PERSPECTIVE

Chemokines and their receptors: important mediators to be aware of in neuroregenerative approaches for spinal cord injury

Curative therapy for spinal cord injury (SCI) remains elusive, however identifying options for tailored treatment strategies is in full swing. Like in the brain there are distinct regions in the adult spinal cord that harbor neural progenitor cells (NPCs) (Horner et al., 2000). This offers the possibility of recruiting these cells in reparative approaches to support endogenous spinal cord regenerative capacities. Hereby, one challenge among many others is to overcome the already restrictive microenvironment of potential endogenous NPCs, which is considerably altered by ongoing secondary lesion cascades by the initial lesion. Research addressing this aspect identified a plethora of mediators that hinder or promote neuroregeneration where at inflammatory mediators are known to play an essential role.

This perspective article—which is based on recent investigations in our lab on a rat SCI model—focus on a highly versatile group of inflammatory mediators, the chemokine-effectors/receptors, with main emphasis in their potential involvement in secondary lesion cascades in the microenvironment of endogenous NPCs after SCI. Beside their well-defined role in inflammatory cascades these chemotactic cytokines were shown to be involved in varied pathological and physiological processes in the central nervous system that also become of importance during lesion cascades induced by SCI. For instance, an interesting observation regarding neuroregeneration is that chemokines are expressed in the developing brain and play important roles in directing cell migration, providing trophic support during cell proliferation and differentiation (for review Asensio and Campbell (1999)). However, little is known to date regarding the influence chemokines might have on endogenous NPC in the adult spinal cord.

To investigate a possible enrolment of these inflammatory mediators in cellular cascades concerning endogenous NPCs the expression profiles of different chemokine effectors/receptors after defined spinal cord lesions were first investigated *in vivo* before addressing specific aspects of NPC properties under SCI- and chemokine-influence *in vitro*: In our SCI paradigm of thoracic force-defined spinal cord impact lesions, which were applied with the Infinite Horizon Impactor (Precision System and Instrumentation, Lexington, KY, USA) (detailed description is provided by Knerlich-Lukoschus et al. (2008)) we investigated chemokine-effector/receptor expression patterns at defined time points (from post-operative days 3, 7, 14 to day 42) after spinal cord lesion of different severity grades (100 kdyn, 150 kdyn, 200 kdny) or sham operation (T_9 laminectomy only). Thereby the whole neuraxis of adult male Long Evans rats (*i.e.*, cervical, thoracic, lesion (T_9), lumbar spinal cord segments and the brain) was analyzed for chemokine-effector/receptor expression *via* real-time RT-PCR, western blotting, immunohistochemistry, and double/triple-immunofluorescence. During their survival time periods, animals underwent standardized behavioral testing for locomotor function, mechanical, and thermal sensitivity to monitor probable SCI related chronic pain equivalents.

In the lesion groups, different chemokines like chemokine (C-C motif) ligand 2 (CCL2), CCL3, chemokine (C-X-C motif) ligand 12 (CXCL12), and their respective receptors chemokine (C-C motif) receptor 2 (CCR2), CCR1, and CXCL4 were strongly induced time and lesion-grade dependent on mRNA-, protein-, and inmmunohistochemical-level in distinct anatomic regions of the spinal cord and brain (Knerlich-Lukoschus et al., 2011a, b). Beside in the lesion rim, dorsal horns, and distinct brain regions, CCL3, CCL2, and CXCL12 and their respective main receptors were found on elevated mRNA- and protein-level in the ventro-lateral spinal cord white matter (Knerlich-Lukoschus et al., 2010), a region that is considered one niche of the adult spinal cord, which harbors endogenous NPCs (Weiss et al., 1996; Horner et al., 2000). We therefore also performed BrdU labeling experiments at defined survival time points to confirm cell proliferation and plot a potential time-dependency after SCI: After SCI, these anatomical regions exhibited significantly elevated subpial cell proliferation which was co-expressed with NPC-markers like nestin, musashi-1, NG2, and radial glia markers like BLBP and 3CB2 (Knerlich-Lukoschus et al., 2010) (**Figure 1A–D**). Interestingly chemokine expression in this region was in part co-localized or co-expressed with these groups of proliferating (BrdU-labled) NPC-marker positive cells (confirmed by double/triple-immunofluorescence labeling) (Knerlich-Lukoschus et al., 2010) (**Figure 1**). These findings suggested that chemokines might be crucial determinants of the microenvironment of spinal cord endogenous NPCs, and prompted further *in vitro* examinations to investigate the potential influence of chemokines on spinal cord derived NPCs (Knerlich-Lukoschus et al., 2014).

For this purpose, NPCs were isolated from thoracic spinal cord segments of adult rats 48 hours after receiving severe thoracic impact SCI (200 kdyn) or sham-laminectomy and cultured according to established protocols for spinal cord derived NPCs ((Weiss et al., 1996; Yamamoto et al., 2001); for further details (Knerlich-Lukoschus et al., 2014)). During establishing this *in vitro* paradigm it became obvious that neurosphere-like cells obtained from lesioned animals exhibited altered proliferation and differentiation capacities compared to sham-derived neurosphere cultures: Accordingly, there was significantly higher cell proliferation measureable in lesioned derived neurosphere cultures (proliferation profiles of the neurosphere-like cultures were established by determining the DNA yield of the analyzed cells by CyQuant proliferation assays). Furthermore, differentiated cultures (under growth factor-deprivation) from lesion animals exhibited significantly higher GFAP-mRNA levels compared to sham-derived cultures (Knerlich-Lukoschus et al., 2014).

This mirrored the previous *in vivo* findings of strong post-traumatic astrogliosis around the initial lesion core (which resulted over time intogliotic scar-like formation on

thoracic level around the developing syrinx), and throughout the ventro-lateral white matter along the whole spinal cord axis (Knerlich-Lukoschus et al., 2010). Thereby CCL2, CCL3, and CXCL12 were co-expressed with astroglial (glial fibrillary acidic protein (GFAP)) and radial glial markers (brain lipid-binding protein (BLBP), 3CB2) in cells that also morphologically resembled radial glial cells with long cell processed spanning from subpialinto the ventro-lateral white matter (**Figure 1B**, **C**). C-X-C chemokine CXCL12 (Stromal cell-derived factor 1-alpha (SDF-1alpha)) and its receptor CXCR4 were additionally in part co-expressed with oligodendroglial markers (CNPase, NG2); otherwise chemokines under investigation were not found in cells co-labeled with inflammatory, microglial, or monocytic markers like CD11b or ED1 or neuronal cell markers (neurofilament (NF), neuronal nuclei antigen (NeuN), III-beta tubulin). This implied that C-C- and C-X-C-chemokines might be involved particularly in post-lesional cellular processes that involve astrocytic and radial gliacells.

Before investigating the effect of externally applied chemokines on spinal cord-derived NPC *in vitro*, the expression level and pattern of different chemokine receptors like CCR1, CCR2, CXCR4, CCR5 on the obtained neurosphere-like cells was establishedby real-time RT-PCR and immunohistochemical analyses (Knerlich-Lukoschus et al., 2010, 2014). Thus, CCR1 was identified as chemokine receptor that was expressed on highest and stable level in both sham- and spinal cord lesion-derived NPC cultures (**Figure 1E**). After differentiation, CCR1-mRNA decreased to significant lower levels compared to expression levels in neurosphere cultures. Applying "external" CCL3—the main ligand of CCR1—to neurosphere cultures during their differentiation cycles led to significant elevated GFAP-mRNA amounts in differentiated sham-derived cell cultures (**Figure 1F**, **G**). Unlike in sham-derived cell cultures, pre-treatment of CCL3 had no significant effect on glial differentiation in spinal cord lesion cultures (Knerlich-Lukoschus et al., 2014).

We interpreted these *in vitro* findings as a result of an *in vivo* preconditioning effect of chemokines on endogenous NPC: After impact lesion there is a strong inflammatory reaction from the very early beginning with induction of pro-inflammatory mediators like cytokines and chemokines on thoracic spinal cord levels (Knerlich-Lukoschus et al., 2008). Consequently it is likely that neurosphere-like cells, which derived from spinal cord-injured animals, were already exposed to these inflammatory mediators and by this means pre-conditioned by microenvironmental changes before isolated for cell-culture experiments (spinal cord segments were taken 48 hours after surgery). In contrast, sham laminectomies did not result in an induction of chemokines and cytokines, and NPCs obtained from these animals were most likely not pre-exposed to these molecules.

In the context of recent literature on chemokines and NPCs these observations in our *in vitro* and *in vivo* SCI settings strongly suggest that CC-chemokines have an impact onendogenous NPCs and specifically onastroglial/radial glial differentiation. Regarding neuroregeneration after SCI, this remained a double-edged sword: In principal, radial glial cells and subgroups of astrocytes can be viewed as pluripotent neural precursors in the adult CNS and are important among others in guiding migrating neurons, regulating axon out-growth and path-finding during white matter patterning (among others (Rakic, 1972)). Thus these cells might be crucial after SCI in that they support endogenous NPCs of neuronal cell fate in their migration and formation of functioning cell circuits, a pre-requisite for functional neuroregeneration. However, on the other hand excessive astrogliosis results into gliotic scarring that is known to hinderformation of such new, functional circuits. This means that balancing these effects of astrogliosis remains an important and challenging aspect, which has to be considered in developing anti-inflammatory-targeted therapies for SCI.

In ongoing and future investigations the intracellular signal transduction pathways of the observed differences in chemokine effects on sham and lesion derived NPC cultures have to be addressed further. Preliminary investigations of CCL3 effects on the EGF signal transduction pathway revealed that pre-treatment with CCL3 induced an activation of MAP Kinase p42 and p44 only in cell cultures derived from sham groups (unpublished preliminary data). This again might be due to chemokine-receptor desensitisation during very early time course after lesion. However, these are preliminary results, which have to be consolidated in further detailed analyses of potentially involved signalling cascades.

Summarizing the presented *in vivo* and *in vitro* studies regarding possible effects of chemokines on endogenous NPCs after SCI, different chemokine subgroups might be involved in distinct regulatory functions of neuro-de- and regeneration, depending on the time-point at which and anatomical region in which these mediators are induced. In regard to their subpial induction in the ventro-lateral white matter C-C-chemokines and their receptors (CCL3/CCR1) are likely involved in cellular processes that comprise astroglial and radial glial cell types. In this sense, chemokines can be viewed as important "determinants" of endogenous NPCs' microenvironment. Consequently, these mediators should also be considered in future development of restorative approaches for SCI.

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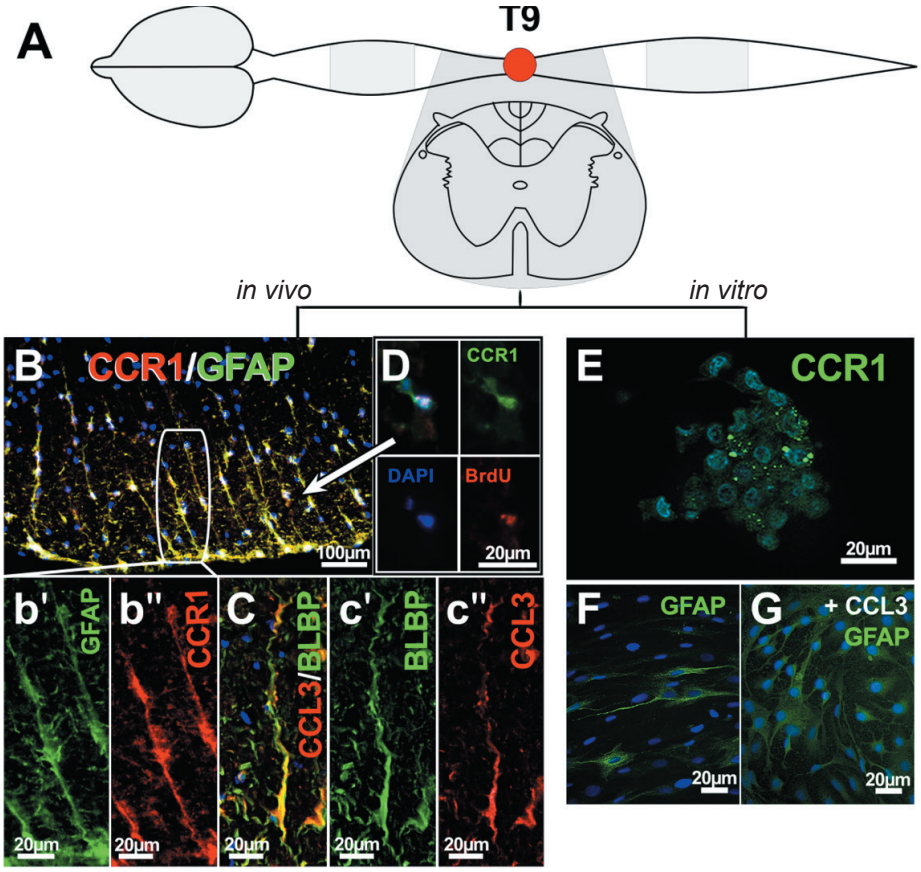

Figure 1 ***In vivo*** **chemokine induction in the ventro-lateral white matter after spinal cord injury (SCI) and chemokine influence on spinal cord-derived neural progenitor cells (NPCs)** ***in vitro*****.**
(A) Impact lesions of different severity grades or sham laminectomies were applied on thoracic level T_9. Cervical, thoracic, and lumbar spinal cord regions were investigated for chemokine expression (grey shaded areas), and thoracic spinal cord segments were taken for NPC cultures. (B, C) Chemokines were induced strongly in the ventro-lateral white matter, exemplarily shown for CCR1 and CCL3 on thoracic level on immunohistochemical level (double/triple-immunofluorescence): (A) Double-immunofluorescence staining for CCR1 and the astroglial marker GFAP in the ventro-lateral white matter after 200 kdyn SCI (CCR1 = red; GFAP = green, co-localization = yellow). b' and b" depict single channel images for GFAP and CCR1 in the outlined region (B). (C) Chemokines were also co-expressed with radial glial markers shown here for BLBP (c', green) and CCL3 (c", red) (co-localization in C = yellow). (D) In the subpial region of the ventro-lateral white matter (arrow B/D) chemokines/-receptors were also co-expressed with BrdU-labeled proliferating cells, exemplarily shown for CCR1 (CCR1 = green, nuclei/DAPI = blue, proliferation/BrdU = red). (E) *In vitro* CCR1 was expressed consistently on neurospheres obtained from thoracic spinal cord segments of sham (shown in D as dotted staining pattern on separated part of a sham neurosphere) and 200 kdyn lesioned animals. Pre-treatment with CCL3 led to significant higher GFAP level in sham derived differentiated cell cultures (G) compared to non CCL3-stimulated controls (F) (for details see (Knerlich-Lukoschus et al., 2010, 2014). CCR1: Chemokine (C-C motif) receptor 1; CCL3: chemokine (C-C motif) ligand 3; GFAP: glial fibrillary acidic protein; BLBP: brain lipid-binding protein.

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