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Bacterial Meningitis: Investigating Injury & Regenerative Therapy in vitro

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Bacterial meningitis (BM) causes brain injury in the hippocampus, a structure critical for memory function. Due to the multifactorial pathogenesis involving the interplay between the brain cells, the bacteria and the host's inflammatory reaction, the majority of research on meningitis is performed in animals. The project (103/06) supported by the 3R Research Foundation Switzerland and carried out by a team of researchers (main investigator Sandra Hofer PhD) lead by Prof. Stephen Leib from the Neuroinfection Laboratory, Inst. for Infectious Diseases, Department of Clinical Research, University of Bern, focused on the establishment of in vitro models that reproduce important pathophysiological processes of brain damage and tissue regeneration in infectious diseases of the brain.

Pathogenesis can be mimikked in cell cultures

Brain injury after bacterial meningitis is characterized by programmed cell death (apoptosis) of cells in the dentate gyrus of the hippocampus.(1) Evidence from clinical and experimental studies suggests that both, the pathogen (bacterial components) and the host inflammatory reaction (e.g. TNF cytokine release and growth factor deprivation), contribute to the development of hippocampal injury. (2) For this purpose we developed a cell-culture system with isolated hippocampus-derived neuronal stem/progenitor cells (NCP). It was possible to differentiate the cultured cells into defined developmental stages (stem cells, immature neurons and mature neurons, Fig 1A). Several days (1, 7, 14 and 21) after differentiation, they were challenged with different stimuli characteristic for bacterial meningitis (Fig 1B), i.e. growth factor deprivation and TNF-α (representing host reactions) or bacterial components (representing a pathogen). Subsequently apoptosis, as observed in meningitis in the dentate gyrus, was documented by cleaved Caspase-3, Annexin-V or Apoptosis Inducing Factor (Fig 1C) . Thus, the newly established cell culture system recapitulated hippocampal neuronal differentiation *in vitro* and reflected the cellular response to typical insults of meningitis.

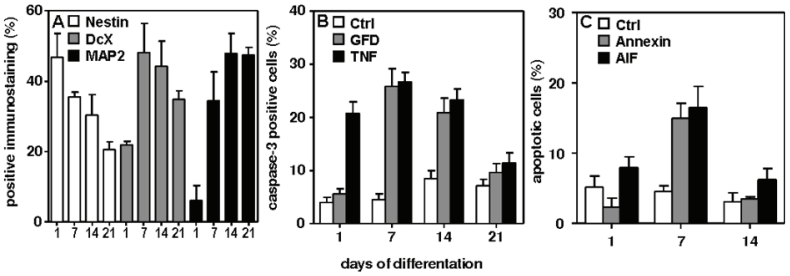

Figure 1:
(A) Neuronal differentiation *in vitro* was documented by a gradual shift in the predominant staining pattern from Nestin (stem cells) at day 1 to Doublecortin (DcX; immature neurons) at 7-14 days to MAP2 (mature neurons) at 21 days (Ctrl=control).

Which cell types are most susceptible to dying from bacterial meningitis?

Experimental studies showed that meningitis impairs hippocampal regeneration.(3) This finding is reproduced in the newly established cell culture model where stem cells and immature neurons showed a higher incidence of apoptotic death than mature neurons after exposure to factors involved in the host pathogen interactions during meningitis (Fig 2 A, B, C). Our data support the view that stem cells and immature neurons are susceptible to apoptosis while mature neurons are more resistant to meningitis-induced death stimuli. This observation may help to understand why neurological deficits persist long after childhood bacterial meningitis and the hippocampal repair potential seems insufficient to compensate for the brain damage. (4) The availability of this *in vitro* system leads to a substantial reduction of animal use as it provides a tool for the screening of pathogenic mechanisms for their relevance prior to conducting large scale studies *in vivo*.

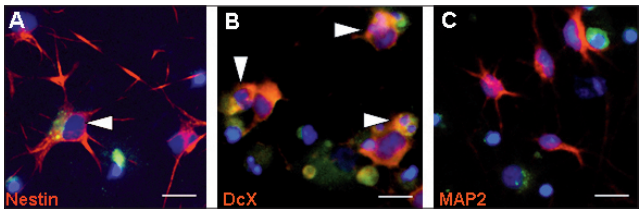

Figure 2:
Immunocytochemical staining revealed that stem cells (A, red = positive for Nestin) and immature neurons (B, red = positive for Doublecortin) are susceptible to apoptosis (White arrow) while mature neurons (C, red = positive for Microtubule Associated Protein) are more resistant to death stimuli characteristic for bacterial meningitis Scale bar = 50 µM.

The *in vitro* findings were validated *in vivo* in an infant rat model of pneumococcal meningitis. The number of immature neurons and stem cells in the hippocampus *in vivo* was found to be proportional to the formation of neurospheres (free floating clusters of immature brain cells) from brain cells isolated from this brain structure. Accordingly, the capacity of hippocampal-derived cells to multiply and form neurospheres was reduced by approx. half in infant rats that survived pneumococcal meningitis compared to their uninfected littermates (Fig 3).

From cells to tissue culture

Organotypic hippocampal slice cultures (OHC) retain the organisation of nervous tissue similar to that *in vivo* and offer a well-validated model for studying different stem cell grafts for their regenerative potential. In a previous study, the hippocampal apoptosis in the subgranular zone of the dentate gyrus was reproduced *in vitro* using the OHC system.(5)

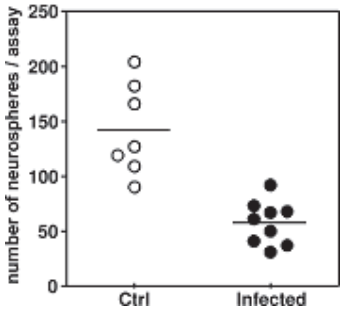

Figure 3
Significantly fewer neurospheres were obtained from the hippocampi of animals with menigitis compared to uninfected controls ($p < 0.05$).

An *in vitro* system of brain tissue regeneration

In the dentate gyrus of the hippocampus neurogenesis continuously occurs throughout life. A potentially attractive treatment option for support/reconstitution of neurogenesis after damage to the stem cell niche (e.g. in meningitis) is the delivery of regenerative cells to the site of cell loss. Currently, different sources of stem cells are being explored for potential use in repairing the brain. In general these explorative studies assessing the potential of different stem cell populations are carried out by performing *in vivo* studies. A large proportion of the cell types evaluated are not suitable for transplantation. To reduce and replace studies in animals, we established an *in vitro* pre-screening system of OHCs injured by challenge with bacterial components to evaluate different neuronal precursor cells (NPCs) for their repair potential in the injured hippocampus.(6)

Regeneration occurs *in vitro*

NPCs from the fetal hippocampus, expressing Green Fluorescence Protein (GFP), were grafted into the dentate gyrus of organotypic hippocampal slice cultures (OHC). The migration and differentiation of grafted cells were examined on cryosections of intact OHCs and in those injured by challenge with bacterial components using immunofluorescence and histomorphometry (Fig 4 A, B). After transplantation NPCs migrated out of the dentate gyrus in intact OHC (Fig 4 A) whereas in injured OHC, NPCs migrated and integrated into the damaged dentate gyrus (Fig 4 B).

Comparable result with *in vivo* studies.

Subsequently, the newly established *in vitro* system was validated by a limited *in vivo* study in an infant rat model of pneumococcal meningitis to demonstrate its appropriateness as an alternative to experiments *in vivo*. Application was done by stereotaxical transplantation of NPCs into the hilus region of intact and injured hippocampal dentate gyrus. Survival and integration were monitored by immunofluorescence and histomorphometry at 1, 2 and 4 weeks after transplantation (Fig 4 C, D).

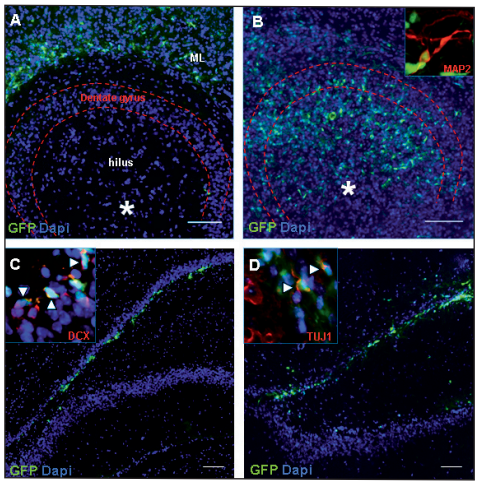

Figure 4: Transplantation of NPCs expressing Green Fluorescent Protein (GFP) in the hippocampus *in vitro* (A, B) and *in vivo* (C, D).

In vitro (A, B) migration of transplanted NPCs (green) after grafting into intact and pneumococci-injured organotypic hippocampal slice cultures (OHCs): A) The grafted NPCs derived from the hippocampus migrated from the area of engraftment (*) into the molecular layer structure (ML) outside of the dentate gyrus region in intact OHC, while B) they migrated to and integrated into the area of cell loss in the dentate gyrus in injured OHC.

In vivo (C, D) NPCs (green) migrated and integrated into the damaged area of the hippocampus at 14 (C) and 28 (D) days after transplantation and expressed markers of neuronal development (DCX, insert C, and TUJ1, insert D). Scale bars 100 µm.

3R relevance

The experimental *in vitro* systems we established allow the reduction and/or replacement of the following *in vivo* investigations: i) testing of pathogenic hypothesis by *in vitro* screening of potential bacteria-derived mediators (e.g. bacterial cell wall components) and potential host factors (e.g. host inflammatory mediators), ii) assessment of the intrinsic properties of the different stages of cell differentiation which contribute to their selective vulnerability, and iii) evaluation of therapeutic approaches that involve the grafting of stem/progenitor cells into brain tissue. Thus, only approaches that proved successful *in vitro* would be considered for further evaluation *in vivo*.

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