by the CXCR7 antagonist, CCX771 (Supp. Info. Fig. 2). Moreover, in both human glioma cell lines, SDF-1 failed to activate Erk and Akt in the additional presence of PTX (Fig. 7; Supp. Info. Table 1), hence, indicating that CXCR7 also couples to $G_{i/o}$ proteins in malignant glia.

DISCUSSION

It has been originally assumed that CXCR7 would represent a silent or a scavenger chemokine receptor which modulates the function of CXCR4 (Boldajipour et al., 2008; Levoye et al., 2009; Naumann et al., 2010; Rajagopal et al., 2010a; Sierro et al., 2007). This view was recently challenged by the demonstration that CXCR7 controls growth, adhesion, and transendothelial migration of several types of tumor cells (Burns et al., 2006; Grymula et al., 2010; Miao et al., 2007; Wang et al., 2008; Zabel et al., 2009) and actively mediates SDF-1-dependent signaling involved in the control of either growth or differentiation of primary astrocytes, Schwann cells, and oligodendroglial precursors (Göttle et al., 2010; Ödemis et al., 2010). Our present studies unravel that SDF-1-bound CXCR7 affects primary rodent astrocytes as well as human glioma cells through PTX-sensitive $G_{i/o}$ proteins.

Astrocytes were previously shown to express CXCR4 and CXCR7 both *in vitro* and *in vivo* (Ödemis et al., 2010; Schönemeier et al., 2008a,b). Confirming earlier reports (Bajetto et al., 2001; Lazarini et al., 2000; Tanabe et al., 1997), we found that treatment of primary rodent astrocytes with SDF-1 results in the activation of PTX-sensitive $G_{i/o}$ proteins, which in turn stimulate increases in $[Ca^{2+}]i$ through a PLC/inositol-1,4,5-triphosphate-dependent mechanism. However, contrasting the previous view that SDF-1 activates G proteins and subsequent increases in $[Ca^{2+}]i$ in astrocytes through CXCR4 (Bajetto et al., 2001; Lazarini et al., 2000; Tanabe et al., 1997), we obtained compelling evidence that these effects are rather brought about by CXCR7. By using $[^{35}S]GTP\gamma S$ binding assay in combination with

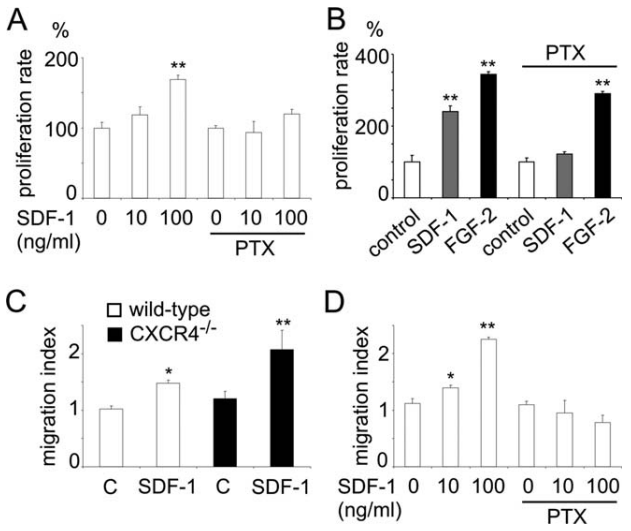

Fig. 6. SDF-1-bound CXCR7 controls glial proliferation and migration through $G_{i/o}$ proteins. (**A**) Demonstration that treatment of cultured rat astrocytes with SDF-1 (24 h) promotes cell proliferation. The proliferative response was absent in cultures co-treated with SDF-1 and PTX (100 ng/mL; 24 h). (**B**) In contrast to SDF-1 (100 ng/mL), PTX had no major effects on the proliferative response of astrocytes to FGF-2 (10 ng/mL; 24 h), hence, excluding putative toxic effects of the compound on cultured astrocytes. (**C**) Nonreplated astrocytes from E18 $CXCR4^{-/-}$ mice and wild-type littermates likewise migrated toward SDF-1 (100 ng/mL), hence, indicating that SDF-1 controls astrocytic migration through CXCR7. (**D**) The migratory response of rat astrocytes toward SDF-1 was abolished following pretreatment with PTX (100 ng/mL; 24 h). Data represent mean ± SD from three independent experiments. $^{*}P < 0.05$; $^{**}P \leq 0.001$; ANOVA with Student-Newman-Keuls; absence *versus* presence of SDF-1.

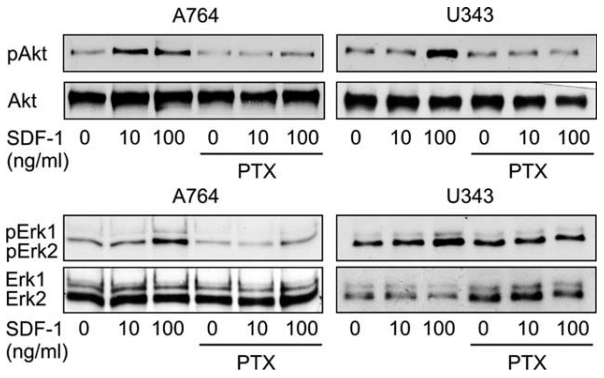

Fig. 7. SDF-1-bound CXCR7 couples to $G_{i/o}$ proteins in glial malignancy. Western blot analysis demonstrated that SDF-1 (10 min)-dependent activation of Akt and Erk in the CXCR7-only expressing human glioma cell lines, A764 and U343, is sensitive to PTX (100 ng/mL). Experiments were replicated four to five times with similar results.

Ca^{2+} imaging, we found that SDF-1-dependent activation of $G_{i/o}$ proteins and subsequent increases in $[Ca^{2+}]i$ persist in astrocytes with depleted CXCR4 expression, but are prevented in astrocytes with depleted expression of CXCR7. Along the same line, we observed that SDF-1-induced increases in $[Ca^{2+}]i$ are sensitive to the CXCR7 antagonist, CCX771 (Zabel et al., 2009), but not to the CXCR4 antagonist, AMD31000 (Hatse et al., 2002). Moreover, we found that PTX, but not depletion of β-arrestin prevents CXCR7-mediated effects of SDF-1 on astrocytic Akt and Erk signaling and/or on astrocytic proliferation and migration. In addition, we show that depletion of β-arrestins, but not PTX, prevents signaling of the alternative CXCR7 ligand, I-TAC (Burns et al., 2006), implying that depending on the ligand, CXCR7 either signals through $G_{i/o}$ proteins or β-arrestins in astrocytes.

We wish to emphasize that our present demonstration of G protein-dependent signaling of SDF-1-bound CXCR7 in astrocytes does not dismiss the previously observed failure of SDF-1-bound CXCR7 to activate G proteins in other cell types, notably HEK-293 cells (Burns et al., 2006; Levoye et al., 2009; Rajagopal et al., 2010a; Sierro et al., 2007). Furthermore, our observation does not preclude the possibility that SDF-1-bound CXCR7 signals through β-arrestin in some cell types (Kalatskaya et al., 2009; Luker et al., 2009; Rajagopal et al., 2010a; Zabel et al., 2009). Our disparate findings rather suggest that depending on the cell type, CXCR7 is differentially integrated into the processing of the SDF-1 signal which eventually determines whether CXCR7 acts as a scavenger chemokine receptor, as a nonclassical, arrestin-coupled GPCR or as in case of astrocytes as a classical, G protein-activating GPCR. In line with this view, we recently demonstrated that in contrast to astrocytes in which SDF-1-induced cell signaling was abrogated following depletion of CXCR7, but not of CXCR4, Schwann cells failed to respond to SDF-1 with cell signaling following depletion of either CXCR4 or CXCR7 (Ödemis et al., 2010).

The previously reported failure of SDF-1-bound CXCR7 to activate G proteins in nonastroglial cells has been primarily attributed to the fact that CXCR7 contains a modified DRYLAIV motif (Thelen and Thelen, 2008). This motif represents a highly conserved region within the second intracellular loop of GPCRs and is assumed to be essential for G protein binding and their subsequent activation. Indeed, several established atypical chemokine receptors such as D6, DARC, and CCX-CKR either lack the DRYLAIV motif or exhibit a modified motif (Graham, 2009). However, other chemokine receptors such as lymphotactin receptors and CXCR6, also showing modifications of the DRYLAIV motif, still signal through G proteins (Thelen and Thelen, 2008). These discrepancies are currently regarded as an indication that at least in case of some GPCRs the DRYLAIV motif is not sufficient for G protein coupling and requires additional sequences (Thelen and Thelen, 2008). Consequently, modifications of the DRYLAIV motif, as exhibited by CXCR7, may not *per se* represent a reliable indicator for the lack of G protein coupling.

Although CXCR4 is traditionally viewed as a classical GPCR (Broxmeyer, 2008), SDF-1-bound CXCR4 failed to stimulate G proteins and subsequent increases in $[Ca^{2+}]i$ in astrocytes. Since CXCR7 reportedly exhibits higher affinity for SDF-1 than CXCR4 (Balabanian et al.,

2005; Crump et al., 1997), it is noteworthy that changes in $[Ca^{2+}]i$ were also undetectable in CXCR7-depleted astrocytes following treatment with higher concentration of the chemokine. This further excludes that putative CXCR4-dependent activation of G protein remained undetectable with the SDF-1 concentrations routinely used in our experiments. The observed lack of SDF-1-bound CXCR4 to activate G proteins in astrocytes points to the possibility that depending on the cell type, either CXCR7 or CXCR4 couples to G proteins.

Agonist binding to GPCR typically results in balanced G protein- and arrestin-mediated cell signaling (Rajagopal et al., 2010b). Biased signaling, through only one of these pathways, is either caused by a biased ligand or a biased receptor. While until now biased receptor function has only been observed with experimentally mutated GPCRs, a recent work demonstrated that CXCR7 acts as a biased receptor in HEK-293 cells and signals exclusively through β-arrestin (Rajagopal et al., 2010a). Notably, in astrocytes signaling of SDF-1-bound CXCR7 was sensitive to PTX, but was not affected by depletion of β-arrestin1 or β-arrestin2. *Vice versa*, signaling of the alternative CXCR7 ligand, I-TAC (Burns et al., 2006), was unaffected by PTX, but was abolished following depletion of β-arrestin2. These findings are consistent with the view that CXCR7 functions as a ligand-biased receptor in astrocytes. Mechanistically, receptor- or ligand-biased signaling of CXCR7 might be determined by whether or not SDF-1-bound CXCR7 couples to G proteins in a given cell type.

It has been previously demonstrated that CXCR4 is expressed in human glial tumors and glial tumor cell lines (Bajetto et al., 2006; Rempel et al., 2000; Zhou et al., 2002). A more recent work provided evidence for the increased expression of CXCR7 in high-grade astrocytomas and further revealed that CXCR7 expression prevails over that of CXCR4 in various human glioma cell lines (Hattermann et al., 2010). The subsequent analysis of two of these cell lines, i.e. A764 and U343, which only express CXCR7 (Hattermann et al., 2010), revealed that similar to primary astrocytes, SDF-1-bound CXCR7 activates Erk and Akt in human glioma cells through PTX-sensitive $G_{i/o}$ proteins. Since Erk and Akt signaling molecules/pathways control glioma proliferation, survival, and migration (Barbero et al., 2003; do Carmo et al., 2010; Zagzag et al., 2006; Zhou et al., 2002), these findings further point to a major role of CXCR7-dependent $G_{i/o}$ signaling in the control of glioma growth and metastasis. We can, however, not dismiss the possibility that this function is taken over by CXCR4 in some gliomas (Calatozzolo et al., 2011), especially in those in which CXCR4 instead of CXCR7 might couple to G proteins.

Our present demonstration of CXCR7 acting as a classical GPCR in primary astrocytes will eventually allow to more precisely define the role of SDF-1 during development and regeneration of the central nervous systems as well as of other organs. The finding that CXCR7 also couples to $G_{i/o}$ proteins in human glioma cell lines point to CXCR7 as an important (additional) target in the therapy of glioma patients. In addition, these findings urge a re-evaluation of the roles of CXCR7 and CXCR4 in other types of tumor cells.

ACKNOWLEDGMENTS

The authors thank Mark E. Penfold (ChemoCentryx) for providing them with CCX771 and CXCR7 antibodies. The expert technical assistance of Ina Hochheim is greatly acknowledged.

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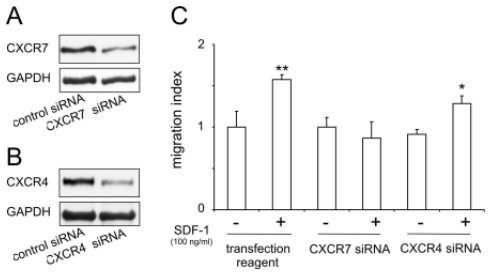
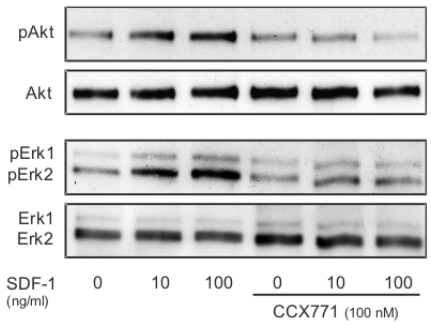
Supplementary Material



Supplementary Figure 1: SDF-1-induced chemotaxis of astrocytes is mediated by CXCR7. (A, B) Cortical rat astrocytes were transfected overnight with either selective CXCR7 siRNA (A) or selective CXCR4 siRNA (B) and analyzed for CXCR7 and CXCR4 expression, respectively, 72 h post-transfection. GAPDH served as loading control. (C) Analysis of astrocytes with RNAi-mediated inhibition of CXCR7 or CXCR4 expression for SDF-1-induced chemotaxis using a modified Boyden chamber. The number of spontaneously migrating cells present in transfection reagent-pretreated (control) cultures was set to 1. Transfection with CXCR7 siRNA, but not with CXCR4 siRNA prevented SDF-1-induced chemotaxis. Data represent mean ±SD from 3 independent experiments. $^{*}p<0.05$, $^{**}p<0.001$; ANOVA with Student-Newman-Keuls; absence vs. presence of SDF-1.



Supplementary Figure 2: SDF-1-dependent activation of Akt and Erk in human glioma cells is sensitive to CCX771. Confluent cultures of human A764 glioma cells were maintained for 6 h with serum-free N2-medium and subsequently stimulated for 15 min with the indicated concentrations of SDF-1 in absence or presence of the CXCR7 antagonist, CCX771. Activation (phosphorylation) of Akt and Erk was assessed by Western blotting using phospho-specific antibodies. To control for protein loading, blot were additionally stained with antibodies recognizing Akt and Erk independent of their phosphorylation status. CCX771 prevented SDF-1-dependent activation of Akt and Erk, hence, confirming that activation of both signalling molecules occurs through CXCR7.

Supporting Information Table 1: Quantification of Western blot analysis. Integrated optical densities of immunoreactive protein bands were measured using the Gel-Pro Analyzer software. Data were normalized to the respective loading control. One way analysis of variance (ANOVA) followed by pairwise multiple comparison procedures (Student-Newman-Keuls method) was used for statistical analysis. Differences with $p<0.05$ were considered significant.

Figure 3A

SDF (ng/ml)	PTX	fold increase pAkt	SD	n	p-value
0	-	1.09	0.06	4	
10	-	1.75	0.40	4	ns
100	-	2.57	0.64	4	0.003
0	+	1.11	0.16	4	
10	+	0.80	0.28	4	ns
100	+	0.86	0.28	4	ns

Figure 3B

SDF (ng/ml)	PTX	fold increase pErk	SD	n	p-value
0	-	1.06	0.10	4	
10	-	1.35	0.10	4	ns
100	-	2.55	0.91	4	0.009
0	+	1.01	0.16	4	
10	+	1.14	0.32	4	ns
100	+	1.07	0.34	4	ns

Figure 3C

SDF (ng/ml)	PTX	fold increase pAkt	SD	n	p-value
0	-	1.02	0.01	4	
10	-	1.29	0.20	4	ns
100	-	3.19	1.64	4	0.026
0	+	1.04	0.18	4	
10	+	0.80	0.26	4	ns
100	+	0.95	0.29	4	ns

Figure 3D

SDF (ng/ml)	PTX	fold increase pErk	SD	n	p-value
0	-	1.06	0.07	4	
10	-	1.46	0.23	4	ns
100	-	2.58	0.88	4	0.007
0	+	0.99	0.20	3	
10	+	0.93	0.24	3	ns
100	+	0.99	0.25	3	ns

Figure 4 A

I-TAC (ng/ml)	PTX	fold increase pAkt	SD	n	p-value
0	-	1.03	0.05	4	
10	-	1.58	0.88	4	ns
100	-	2.49	0.80	4	0.036
0	+	1.04	0.04	4	
10	+	1.19	0.60	4	ns
100	+	2.44	0.92	4	0.03

Figure 4 B

I-TAC (ng/ml)	PTX	fold increase pErk	SD	n	p-value
0	-	1.01	0.03	4	
10	-	1.68	0.23	4	ns
100	-	4.08	2.07	4	0.014
0	+	1.22	0.04	3	
10	+	1.18	0.44	3	ns
100	+	2.51	0.18	3	0.001

Figure 4 C

siRNA	% inhibition	SD	n
CXCR3	78	4	3

Figure 4 D

I-TAC (ng/ml)	siRNA	fold increase pAkt	SD	n	p-value
0	control	1.04	0.06	4	
10	control	1.86	0.14	4	0.001
100	control	1.67	0.29	4	<0.001
0	CXCR3	0.99	0.09	4	
10	CXCR3	1.25	0.23	4	ns
100	CXCR3	1.68	0.26	4	0.003

Figure 4 E

I-TAC (ng/ml)	siRNA	fold increase pErk	SD	n	p-value
0	control	1.08	0.08	4	
10	control	1.71	0.24	4	0.011
100	control	2.22	0.42	4	<0.001
0	CXCR3	1.08	0.08	4	
10	CXCR3	1.79	0.46	4	0.049
100	CXCR3	1.74	0.42	4	0.028

Figure 5 A

siRNA	% inhibition	SD	n
ßarr1 (50 nM)	81	4	3

Figure 5B

siRNA	% inhibition	SD	n
ßarr2 (50 nM)	82	7	3

Figure 5C

I-TAC (ng/ml)	siRNA	fold increase pAkt	SD	n	p-value
0	control	1.01	0.02	4	
10	control	1.31	0.21	4	ns
100	control	2.77	0.69	4	0.001
0	ßarr1	0.72	0.28	4	
10	ßarr1	1.48	0.62	4	ns
100	ßarr1	3.82	0.95	4	<0.001

Figure 5 D

I-TAC (ng/ml)	siRNA	fold increase pErk	SD	n	p-value
0	control	1.02	0.01	3	
10	control	1.88	0.30	3	0.016
100	control	2.15	0.46	3	0.012
0	ßarr1	1.18	0.21	3	
10	ßarr1	1.38	0.12	3	ns
100	ßarr1	1.86	0.24	3	0.013

Figure 5 D

I-TAC (ng/ml)	siRNA	fold increase pErk	SD	n	p-value
0	control	1.02	0.01	3	
10	control	1.88	0.30	3	0.016
100	control	2.15	0.46	3	0.012
0	ßarr1	1.18	0.21	3	
10	ßarr1	1.38	0.12	3	ns
100	ßarr1	1.86	0.24	3	0.013

Figure 5 F

I-TAC (ng/ml)	siRNA	fold increase pErk	SD	n	p-value
0	control	1.01	0.04	5	
10	control	2.43	0.84	5	0.005
100	control	2.11	0.51	5	0.01
0	ßarr2	1.00	0.27	4	
10	ßarr2	0.81	0.19	4	ns
100	ßarr2	0.96	0.42	4	ns

Figure 5 G

SDF-1 (ng/ml)	siRNA	fold increase pAkt	SD	n	p-value
0	control	1.04	0.01	3	
10	control	1.56	0.22	3	0.018
100	control	3.41	0.26	3	<0.001
0	ßarr1	1.12	0.14	3	
10	ßarr1	1.59	0.41	3	ns
100	ßarr1	2.56	0.74	3	0.028

Figure 5 H

SDF-1 (ng/ml)	siRNA	fold increase pErk	SD	n	p-value
0	control	1.03	0.02	3	
10	control	1.19	0.10	3	ns
100	control	3.40	1.15	3	0.011
0	ßarr1	1.10	0.13	3	
10	ßarr1	1.65	0.08	3	ns
100	ßarr1	2.75	0.35	3	0.001

Figure 5 I

SDF-1 (ng/ml)	siRNA	fold increase pAkt	SD	n	p-value
0	control	1.03	0.02	3	
10	control	1.34	0.17	3	ns
100	control	2.42	0.23	3	0.001
0	ßarr2	0.93	0.14	3	
10	ßarr2	1.28	0.22	3	ns
100	ßarr2	2.97	1.04	3	0.016

Figure 5 J

SDF-1 (ng/ml)	siRNA	fold increase pErk	SD	n	p-value
0	control	1.01	0.003	3	
10	control	1.38	0.37	3	ns
100	control	2.92	1.02	3	0.022
0	ßarr2	0.99	0.02	3	
10	ßarr2	1.41	0.20	3	ns
100	ßarr2	2.87	0.94	3	0.015

Figure 7 **A764**

SDF-1 (ng/ml)	PTX	fold increase pAkt	SD	n	p-value
0	-	1.01	0.01	5	
10	-	1.77	0.67	5	ns
100	-	2.25	0.73	5	0.013
0	+	1.10	0.14	5	
10	+	0.97	0.17	5	ns
100	+	0.89	0.27	5	ns

A764

SDF-1 (ng/ml)	PTX	fold increase pErk	SD	n	p-value
0	-	1.02	0.03	4	
10	-	1.24	0.51	4	ns
100	-	2.09	0.19	4	0.003
0	+	1.07	0.09	4	
10	+	0.97	0.07	4	ns
100	+	0.96	0.14	4	ns

U343

SDF-1 (ng/ml)	PTX	fold increase pAkt	SD	n	p-value
0	-	1.01	0.01	4	
10	-	1.26	0.21	4	ns
100	-	2.87	0.97	4	0.004
0	+	1.10	0.19	4	
10	+	1.10	0.23	4	ns
100	+	1.44	0.19	4	ns

U343

SDF-1 (ng/ml)	PTX	fold increase pErk	SD	n	p-value
0	-	1.04	0.02	4	
10	-	1.25	0.60	4	ns
100	-	2.61	0.91	4	0.016
0	+	0.96	0.14	4	
10	+	0.86	0.39	4	ns
100	+	0.86	0.38	4	ns

3.3. Grk2 is an Essential Regulator of CXCR7 Signalling in Astrocytes

Titel:	Grk2 is an Essential Regulator of CXCR7 Signalling in Astrocytes
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eingereicht am:	07.08.2012
akzeptiert am:	17.08.2012
Veröffentlichung:	Cellular Molecular Neurobiology 33:111-118 (2013)

Cell Mol Neurobiol (2013) 33:111–118
DOI 10.1007/s10571-012-9876-5

ORIGINAL RESEARCH

Grk2 is an Essential Regulator of CXCR7 Signalling in Astrocytes

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Received: 7 August 2012 / Accepted: 17 August 2012 / Published online: 2 September 2012

Abstract We previously demonstrated that in astrocytes, SDF-1/CXCL12 exclusively signals through CXCR7 despite the additional presence of the alternate SDF-1/CXCL12 receptor, CXCR4. In addition, we provided evidence that astrocytic CXCR7-signalling involves a G protein-dependent mechanism. This is insofar remarkable as in all other cell types studied to date, CXCR7 either acts as a scavenger chemokine receptor, a modulator of CXCR4, or a non-classical chemokine receptor, signalling through ß-arrestin. To begin to unravel the molecular framework impinging the selective function of CXCR7 on a given cell type, we have now analysed the role of G protein-coupled receptor kinases (Grks) in astrocytic CXCR7 signalling. We demonstrate that Grk2 mediates signalling of SDF-1/CXCL12-bound CXCR7 as suggested by the finding that SDF-1/CXCL12-induced activation of Erk1/2 and Akt is abrogated following RNAi-mediated inhibition of Grk2, but not of Grk3, Grk5, or Grk6. We further unravel that Grk2 additionally controls signalling of SDF-1/CXCL12-bound CXCR7 in astrocytes by mediating internalization and subsequent silencing of CXCR7. Finally, we demonstrate that Grk2 is likewise expressed by microglial cells and Schwann cells, cell types in which CXCR7 does not act as a classical chemokine receptor. In conclusion, our findings establish that Grk2 tightly controls CXCR7 signalling in astrocytes, but does not imprint the cell type-specific function of this chemokine receptor.

Keywords SDF-1/CXCL12 · RANTES/CCL5 · Cell signalling · Receptor internalization

Introduction

The chemokine, SDF-1/CXCL12, regulates development of the hematopoietic, cardiovascular, and neuromuscular system and, in addition, controls brain morphogenesis (Ma et al. 1998; Zou et al. 1998; Tachibana et al. 1998; Li and Ransohoff 2008; Ödemis et al. 2005). SDF-1/CXCL12 further promotes growth and metastasis of various types of tumours (Maksym et al. 2009; Sun et al. 2010). It has long been assumed that SDF-1/CXCL12 would affect its target cells by selectively interacting with the chemokine receptor, CXCR4. However, a more previous work unravelled that SDF-1/CXCL12 also binds to the chemokine receptor, CXCR7 (Balabanian et al. 2005). Subsequent studies indicated that CXCR7 might have diverse functions in different cell types and biological conditions. During development, CXCR7 was shown to control CXCR4-mediated migration of primordial germ cells by sequestering extracellular SDF-1/CXCL12 and, hence, shaping the extracellular chemokine gradient (Boldajipour et al. 2008). Likewise, CXCR7 exhibits several characteristics of a scavenger chemokine receptor in MDCK cells including a constant cycling of the receptor between the plasma membrane and intracellular compartments and a non-saturating binding of SDF-1/CXCL12 (Naumann et al. 2010). With exception of astrocytes (Ödemis et al. 2012), CXCR7 fails to activate G proteins and subsequent Ca^{2+}-induced chemotaxis in all cell types examined to date, including HEK-293 cells and breast

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Electronic supplementary material The online version of this article (doi:10.1007/s10571-012-9876-5) contains supplementary material, which is available to authorized users.

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tumour cells (Burns et al. 2006; Sierro et al. 2007; Levoye et al. 2009). However, CXCR7 associates ß-arrestin in several types of cells, such as hamster ovary cells, HEK-293 cells, and T cells and, subsequently, functions as a non-classical chemokine receptor by signalling through ß-arrestin (Luker et al. 2009; Kalatskaya et al. 2009; Zabel et al. 2009; Rajagopal et al. 2010; Ray et al. 2012). Finally, studies with HEK-293 cells demonstrated that CXCR7 can modulate the function of CXCR4 by forming heterodimers (Levoye et al. 2009; Sierro et al. 2007). In contrast to CXCR4 which binds G proteins in HEK-293 cells, CXCR4/CXCR7 heterodimers preferentially show recruitment of ß-arrestins (Décaillot et al. 2011).

G protein-coupled receptor kinases (Grks) represent serine/threonine kinases which together with arrestins desensitize G protein-coupled receptors (GPCR) (Reiter and Lefkowitz 2006; Premont and Gainetdinov 2007). The Grk family comprises seven members, termed Grk1–7. Grk1 and Grk7 are confined to retinal rods and cones (Reiter and Lefkowitz 2006). With exception of Grk4 which shows a limited expression in some organs, such as kidney and testis, all other non-visual Grk isoforms are widely expressed in mammalian tissue (Reiter and Lefkowitz 2006). Grks phosphorylate agonist-activated GPCRs which in turn allows for binding of arrestins to the receptor protein. Arrestin binding prevents G protein coupling to the receptor and subsequent G protein-dependent cell signalling. In addition, arrestin-bound GPCRs become a target for clathrin-mediated endocytosis. More recently, evidence emerged that Grks and especially Grk2 are multidomain proteins with a variety of cellular functions (Penela et al. 2010). Indeed, Grk2 phosphorylates an array of non-receptor proteins, such as tubulin, synucleins, and phosducin. Moreover, Grk2 modulates cell signalling in a phosphorylation-independent manner by associating with a variety of signalling molecules, including PI3K, MEK, and Akt (Ferguson 2007; Ribas et al. 2007; Evron et al. 2012).

In an attempt to determine whether the Grk-arrestin system would impinge the function of CXCR7 on a given cell type, we have now analysed the role of Grks in astrocytic CXCR7 signalling. We found that the specific function of CXCR7 in astrocytes does not correlate with a unique expression pattern of Grks. We, however, also found that in astrocytes Grk2 controls internalization of CXCR7 and in addition acts as a crucial mediator of CXCR7 signalling.

Materials and Methods

Cell Cultures

Enriched astroglial cultures, containing >90 % GFAP-expressing cells, were established from the cerebral hemispheres of postnatal days 2–3 Sprague Dawley rats according to a previously established protocol (Figiel et al. 2003). Schwann cell cultures were prepared from postnatal day 0–1 rats according to Brockes et al. (1979). Enriched microglial cultures were established according to a previously established protocol (Giulian and Baker 1986). Specifically, microglial cells were isolated from confluent cultures of cerebral hemispheres of postnatal days 2–3 Sprague Dawley rats by gently shaking culture dishes. Detached cells were collected and further grown in DMEM and 10 % FCS. If not stated otherwise, cultures were switched to serum-free N2-medium 24 h prior to experiments. For studying activation of signalling pathways, culture medium was additionally supplemented for the indicated time with SDF-1α/CXCL12 (1–100 ng/ml; Almac, Edinburgh, UK) or RANTES/CCL5 (100 ng/ml; Peprotech, Rocky Hill, NJ).

RNA Interference

Predesigned rat Grk2, Grk3, Grk5, Grk6, and control siRNA were purchased from Qiagen (Hilden, Germany). One hour prior to transfection, culture medium was replaced by 400 µl MEM containing 2 % fetal calf serum. Transfection was performed with the siPORT^{TM} Amine Transfection Agent (Ambion, Huntingdon) according to the manufacturer's recommendations. For transfection, cultures additionally received 100 µl of serum-free MEM-medium containing either the selective siRNA or control siRNA, and 2 µl of transfection reagent, and were incubated overnight. Transfected cultures were further maintained for 24 h with MEM containing 10 % fetal calf serum and for another 24 h with N2.

Western Blot Analysis

Cultured cells were lysed by ultrasonification in 62.5 mM Tris–HCl, containing 2 % SDS and 10 % sucrose. Proteins were denatured at 95°C for 10 min and further diluted in sample buffer (500 mM Tris–HCl, pH 6.8, containing 4 % SDS, 10 % glycerol, and 2 % ß-mercaptoethanol). For detection of phosphorylated proteins, sample buffer was additionally supplemented with sodium orthovanadate (10 mM; Sigma-Aldrich). Protein content of lysates was determined using the BCA protein assay kit (Thermo Scientific, Waltham, MA) and bovine serum albumin as a standard. Proteins (10 µg/lane) were separated by SDS-(10 %) polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. Upon blocking non-specific binding sites with 3 % bovine serum albumin for 60 min, blots were incubated overnight at 4°C with one of the following antibodies: anti-Grk2, anti-Grk3, anti-Grk5, anti-Grk6 (all 1:1000, all Santa Cruz), phosphospecific anti-Erk 1/2 (1:2000; Cell Signaling Technology, Danvers, MA);

phosphospecific anti-Akt (1:2000, Invitrogen, Darmstadt, Germany). Antibody labelling was detected by incubating cultures for 2 h at room temperature with appropriate horseradish peroxidase-labelled secondary antibodies (1:1000; Jackson ImmunoResearch, West Grove, PA) and visualized with the enhanced chemiluminescence kit (Serva, Heidelberg, Germany). To control for protein loading, blots were additionally stained with antibodies against either GAPDH (1:10000; BD Transduction Laboratories, San Jose, CA), non-phosphorylated Erk1/2 (1:1000, Cell Signaling Technology), or non-phosphorylated Akt (1:1000, Cell Signalling Technology). For protein quantification, integrated optical densities of immunoreactive protein bands were measured using the Gel-Pro Analyzer software 3.1. (Media Cybernetics), and protein levels were normalized to loading controls. In case of Erk, protein levels are expressed as the sum of Erk1 and Erk2.

CXCR7 Cell Surface Expression

Cell surface expression of CXCR7 was quantified by flow cytometric analysis. Cultured astrocytes were suspended in PBS supplemented with 0.3 % bovine serum albumin and 0.1 % sodium azide and subsequently incubated for 20 min at 4°C with CXCR7 antibodies (1:500; Acris; order no., AP17961PU-N) and for another 20 min with fluorescein isothiocyanate-labelled secondary antibodies (1:500; Jackson Laboratories, West Grove, PA). Flow cytometric analysis was performed with a FACScan (Becton–Dickinson). The forward narrow angle light scatter was used to exclude dead and aggregated cells.

Statistics

Data, obtained from at least three independent experiments, are given as mean ± SD. One-way analysis of variance (Anova) followed by pairwise multiple comparison procedures (Student–Newman–Keuls method) was used for statistical analysis. Differences with $P \leq 0.05$ were considered significant.

Results

We have previously provided evidence that SDF-1/CXCL12 signals to astrocytes exclusively through CXCR7 (Ödemis et al. 2010, 2012). In addition, we found that in astrocytes signalling of SDF-1/CXCL12-bound CXCR7 involves a G protein-dependent mechanism (Ödemis et al. 2012). Finally, we demonstrated that in Schwann cells and microglial cells, SDF-1/CXCL12 signalling requires both CXCR4 and CXCR7 (Ödemis et al. 2010; Lipfert et al. submitted). To gain insight into the molecular mechanisms defining the function of CXCR7 in different cell types, we initially asked whether the diverse function of CXCR7 seen in astrocytes, Schwann cells, and microglial cells would be dictated by differences in the expression pattern of the non-visual Grk isoforms, Grk2, Grk3, Grk5, and Grk6. Western blotting demonstrated that cultured cortical astrocytes express high levels of both Grk2 and Grk6 and somewhat lower levels of Grk3 and Grk5 (Fig. 1). Likewise, cultured primary Schwann cells expressed Grk2, -3, -5, and -6 at similar high levels. In contrast, cultured primary microglial cells exhibited high- and low-expression levels of Grk2 and Grk3, respectively, whereas Grk5 and Grk6 expressions were extremely low or even absent (Fig. 1). Since these experiments did not show an obvious correlation between CXCR7 function and Grk expression, we next sought to determine whether Grks are essential for signalling of SDF-bound CXCR7 in astrocytes. To address this issue, we inhibited expression of distinct Grk isoforms by RNAi and subsequently analysed the cells for SDF-1/CXCL12-induced phosphorylation (activation) of Erk1/2 and Akt. Erk1/2 and Akt are prototypical signalling molecules activated by SDF-1/CXCL12 (Ödemis et al. 2010, 2012). Transient transfection of cultured astrocytes with selective Grk2 siRNA resulted in an 87 ± 3.7 % decrease ($n = 5$) in Grk2 protein levels after 48 h (Supplementary Fig. 1). We have previously demonstrated that treatment of cultured astrocytes with SDF-1/CXCL12 at 100 ng/ml allows for a maximal phosphorylation of both Erk1/2 and Akt after 10 min (Ödemis et al. 2010). Corroborating these findings, SDF-1/CXCL12-dependent phosphorylation of Erk1/2 and Akt in

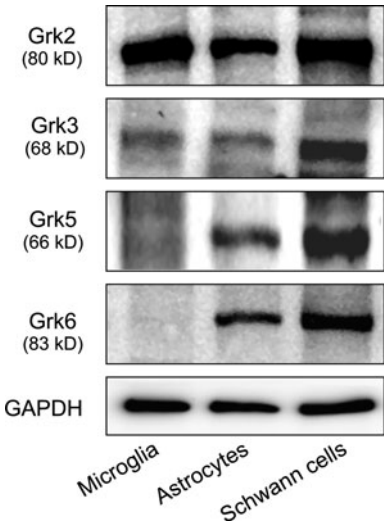

Fig. 1 Comparative analysis of Grk expression in various neural cell types. Cultures of primary astrocytes, microglial cells, and Schwann cells were set up as described under 'Materials and Methods' section. Expression of the non-visual Grk isoforms, Grk2, Grk3, Grk5, and Grk6 were determined by Western blotting in subconfluent cultures from each cell type. GAPDH served as loading control. To visualize putative differences in the expression levels of the various Grk isoforms, blots were exposed for an identical time

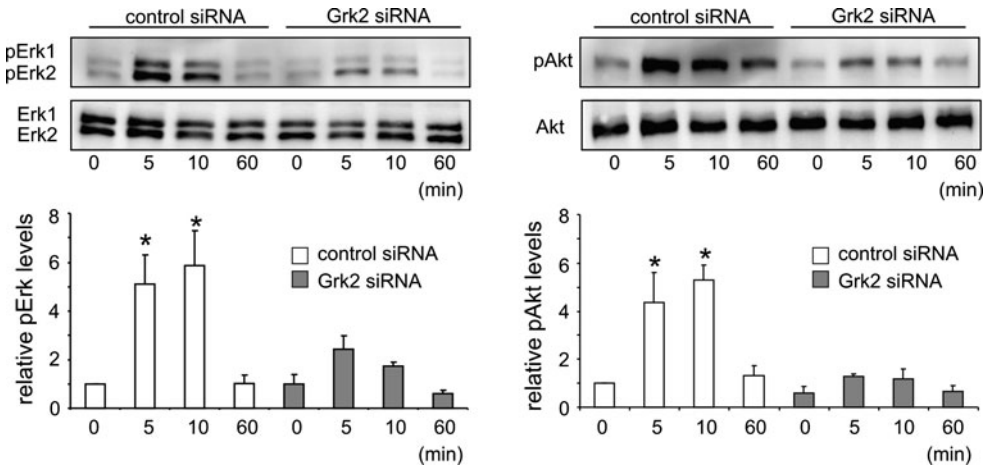

Fig. 2 Grk2 is essential for signalling of SDF-1-bound CXCR7 in astrocytes. Cultured astrocytes were transiently transfected with either selective Grk2 siRNA or control siRNA. 48 h post-transfection, cells were treated with SDF-1 (100 ng/ml) for the indicated time. Levels of phosphorylated (p)Erk1/2 and phosphorylated (p)Akt were subsequently determined by Western blotting and the use of phospho-specific antibodies. To control for protein loading, blots were additionally stained with antibodies recognizing Erk1/2 and Akt independent of their phosphorylation status. Immunoreactive protein bands were quantified by measuring integrated optical densities, and the ratio of the amount of phosphorylated to non-phosphorylated proteins was calculated. The ratio determined in untreated astrocytes transfected with control siRNA was set to 1. Bars represent average pErk1/2 and pAkt levels (±SD) as determined in 3–4 independent experiments. Depletion of Grk2 prevents SDF-1/CXCL12-induced increases in both pErk1/2 and pAkt levels. $^*P < 0.001$

control astrocytes was detectable after 5 min and peaked after 10 min (Fig. 2). After 60 min, phosphorylation of both signalling molecules returned to control levels (Fig. 2). In astrocytes with siRNA-mediated depletion of Grk2, SDF-1/CXCL12 completely failed to phosphorylate Akt up to 60 min. In addition, Grk2-depleted astrocytes showed only a subtle and statistically not significant increase in Erk1/2 phosphorylation after 5 and 10 min of SDF-1/CXCL12 treatment. We further wish to underline that treatment of cultured astrocytes with SDF-1/CXCL12 remained without effects on total Erk and Akt protein levels. To determine whether astrocytes depend on additional Grk isoforms for SDF-1/CXCL12 signalling, we focussed next on Grk6 which as shown above is expressed by astrocytes to similar high levels as Grk2. Transient transfection of cultured astrocytes with Grk6 siRNA resulted in a 69.5 ± 10 % ($n = 4$) decline in Grk6 protein levels after 48 h (Supplementary Fig. 1). Grk6-depleted astrocytes fully responded to SDF-1/CXCL12 with phosphorylation of Erk1/2 and Akt (Fig. 3). By focussing on Erk1/2 phosphorylation (activation) as a read-out, we then further assessed whether Grk3 or Grk5 is required for SDF-1/CXCL12 signalling in astrocytes. RNAi allowed for a 80 ± 1.9 % ($n = 3$) and 54 ± 3.3 % ($n = 3$) inhibition of Grk3 and Grk5 expression, respectively (Supplementary Fig. 1). Treatment of Grk3-depleted astrocytes with SDF-1/CXCL12 (100 ng/ml) resulted in a 3.4 ± 0.6-fold increase ($n = 3$) in Erk1/2 phosphorylation. This increase in Erk1/2 phosphorylation

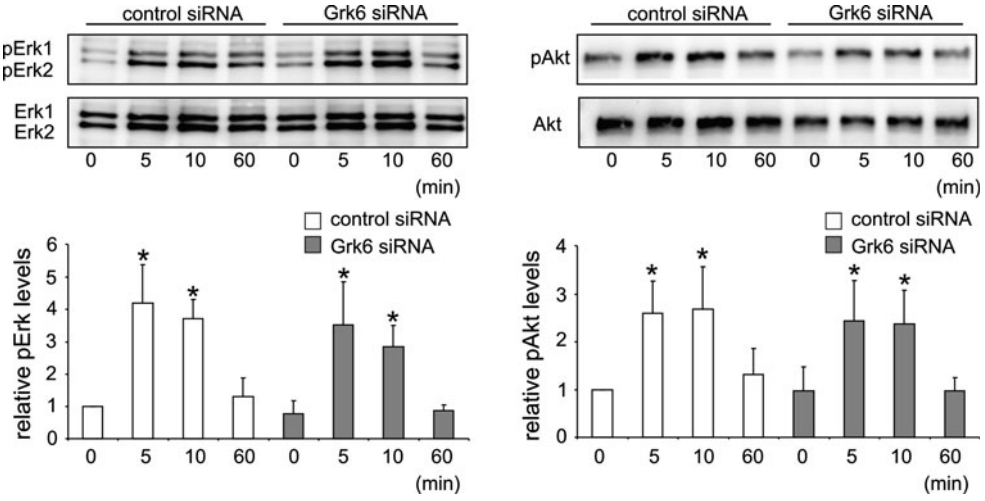

Fig. 3 Grk6 is dispensable for CXCR7 signalling in astrocytes. Astrocytes were transiently transfected with Grk6 siRNA. 48 h post-transfection, cells were treated with SDF-1/CXCL12 (100 ng/ml) for up to 60 min and levels of pErk1/2 and pAkt were quantified by Western blotting as described in Fig. 2. Bars show average pErk1/2 and pAkt levels (±SD) as determined in 3–6 independent experiments. Astrocytic SDF-1/CXCL12-signalling remained unaffected by depletion of Grk6. $^*P < 0.005$

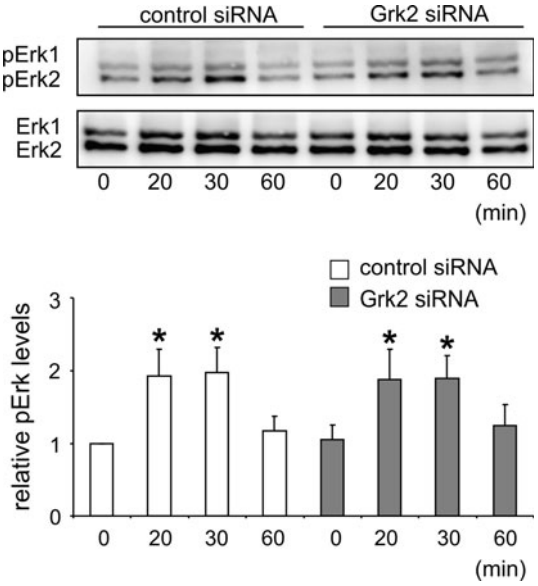

Fig. 4 Grk2 does not modulate RANTES/CCL5-dependent signalling in astrocytes. Astrocytes were transiently transfected with Grk2 siRNA or control siRNA. 48 h post-transfection, cells were treated with RANTES/CCL5 (100 ng/ml) for up to 60 min, and levels of pErk1/2 were quantified by Western blotting as described in Fig. 2. Bars show average pErk1/2 levels (±SD) as determined in 6 independent experiments. RANTES/CCL5 resulted in maximal increases in pErk1/2 levels after 20–30 min in astrocytes treated with control siRNA. RANTES/CCL5-induced phosphorylation of Erk1/2 remained virtually unaffected in astrocytes transfected with Grk2 siRNA. $*P < 0.001$

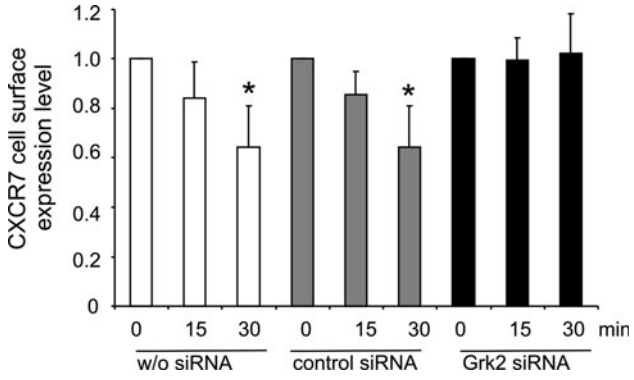

Fig. 5 Grk2 mediates internalization of CXCR7 in astrocytes. Astrocytes were transiently transfected with either control siRNA or Grk2 siRNA. After 48 h, transfected astrocytic cultures as well as non-transfected sister cultures were treated for up to 30 min with SDF-1/CXCL12 (100 ng/ml). Cell surface expression levels of CXCR7 present at the end of the respective treatment period were determined by flow cytometry as described under Materials and Methods. CXCR7 cell surface expression levels present prior to the treatment with SDF-1/CXCL12 were set to 1. Bars show average expression levels ±SD as determined in 6–10 independent experiments. SDF-1/CXCL12 reduced CXCR7 cell surface expression levels in non-transfected astrocytes and astrocytes transfected with control siRNA after 30 min. A similar SDF-1/CXCL12-induced decrease in CXCR7 cell surface expression was undetectable in astrocytes transfected with Grk2 siRNA. $*P < 0.001$, SDF-1-treatment vs. prior to treatment

was similar to that elicited by SDF-1/CXCL12 (100 ng/ml, 10 min) in sister cultures transfected with control siRNA (3.6 ± 0.9-fold, $n = 3$). Likewise, SDF-1/CXCL12 (100 ng/ml) resulted in a similar 3.3 ± 0.9-fold ($n = 5$) and 3.4 ± 0.7-fold ($n = 5$) increase in Erk1/2 phosphorylation in astrocytes transfected with Grk5 siRNA and control siRNA, respectively. Collectively, these findings suggest that in astrocytes signalling of SDF-1/CXCL12-bound CXCR7 selectively depends on Grk2.

To further determine whether Grk2 is equally essential for signalling of other chemokine receptors, we assayed cultured astrocytes for RANTES/CCL5-dependent cell signalling. RANTES/CCL5 binds to the chemokine receptors, CCR5, CCR1, and CCR3 (Jones et al. 2011). Treatment of cultured control astrocytes with RANTES/CCL5 at 100 ng/ml resulted in the phosphorylation of Erk1/2 (Fig. 4). Erk1/2 phosphorylation was maximal after 20–30 min and declined up to 60 min (Fig. 4). A similar delayed response of astrocytic Erk signalling to RANTES/CCL5 has been reported previously (Luo et al. 2002). RANTES/CCL5 (100 ng/ml)-induced Erk1/2 phosphorylation fully persisted in astrocytes with RNAi-mediated inhibition of Grk2 expression (Fig. 4). These findings imply that Grk2 does not represent a common modulator of chemokine signalling in astrocytes.

According to the classical concept, Grks together with arrestins control internalization of GPCRs and, hence, the duration of receptor signalling (Reiter and Lefkowitz 2006; Premont and Gainetdinov 2007). This prompted us to examine whether depletion of Grk2 in cultured astrocytes would affect internalization of CXCR7. To this end, we determined CXCR7 cell surface expression following treatment with SDF-1/CXCL12 by flow cytometry. We previously demonstrated that the majority of cultured astrocytes express CXCR7 (Ödemis et al. 2010). SDF-1/CXCL12 (100 ng/ml) resulted in a 36 % decline of CXCR7 cell surface expression levels after 30 min (Fig. 5). The SDF-1/CXCL12-induced decline in CXCR7 cell surface expression was abrogated in astrocytes with siRNA-mediated inhibition of Grk2 expression. In contrast, the SDF-1/CXCL12-induced decline in CXCR7 cell surface expression persisted in astrocytes transfected with control siRNA (Fig. 5). These findings establish that Grk2 controls internalization of CXCR7 in astrocytes.

Discussion

SDF-1/CXCL12 binds to the chemokine receptors, CXCR4 and CXCR7 (Thelen and Thelen 2008). While CXCR4 represents a classical GPCR, CXCR7 either acts as a

scavenger chemokine receptor (Boldajipour et al. 2008; Naumann et al. 2010) or a modulator of CXCR4 (Levoye et al. 2009; Sierro et al. 2007) in some cells, whereas in other cells CXCR7 represents an atypical chemokine receptor, signalling through ß-arrestin (Luker et al. 2009; Kalatskaya et al. 2009; Zabel et al. 2009; Rajagopal et al. 2010; Ray et al. 2012). One exception is astrocytes in which CXCR7 functions as a classical G protein-coupled chemokine receptor (Ödemis et al. 2012). In an attempt to unravel the molecular set-up which allows CXCR7 to signal through G proteins in astrocytes, we have now analysed the role of Grks in astrocytic CXCR7 function. We demonstrate that in astrocytes SDF-1/CXCL12 signalling is tightly controlled by Grk2, but not by other Grk isoforms. We further provide evidence that despite its central role in SDF-1/CXCL12 signalling, Grk2 does not determine the function of CXCR7 as a GPCR in astrocytes.

Evidence for a central role of Grk2 but not of Grk3, Grk5, and Grk6 in astrocytic CXCR7 function emerged from the analysis of SDF-1/CXCL12 signalling in astrocytes with RNAi-mediated inhibition of the various Grk isoforms. These analyses further unravelled that Grk2 affects astrocytic CXCR7 function by two independent mechanisms. One mechanism involves the Grk2-mediated internalization of CXCR7 as implied by the finding that SDF-1/CXCL12-induced depletion of CXCR7 cell surface expression levels is abrogated following inhibition of Grk2 expression. Receptor internalization is viewed as a crucial step eventually leading to receptor silencing (Moser et al. 2010). It is further of note that Grks together with arrestins not only function in a receptor-specific, but also in a cell type-specific manner. Consequently, no prediction is possible to whether Grk2 likewise controls CXCR7 internalization in other cell types. In this respect, it will be especially interesting to see whether Grk2 is also involved in the internalization of CXCR7 in cells in which CXCR7 functions as a scavenger receptor (Boldajipour et al. 2008; Naumann et al. 2010). In addition to regulating CXCR7 internalization, Grk2 seems to act as a mediator of CXCR7-dependent SDF-1/CXCL12 signalling in astrocytes. Consistent with this alternate function, we found that SDF-1/CXCL12 signalling in astrocytes is abrogated following inhibition of Grk2 expression. We wish to emphasize that this positive regulation of CXCR7-dependent SDF-1/CXCL12 signalling by Grk2 cannot be explained by its effects on CXCR7 internalization. In fact, as noted above, inhibition of Grk2 expression in astrocytes is associated with a loss in SDF-1/CXCL12-dependent internalization of CXCR7 which in a consequence should prolong, but not prevent CXCR7 signalling. Grk2 reportedly can affect cell signalling by phosphorylation of non-receptor proteins or the phosphorylation-independent activation of signalling molecules (Penela et al. 2010; Evron et al. 2012). By which of these mechanisms, Grk2 mediates SDF-1/CXCL12 signalling in astrocytes remains to be established. It is further of note that Grks not only function as positive regulators of CXCR7, but also of the alternate SDF-1/CXCL12 receptor, CXCR4, and might, thus, represent a general mechanism controlling the function of chemokines in health and disease. In fact, depending on the cell type, CXCR4 is a substrate for Grk6 and/or Grk3 (Vroon et al. 2004; Busillo et al. 2010; Balabanian et al. 2008). Moreover, in HEK-293 cells, SDF-1/CXCL12-induced activation of Erk1/2 decreases following depletion of either Grk6 or Grk3 (Busillo et al. 2010). Likewise, SDF-dependent chemotaxis is impaired in lymphocytes from Grk6-deficient animals (Fong et al. 2002). An emerging future issue will be to determine whether tuning of CXCR7 and CXCR4 signalling by Grks will have implications for tumour growth and metastasis. In this respect, previous work already revealed that CXCR7 is expressed by human glioma cells and additionally provided evidence that CXCR7 expression levels increase with the degree of glioma malignancy (Hattermann et al. 2010). Work from our group further demonstrated that as in primary astrocytes, CXCR7 mediates SDF-1/CXCL12-dependent signalling in human glioma cells by a G protein-dependent mechanism (Ödemis et al. 2012). Moreover, a large body of literature indicates that CXCR4 is expressed in tumour cells from various organs and affects tumour growth and metastasis (Furusato et al. 2010).

In contrast to signalling of SDF-1/CXCL12-bound CXCR7, RANTES/CCL5-dependent signalling remained unaffected in astrocytes with depleted Grk2 levels. RANTES/CCL5 binds to the chemokine receptors, CCR5, CCR1, and CCR3 (Jones et al. 2011), which are reportedly all expressed by astrocytes (Dorf et al. 2000). These findings further imply that Grk2 discriminates between signalling of CXCR7 and other chemokine receptors and, thereby, contributes to the diversity of GPCR signalling.

Notably, while our present studies establish that Grk2 is crucially involved in signalling of SDF-1/CXCL12-bound CXCR7 in astrocytes, we did not obtain evidence for the involvement of the Grk-arrestin system in defining the function of CXCR7 in a given cell type, in terms that a specific CXCR7 function would correlate with a selective expression pattern of Grks. In fact, we found that primary astrocytes as well as primary Schwann cells express Grk2, Grk3, Grk5, and Grk6. However, in astrocytes SDF-1/CXCL12 signals through CXCR7, whereas in Schwann cells, SDF-1/CXCL12 signalling requires the presence of both CXCR4 and CXCR7 (Ödemis et al. 2010). In a more recent work, we further found that similar to Schwann cells, CXCR4 and CXCR7 form a functional receptor unit in primary microglial cells (Lipfert et al. submitted). However, unlike Schwann cells, primary microglia only expressed Grk2 and Grk3 at detectable levels.

In summary, our findings unravel a tight control of CXCR7-dependent SDF-1/CXCL12 signalling in astrocytes by Grk2 which might have implications for the function of astrocytic CXCR7 under both physiological and pathophysiological conditions.

Acknowledgments We thank Patrick Küry for providing us with primary Schwann cells. This work was supported by the Deutsche Forschungsgemeinschaft (En 187/5-9).

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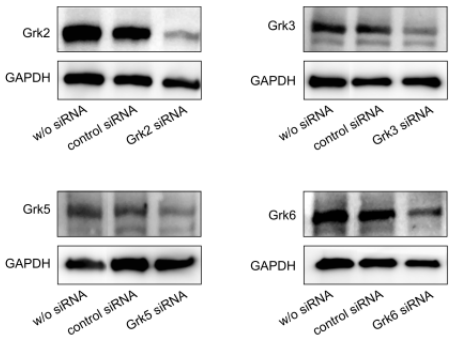
Supplementary Material



Supplementary Figure 1: Characterization of RNAi-mediated inhibition of Grk expression in cultured astrocytes. Cultured astrocytes were transfected overnight with selective Grk2 siRNA, Grk3 siRNA, Grk5 siRNA, or Grk6 siRNA as well as with control siRNA. Transfected cells and non-transfected controls were further maintained for 24 h with MEM containing 10% fetal calf serum, followed by another 24 h with N2-medium and were subsequently subjected to Western blot analysis. GAPDH served as a loading control.

4. Zusammenfassung

Publikationsdissertation zur Erlangung des akademischen Grades Dr. rer. med.

Die Rolle und Funktionsweise der Chemokinrezeptoren CXCR4 und CXCR7 in Mikroglia und Astrozyten

eingereicht von:	Diplom-Trophologin Jana Lipfert
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eingereicht im:	Februar 2013

Das Chemokin SDF-1 gehört zur Gruppe der CXC-Chemokine und ist an vielen physiologischen und pathologischen Prozessen im Organismus beteiligt. Es spielt eine entscheidende Rolle bei Immunreaktionen, während der Hämatopoese und bei der Entwicklung des Herzens, der Extremitätenmuskulatur und des peripheren und zentralen Nervensystems. Zudem ist SDF-1 ein entscheidender Faktor bei der Invasion und Metastasierung von Tumorzellen. Lange Zeit galt der Chemokinrezeptor CXCR4 als einziger Rezeptor für SDF-1. Erst vor einigen Jahren entdeckte man CXCR7, der bis dato als ein sogenannter *orphan*-Rezeptor galt, als einen alternativen Rezeptor für das Chemokin SDF-1. Es zeigte sich, dass CXCR7 von vielen unterschiedlichen Zelltypen expremiert wird, aber offensichtlich nicht wie CXCR4 als typischer G-Protein-gekoppelter Rezeptor agiert.

Da alle Zelltypen des ZNS sensitiv für SDF-1 sind, sollte in dieser Arbeit untersucht werden, ob CXCR7 auch von Mikroglia, den immunkompetenten Zellen des ZNS, expremiert wird und welche Funktion der Rezeptor beim SDF-1-Signalverhalten inne

hat. Es zeigte sich, dass primäre Mikroglia CXCR4 und CXCR7 sowohl auf RNA- als auch auf Proteinebene expremiert. Die Analyse von membranständigem CXCR4 und CXCR7 machte eine Kolokalisation der Rezeptoren auf der Mehrheit der Mikrogliazellen deutlich. Die Stimulation von primärer Mikroglia mit SDF-1 ergab im Westernblot eine Aktivierung der Signalmoleküle Erk1/2 und Akt. Zudem wurde ein mitogener und chemotaktischer Effekt von SDF-1 auf Mikroglia nachgewiesen. Um zu überprüfen, über welchen der beiden Chemokinrezeptoren SDF-1 seine Effekte vermittelt, wurden pharmakologische Rezeptorantagonisten genutzt. Der Einsatz der CXCR4-Inhibitoren AMD3100 und T140 und der CXCR7-Inhibitoren CCX771 und CCX733 ergab eine Beteiligung beider Chemokinrezeptoren an allen untersuchten SDF-1-Effekten. In einem nächsten Schritt wurde untersucht, ob der Aktivierungszustand von Mikroglia einen Einfluss auf die Expression von CXCR4 und CXCR7 hat. In vitro zeigte sich nach Behandlung der Mikroglia mit Lipopolysacchariden (LPS) eine Zunahme von sowohl CXCR4 als auch CXCR7. Als in vivo-Modell wurden Hirnschnitte von MCAO-Ratten genutzt. Bei der MCAO wird Ratten die Arteria cerebri media verschlossen und so künstlich eine Ischämie verursacht. Nach Ischämie kam es zu einer Einwanderung von Mikroglia in die Randzone des betroffenen Bereichs und dort zu einem Übergang von ramifizierter in aktivierte Mikrolgia. In ramifizierter Mikroglia konnte nur eine sehr schwache CXCR4- und keine CXCR7-Expression nachgewiesen werden. In aktivierter Mikroglia war dagegen eine deutliche Expression beider Rezeptoren zu erkennen. Auch der Ligand von CXCR4 und CXCR7, SDF-1, wird nach Verschluss der Arterie im Ischämiegebiet vermehrt expremiert [96]. Die Hochregulation beider Rezeptoren in aktivierter Mikroglia scheint deshalb für deren Wanderung in Richtung SDF-1 und damit zum Ischämiegebiet wichtig zu sein. Daneben könnte SDF-1 über CXCR4 und CXCR7 auch in vivo mitogen auf Mikroglia wirken, denn nach MCAO kommt es zu einer starken Proliferation von Mikroglia [99]. Welche weiteren Funktionen CXCR4 und CXCR7 in aktivierter Mikroglia haben, bleibt Aufgabe weiterer wissenschaftlicher Analysen.

Die Ergebnisse dieser Untersuchungen lassen schlussfolgern, dass in primärer Mikroglia sowohl CXCR4 als auch CXCR7 für die Vermittlung der SDF-1-Effekte essentiell sind und das beide Rezeptoren nach Aktivierung der Mikroglia in vitro und in vivo verstärkt expremiert werden.

In einer vorangegangenen Arbeit [71] wurde bereits gezeigt, dass in primären Astro-

zyten CXCR7 als alleiniger funktioneller Rezeptor für SDF-1 fungiert. Die vorliegenden Untersuchungen sollten nun dazu dienen, die molekularen Mechanismen, über die CXCR7 agiert, aufzuklären. Erstmals erfolgte hier der Nachweis einer Kopplung von $G_{i/o}$-Proteinen an den SDF-1-aktivierten CXCR7. Die Bindung beziehungsweise Aktivierung von $G_{i/0}$-Proteinen wurde mit Hilfe des [^{35}S]-GTPγS Bindungs Assays, der fluorometrische $[Ca^{2+}]_i$-Messung und durch den Einsatz des $G_{i/0}$-Protein-Blockers Pertussistoxin (PTX) nachgewiesen. Bei allen diesen Untersuchungen zeigte sich, dass die Depletion des Chemokinrezeptors CXCR4 keinerlei Einfluss auf die SDF-1-Effekte in Astrozyten hatte. Wurde dagegen CXCR7 durch siRNA inhibiert, blieb die G-Protein-Aktivierung und der intrazelluläre Calciumanstieg nach Stimulation mit SDF-1 aus.

Da CXCR7 neben SDF-1 noch I-TAC als zweiten Liganden bindet, wurde auch das I-TAC-Signalverhalten in primären Astrozyten untersucht. Im Gegensatz zu SDF-1 führte I-TAC zu keiner Aktivierung von G-Proteinen durch CXCR7 und ebenfalls zu keinem intrazellulären Calciumanstieg. Die durch I-TAC induzierte Aktivierung der Signalmoleküle Erk1/2 und Akt wurde dagegen über β-Arrestin2 vermittelt. In Astrozyten ist somit CXCR7 ein ligandenabhängiger Rezeptor, der nach SDF-1-Bindung G-Proteine aktiviert und nach I-TAC-Bindung seine Effekte über β-Arrestin2 mediiert.

Um die molekularen Mechanismen des SDF-1-Signalverhaltens in Astrozyten weiter aufzuklären, wurden Grk2, Grk3, Grk5 und Grk6 mit siRNA inhibiert und die Internalisierung von CXCR7 sowie die Aktivierung der Signalmoleküle Erk1/2 und Akt analysiert. Grks phosphorylieren aktivierte GPCRs und sind so für deren Desensitivierung und Internalisierung wichtig. Zudem können Grks auch viele weitere Moleküle regulierend beeinflussen. Die Untersuchungen zeigten, dass in primären Astrozyten allein Grk2 für die Internalisierung des SDF-1-aktivierten CXCR7 essentiell ist. Überraschenderweise führte die Depletion von Grk2 nicht zu einer Verlängerung des SDF-1-Signals, sondern zu einem Ausbleiben der Aktivierung von Erk1/2 und Akt, was eine Interaktion von Grk2 mit aktivierten G-Proteinen vermuten lässt.

Darüber hinaus ergab die Untersuchung der humanen Gliomzelllinien U343 und A764, die beide CXCR7, aber keinen CXCR4 expremieren, eine SDF-1-induzierte Aktivierung der Signalmoleküle Erk1/2 und Akt. Dieser Effekt konnte wie bei primären Astrozyten durch PTX vollständig unterbunden werden, so dass auch hier offensicht-

lich eine Aktivierung von $G_{i/0}$-Proteinen durch den SDF-1-gebundenen CXCR7 stattfindet. Die Induktion der Erk- und Akt-Signalwege ist für die Migration, Proliferation und das Überleben von Gliomzellen essentiell [100–103]. Es gibt aber auch Gliomzelllinien, bei denen CXCR4 die proliferativen Effekte von SDF-1 mediiert [104]. Auch die Zellen vieler anderer Krebsarten wie Blasen- [105], Gallenblasen- [106], Prostata- [60], Gebärmutterhals- [107] und Brustkrebs [108] expremieren verstärkt CXCR4 und/oder CXCR7. Für die Entwicklung von Therapien für Krebspatienten ist es daher einerseits wichtig zu wissen, welchen der beiden Chemokinrezeptoren SDF-1 aktiviert und andererseits welche Signalmoleküle infolgedessen aktiviert werden.

Betrachtet man die Ergebnisse dieser Untersuchungen im Kontext mit bisherigen Arbeiten zu dem Chemokin SDF-1 und seinen Rezeptoren CXCR4 und CXCR7, wird einmal mehr eine zelltypspezifische Funktionsweise dieses Systems deutlich. In primärer Mikroglia, wie auch in Schwannschen Zellen [71] sind sowohl CXCR4 als auch CXCR7 für die Vermittlung der SDF-1-Effekte notwendig. Wie genau diese "Rezeptoreinheit" arbeitet, also beispielsweise über welche Bindungsstellen Heterodimere interagieren, bleibt Aufgabe weiterer Untersuchungen. In Muskelzellen mediiert SDF-1 seine Effekte über CXCR4, während CXCR7 nur als ein *Scavanger*-Rezeptor für das Chemokin dient [66]. In Astrozyten dagegen agiert nur CXCR7 als funktioneller Rezeptor für SDF-1. Während in anderen Zelltypen ebenfalls eine Bindung von SDF-1 an CXCR7 gezeigt wurde, konnte bisher keine G-Protein-Kopplung an den Rezeptor nachgewiesen werden [21, 55, 68]. Stattdessen erfolgte das SDF-1-*Signalling* über CXCR7 durch β-Arrestine [69, 75–77]. In primären Astrozyten zeigte sich aber nicht nur eine Kopplung des SDF-1-aktivierten CXCR7 an $G_{i/0}$-Proteine, sondern auch eine ligandenabhängige Arbeitsweise des Rezeptors. Bindet I-TAC an CXCR7 führt dies nicht zu einer Aktivierung von Signalmolekülen über G-Proteine, sondern über β-Arrestin2.

Weitere Untersuchungen sind notwendig, um mögliche "Marker" zu finden, die eine Vorhersage zulassen, über welchen Rezeptor und über die Aktivierung welcher Effektormoleküle SDF-1 seine Effekte in einer bestimmten Zelle vermittelt. Mit dem Nachweis von CXCR7 als einen funktionellen Rezeptor für SDF-1, sollten zudem frühere Untersuchungen, die CXCR4 als einzigen Rezeptor für SDF-1 voraussetzten, nochmals überprüft werden.

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A. Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren.

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	"Adipokine als Mediatoren der Adipositas-vermittelten endothelialen Dysfunktion" unter Betreuung von Prof. Dr. Mathias Faßhauer
seit März 2010	**Institut für Anatomie, Medizinische Fakultät, Universität Leipzig** Anfertigung der vorliegenden Dissertation in der Arbeitsgruppe Molekulare Neuroantatomie unter Betreuung von Prof. Dr. Jürgen Engele

Auslandsaufenthalte

Okt. 2007-Feb. 2008	**Institute of Food Research, Norwich, UK** Stipendium über das LEONARDO DA VINCI-Projekt Mitarbeit an einer Humanstudie zum Thema "Selen und Immunstatus"

C. Publikationen und Poster

Publikationen

- J. Lipfert, V. Ödemis, D. Wagner, J. Boltze and J. Engele. CXCR4 and CXCR7 form a functional receptor unit for SDF-1/CXCL12 in primary rodent microglia. In: Neuropathol Appl Neurobiol, doi: 10.1111/nan.12015.
- J. Lipfert, V. Ödemis and J. Engele. Grk2 is an Essential Regulator of CXCR7 Signalling in Astrocytes. In: Cell Mol Neurobiol 33 (2013), pp. 111-118.
- V. Ödemis, J. Lipfert, R. Kraft, G. Abraham, K. Hattermann, R. Mentlein and J. Engele. The presumed atypical chemokine receptor CXCR7 signals through G(i/o) proteins in primary rodent astrocytes and human glioma cells. In: Glia 60.3 (2012), pp. 372-381.
- V. Ödemis, J. Lipfert and J. Engele. The chemokine CXCL11/I-TAC signals to primary rat cortical astrocytes and human glioma cells via the CXCL12/SDF-1 receptor CXCR4. submitted
- G. Sommer, S. Kralisch, J. Lipfert, S. Weise, K. Krause, B. Jessnitzer, U. Lössner, M. Blüher, M. Stumvoll and M. Fasshauer. Amyloid precursor protein expression is induced by tumor necrosis factor alpha in 3T3-L1 adipocytes. In: J Cell Biochem 108.6 (2009), pp. 1418-1422.
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- S. Kralisch, S. Weise, G. Sommer, J. Lipfert, U. Lössner, M. Blüher, M. Stumvoll and M. Fasshauer. Interleukin-1beta induces the novel adipokine chemerin in adipocytes in vitro. In: Regul Pept 154.1-3 (2009), pp. 102-106.

Poster

- J. Lipfert, V. Ödemis and J. Engele: Expression and function of the SDF-1 chemokine receptors CXCR4 and CXCR7 in primary rat microglia. 9th Leipzig Research Festival for Life Science (2010).
- J. Lipfert, V. Ödemis and J. Engele: The presumed atypical chemokine receptor CXCR7 controls astrocytic migration and proliferation through $G_{i/0}$ protein coupling. 10th European Meeting on glial Cells in Health and Disease, Prague and 10th Leipzig Research Festival for Life Science (2011).
- J. Lipfert, V. Ödemis and J. Engele: I-TAC signals through the SDF-1 receptor CXCR4 in primary rodent astrocytes and human glioma cells. 11th Leipzig Research Festival for Life Science (2012).

D. Danksagung

Besonders möchte ich Herrn Prof. Dr. Jürgen Engele danken, der mir durch die Aufnahme in seine Arbeitsgruppe die Bearbeitung dieses interessanten Themas ermöglichte.

Zudem geht mein Dank an Dr. Veysel Ödemis und Nicole Lakowa für die Einarbeitung in verschiedene Arbeitstechniken, für ihre ständige Hilfsbereitschaft und für die freundschaftliche Arbeitsatmosphäre.

Herzlich bedanken möchte ich mich auch bei PD Dr. Robert Kraft, der mir das Anlegen von primären Mikrogliakulturen beibrachte und darüber hinaus für die Calciummessungen zuständig war. Bei Dr. Peter Hajek und Dr. Getu Abraham bedanke ich mich für die Durchführung des [^{35}S]-GTPγS Bindungs Assays. Mein Dank gilt zudem Dr. Daniel-Christoph Wagner, der mir die MCAO-Schnitte zur Verfügung stellte. Dr. Kirsten Hattermann und Prof. Dr. Patrick Küry danke ich für die Bereitstellung der Gliomzelllinien bzw. Schwannschen Zellen.

Mein Dank gilt auch allen anderen Mitarbeitern der Anatomie, die mich sehr offen und freundlich am Institut aufgenommen haben. Besonders danken möchte ich dabei Dr. Elke Brylla, die in schwierigen Zeiten immer ein offenes Ohr für mich hatte. Ein großer Dank geht auch an Angela Ehrlich und Constanze Hobusch, die mir stets bei praktischen Fragen weiterhelfen konnten und in deren gut gefüllten und perfekt geordneten Tiefkühlschrank ich mich des Öfteren bedienen durfte.

Frau Evelyn Finsterbusch vom Medizinisch-Experimentellen Zentrum danke ich für ihr unkompliziertes, stets freundliches Gemüt, mit dem sie mich immer zum Lächeln brachte.

Nicht zuletzt bedanke ich mich bei meiner Familie und bei meinen Freunden für ihre Unterstützung und Aufmunterung während der letzten drei Jahre. Ohne meinen Freund Daniel als steten Ruhepol, hätte ich diese Zeit sicher nur mit ein paar grauen Haaren mehr überstanden.