Bacterial Meningitis Impairs Hippocampal Neurogenesis

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Abstract
Bacterial meningitis causes persisting neurofunctional sequelae. The occurrence of apoptotic cell death in the hippocampal subgranular zone of the dentate gyrus characterizes the disease in patients and relates to deficits in learning and memory in corresponding experimental models. Here, we investigated why neurogenesis fails to regenerate the damage in the hippocampus associated with the persistence of neurofunctional deficits. In an infant rat model of bacterial meningitis, the capacity of hippocampal-derived cells to multiply and form neurospheres was significantly impaired compared to that in uninfected littersmates. In an in vitro model of differentiating hippocampal cells, challenges characteristic of bacterial meningitis (i.e. bacterial components, tumor necrosis factor [20 ng/mL], or growth factor deprivation) caused significantly more apoptosis in stem/progenitor cells and immature neurons than in mature neurons. These results demonstrate that bacterial meningitis injures hippocampal stem and progenitor cells, a finding that may explain the persistence of neurofunctional deficits after bacterial meningitis.

Key Words: Apoptosis, Bacterial meningitis, Dentate gyrus, Differentiation, Hippocampal injury, Neurosphere assay, Stem/progenitor cells.

INTRODUCTION
Survivors of bacterial meningitis experience permanent neurologic sequelae including impairment of learning and memory in up to 50% of cases (1–3). Importantly, the disabilities persist into adulthood (2, 4–6). Patients who die of bacterial meningitis show neuronal apoptosis in the subgranular zone of the hippocampal dentate gyrus, a brain structure involved in learning and memory function (7, 8). Identical histopathologic features are observed in corresponding animal models (9–11). Moreover, the severity of learning dysfunction is associated with the extent of hippocampal cell (HC) apoptosis in experimental pneumococcal meningitis (9, 12, 13).

The dentate gyrus is a site of a stem cell niche with continuous formation of new neurons (14). Neurogenesis occurs lifelong in predominantly 2 brain regions: the subventricular zone lining the lateral ventricles and the subgranular zone, part of the dentate gyrus of hippocampus (15, 16). In the subgranular zone, the stem cells proliferate and differentiate into progenitor cells. These newly formed cells subsequently migrate into the granule layer of the dentate gyrus, differentiate into mature, functional neurons, and become integrated into the hippocampal network. Therefore, the hippocampus is potentially well equipped for repair. However, because neurologic sequelae of bacterial meningitis related to hippocampus function persist throughout childhood into adulthood, the capacity for self-repair seems insufficient to compensate for the brain damage (2).

We hypothesize that the regenerative capacity of the hippocampus is compromised by bacterial meningitis. In this study, we characterized the cellular targets of apoptosis in the dentate gyrus in organotypic HC cultures and in experimental pneumococcal meningitis with the aim of assessing whether bacterial meningitis impairs neurogenesis in the hippocampus.

MATERIALS AND METHODS
Animal Model of Meningitis
All animal studies were approved by the Animal Care and Experimentation Committee of the Canton Bern, Switzerland. They followed the Swiss national guidelines for the performance of animal experiments and were conducted in accordance with the US Public Health Service’s Policy on Human Care and Use of Laboratory Animals. Eleven-day-old Wistar rats (Charles River, Germany) were injected intracisternally with 10 µL of saline containing 1 × 10^6 colony-forming units (cfu)/mL of a clinical isolate of Streptococcus pneumoniae (n = 9) (9, 12, 13, 17). Sham-infected, control littersmates (n = 7) were injected with an equal volume of sterile, pyrogen-free saline. Cerebrospinal fluid (CSF) samples were obtained 18 hours later by puncture of the cisterna magna to document infection. The number of bacteria in the CSF was determined by plating serial dilutions of 10 µL of CSF on blood agar plates (5.17 ± 1.17 × 10^3 cfu/mL). All animals received ceftriaxone (Roche Pharma, Switzerland 100 mg/kg body weight twice a day subcutaneously). At 48 hours after infection, the rats were killed with 100 mg/kg pentobarbital intraperitoneally, brains were removed, and the hippocampi were dissected and processed for isolation and quantitative assessment of neurosphere-forming cells.

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Hippocampal Cell Culture and Differentiation

Hippocampal cells of rats from P5 and P11 were dissected and dissociated as previously described (18, 19). For the induction of differentiation, single cells (P5) were plated onto poly-L-lysine-coated coverslips (Becton Dickinson AG, Allschwil, Switzerland) at a density of $5 \times 10^4$ cells per coverslip. Defined growth medium conditions (Dulbecco modified Eagle medium [DMEM]/F12, 1:1; Gibco Life Technologies, Basel, Switzerland), HEPES (15 mmol/L), 10$\mu$g/mL penicillin-streptomycin-amphotericin B (Bioswisstec AG, Schaffhausen, Switzerland), B27 supplement (Gibco Life Technologies), 20 ng/mL fibroblast growth factor (FGF; PeproTech EC Ltd, London, UK), and 20 ng/mL epidermal growth factor (EGF; PeproTech EC Ltd) were used including 20 ng/mL brain-derived neurotrophic factor (BDNF; PeproTech EC Ltd) (20). Cells were cultured at 37°C with 5% CO$_2$ for 21 days.

Collagen-Based Neurosphere Assay

The assay was performed using a protocol adapted from the procedure of Ma et al (20). After 24 hours in culture, the HC-derived cell suspension was centrifuged for 5 minutes at 600 $\times$ g, and the pellet was resuspended in 1 mL of ice-cold DMEM/F12. Viable cells were counted using trypan blue exclusion. Four wells of a 24-well tissue culture plate coated with poly-HEMA (Sigma, Buchs, Switzerland) were used per condition.

To assess the vulnerability of stem/progenitor cells to death triggers, cells were challenged with 3 death factors at defined time points. Five hundred microliters of a semi-solid collagen growth matrix (3 mg/mL collagen (Sigma), PBS pH 7.4, and growth medium including 4$\mu$g/mL heparin (Stem Cell Technologies, Grenoble, France) containing $5 \times 10^4$ cells were plated on each well and incubated for 1 hour at 37°C and 5% CO$_2$ in a humidified atmosphere. Once the gel has solidified, 500$\mu$L of neurosphere growth medium was added on the top of each gel. The cultures were kept for 21 days. Half of the growth medium was replaced by

![Graphs](http://jnen.oxfordjournals.org/)

**FIGURE 1.** In vitro model of hippocampal cell (HC) differentiation. (A) The differentiation stage of HC was identified by immunoassaying for Nestin (stem cells), doublecortin (DcX) (immature neurons), and microtubule-associated protein 2 (MAP2) (neurons) at 1, 7, 14, and 21 days. Neuronal differentiation in vitro was documented by a gradual shift in the predominant staining pattern from Nestin at 1 day to DcX at 7 to 14 days to MAP2 at 21 days. (B) Growth factor deprivation (GFD) and tumor necrosis factor (TNF) induce caspase 3-dependent apoptosis. Tumor necrosis factor induced apoptosis early, at 1 to 14 days of differentiation. Apoptosis induced by GFD was most prominent at 7 and 14 days ($p < 0.05$). (C) Bacterial components (BC) induce caspase 3-independent apoptosis. Hippocampal cells were exposed to BC for 2 hours. Cell death induced by BC was compared with control cells using annexin V or apoptosis-inducing factor (AIF) staining. Apoptosis induced by BC was most abundant at 7 days of differentiation ($p < 0.05$). Overall, the vulnerability of cells to undergo death after both challenges peaked at 7 days. $p$ values were calculated by 1-way analysis of variance followed by Tukey multiple comparison post hoc test. Data are presented as mean $\pm$ SEM of 3 or more independent experiments.
fresh growth medium twice a week. Quantification of the neurospheres was performed by mounting the gels on glass slides after fixation in 10% paraformaldehyde in PBS. Subsequently, dried gels were stained with cresyl violet, and numbers of neurospheres per gel were counted using ImageJ 1.37v (Wayne Rasband; National Institutes of Health, Bethesda, MD).

**Induction of Apoptosis**

Hippocampal cells (P5) were exposed to death stimuli characteristic of bacterial meningitis. These consisted of application of bacterial components (BCs) for 2 hours or 20 ng/mL tumor necrosis factor (TNF; Sigma) for 24 hours to the medium, or growth factor deprivation (GFD), that is, removal of FGF, EGF and BDNF for 24 hours at 1, 7, 14, and 21 days after initiation of differentiation. For exposure to BC, a clinical isolate of *S. pneumoniae* (serotype 3) was cultured overnight in a brain heart infusion medium. The bacterial suspension was diluted and grown to logarithmic phase for 4.5 hours. After washing with NaCl, bacterial suspension was resuspended to a defined concentration of 1.7 × 10⁷ cfu/mL in DMEM/F12 containing 10 μg/mL streptomycin and penicillin to cause bacteriolysis. After 4 hours of incubation at 37°C with 5% CO₂, the suspension was centrifuged at 3000 × g for 10 minutes. The supernatant containing the BCs was added at a volume of 1:5 to the medium of the HC for 2 hours; a longer exposure of BCs resulted in necrosis of the HC. Bacterial component–induced cell death was observed using phase-contrast microscopy. For application of TNF, 20 ng/mL TNF was added to the medium for 24 hours at 37°C with 5% CO₂ (21, 22). For GFD, FGF, EGF, and BDNF (20 ng/mL each) were removed from the culture medium. staurosporine (STS; 200 nmol/L for 2 hours; Sigma) was used as a positive control for induction of apoptosis. Control cells were handled identically in growth medium.

For caspase inhibition, z-VAD-FMK (50 μM; Sigma) was used at 7 days after differentiation. After the challenge, coverslips were fixed and stained as follows: for neuronal markers (Nestin, mouse-monoclonal, 1:1000; ProSci, Inc, Lausen, Switzerland), apopto sis-inducing factor (AIF, rabbit-polyclonal, 1:100; ProSci, Inc, Lausen, Switzerland) and apoptosis markers (caspase 3, rabbit-polyclonal, 1:400; Cell Signaling Technology, Allschwil, Switzerland) apoptosis–inducing factor (AIF, rabbit-polyclonal, 1:100; ProSci, Inc, Lausen, Switzerland), or used for annexin V (1.8 μg/mL) and propidium iodide (PI) (5 μg/mL; Sigma) staining using standard protocols. Stained cells were subsequently analyzed at 40× magnification and photographed with a Zeiss fluorescence microscope (Axiophot or Axioper 135; Zeiss, West Germany) and analyzed with Openlab 4.0.3 (Improvision, UK) and ImageJ 1.37v.

**Western Blots**

Hippocampal cells (P5) were induced to form neurospheres, which were then plated on 60-mm dishes and driven into differentiation as described above. Western blots were performed from control and challenged neurosphere-derived cells. Cells were harvested and lysed with 30 μL of radioimmunoprecipitation assay buffer. Proteins were separated in an electrophoresis chamber (Mini Protein II; Bio-Rad, Cressier, France) and transferred to a polyvinyl difluoride membrane (Millipore AG, Zug, Switzerland). After blocking with 5% milk, the primary antibodies to the following were applied at 4°C overnight for immunoblotting: Bax (rabbit antibody kindly provided by Prof. Ch. Borner, PhD, Albert-Ludwigs-University, Freiburg, Germany, at a dilution of 1:5000), Bim (rabbit antibody, 1:1000; Millipore), and Mcl-1 (rat antibody, gift from Th. Kaufmann, PhD, Institute of Pharmacology, University of Berne, Switzerland, 1:2000). Blots were incubated with Immobilon Western Chemiluminescent HPR Substrate (Millipore AG), and bands were visualized by exposure to Fuji RX photo film (Fujifilm Corporation, Tokyo, Japan).

**Statistical Analysis**

Multigroup comparisons were performed by 1-way analysis of variance (ANOVA) followed by Tukey post hoc test or 2-way ANOVA and Student t-test to compare the means of 2 groups of data using Prism 5.01 (level of significance set to p < 0.05). For Western blots, the relative amount of each protein was determined quantitatively by ImageJ 1.37v and normalized to the expression of β-actin. Data are presented as mean ± SEM, and the numbers of different experiments are indicated for each.

**RESULTS**

**Neuronal Maturation of HC Stem/Progenitor Cell In Vitro**

Hippocampal cells were differentiated by culture on poly-L-lysine–coated coverslips for 1, 7, 14, and 21 days with 20 ng/mL BDNF. At defined time points, the ratios of the different developmental stages (stem/progenitors, immature, and mature neurons) relative to the total cell population were calculated based on counts of cells positive for Nestin, DcX, and MAP2, divided by the number of nuclei stained with DAPI. Stem and progenitor cells staining positive for Nestin were the predominant cell population on the first day (47% ± 12%) after starting the differentiation process; this percentage decreased as a function of cellular differentiation over time to a minimum of 21% ± 4% at 21 days (Fig. 1A). After 7 days of differentiation, 48% ± 14% of the cells were immature neurons, indicated by positive staining for DcX. At 21 days, the percentage of DcX-positive cells (35% ± 4%) decreased and 47% ± 3% of the cells were positive for MAP2, a marker of mature neurons.

**Caspase 3–Dependent Apoptosis of HC by TNF and GFD**

The concentration of TNF (20 ng/mL) used corresponded to that found in the CSF of infected animals with acute bacterial meningitis (9). Higher concentrations (i.e. 200 or 2,000 ng/mL) led to apoptosis in all cell types (data not shown). No significant difference in apoptosis was observed after challenge for 24 or 48 hours (p > 0.05). Exposure of TNF induced apoptosis at Day 1, peaked at
FIGURE 2. The stage of cellular differentiation (Nestin, doublecortin [DcX], microtubule-associated protein 2 [MAP2]) determines the vulnerability to undergo apoptosis (annexin V and apoptosis-inducing factor [AIF]) after challenge with death triggers. (A–C) Bacterial components (BC) induce necrosis (propidium iodide) and apoptosis. Apoptotic cells (Arrowhead 1) showed characteristic morphology with shrunken and condensed nuclei (A), and the appearance of annexin V/propidium iodide (Arrowhead 2; B) and AIF (Arrowhead 1; C). Healthy cells (Arrowhead 3) do not show any of these characteristics. (D–F) Tumor necrosis factor and growth factor deprivation induced apoptosis in cells staining positive for doublecortin (DcX) and Nestin but not for microtubule associated protein 2 (MAP2). (G–L) After exposure to BC, AIF colocalized in mature neurons (MAP2; I, L), immature neurons (DcX; H, K), and stem/progenitor cells (Nestin; G, J). Scale bar = 50 μm.
Day 7 (29% ± 3%), and decreased thereafter with the differentiation process, as determined by the ratio of cleaved caspase 3–positive cells (Fig. 1B). Growth factor deprivation for 24 hours led to apoptosis that peaked at Day 7 (27% ± 7%) and decreased on Day 21 (Fig. 1B). The caspase inhibitor z-VAD-FMK attenuated apoptosis induced by TNF (p < 0.05), GFD (p < 0.05), and STS (p < 0.05) at Day 7 after initiation of differentiation.

Caspase 3–Independent Apoptosis and Necrosis of HC After Challenge With BC

A bacterial concentration of 1.7 × 10⁷ cfu/mL (a number in the range of that found in CSF of patients with bacterial meningitis) was used for challenge with BC. Direct exposure of living bacteria to HC for 2, 4, and 6 hours triggered necrosis; lower BC concentrations (dilutions of 1:10 and 1:20) failed to induce apoptosis (data not shown). Bacterial component challenge of cells led to low levels of caspase 3–dependent apoptosis similar to that seen in control cultures (5% ± 3%) (Fig. 1B). Cell death due to BC is a consequence of both necrosis and apoptosis. Importantly, similar levels of necrosis (cells staining positive for both annexin V/propidium iodide) were induced in all the different developmental stages assessed (Fig. 2B). The susceptibility of cells to undergo apoptosis (cells staining positive exclusively for annexin V [Fig. 2B] or AIF [Fig. 2C]) were maximal at Day 7 after initiation of differentiation (Fig. 1C). In contrast to TNF and GFD, BC induced apoptosis that decreased earlier, that is, on Day 14 of differentiation. Similar levels of relocalized AIF and annexin V staining were found in cells challenged with BC (15% ± 8% and 16% ± 13%, respectively). The caspase inhibitor z-VAD-FMK had no significant effect on BC-induced apoptosis in HC, whereas it significantly reduced STS-induced apoptosis 7 days after differentiation (p < 0.05).

FIGURE 2B. Effect of death triggers on cellular differentiation stages. Hippocampal cells were challenged with death stimuli characteristic of bacterial meningitis, that is, tumor necrosis factor (TNF) (upper panel), bacterial components (BC) (middle panel), and growth factor deprivation (GFD) (lower panel) at 1, 7, 14, and 21 days of differentiation. After TNF, immature neurons (DcX) and stem/progenitor cells (Nestin) were found to be significantly more susceptible to undergo apoptosis versus mature neurons (MAP2) (p < 0.0005). Bacterial components and GFD affected both differentiation stages (p values not significant). Data are presented as mean ± SEM of 3 or more independent experiments. p values were calculated by 2-way analysis of variance.

Stem and Progenitor Cells, But Not Mature Neurons, Are Susceptible to Undergo Apoptosis After Challenge by Death Triggers

We investigated which differentiation stage (i.e. stem and progenitor cells, immature neurons, or mature neurons) was most susceptible to undergo apoptosis by the 3 death stimuli relevant in bacterial meningitis (9, 23–26). The fraction of cells staining positive for both cleaved caspase 3 (TNF and GFD) or AIF (BC) and the marker of the differentiation stage was calculated relative to the number of apoptotic cells. Immature

FIGURE 3. Effect of death triggers on cellular differentiation stages. Hippocampal cells were challenged with death stimuli characteristic of bacterial meningitis, that is, tumor necrosis factor (TNF) (upper panel), bacterial components (BC) (middle panel), and growth factor deprivation (GFD) (lower panel) at 1, 7, 14, and 21 days of differentiation. After TNF, immature neurons (DcX) and stem/progenitor cells (Nestin) were found to be significantly more susceptible to undergo apoptosis versus mature neurons (MAP2) (p < 0.0005). Bacterial components and GFD affected both differentiation stages (p values not significant). Data are presented as mean ± SEM of 3 or more independent experiments. p values were calculated by 2-way analysis of variance.
neurons and stem and progenitor cells were found to be most susceptible to undergo apoptosis after TNF and GFD, and mature neurons were particularly insensitive (Figs. 2D–F). Bacterial components affected all differentiation stages including mature neurons (Figs. 2G–L). Two-way ANOVA revealed significant differences in vulnerability between the different groups for the 3 death triggers. Although both parameters (“stages” and “days after differentiation”) significantly contributed to the observed variation by TNF challenge, the main determinant explaining the variation by BC and GFD was “days after differentiation” (2-way ANOVA, p < 0.005; Fig. 3).

Death Triggers Impair HC Neurogenesis In Vitro

The potential of HC-derived cells to multiply and form new colonies in the neurosphere assay was compared between cells challenged in vitro with the 3 death triggers. After GFD (84 ± 39, n = 10), no difference in the number of newly formed colonies was observed compared with cells without challenge (84 ± 37, n = 10) (p > 0.05) (Fig. 4A). In contrast, after application of BC (40 ± 29, n = 9) and TNF (37 ± 19, n = 10), significantly fewer colonies were obtained versus control cells and cells challenged with GFD (p < 0.005).

Bacterial Meningitis Impairs HC Neurogenesis In Vivo

To assess whether experimental pneumococcal meningitis reduces the number of neurogenic cells in the hippocampus of infant rats, stem and progenitor cells of individual hippocampi were enumerated using the neurosphere assay. At 3 weeks after plating, significantly fewer colonies developed from HC of animals after pneumococcal meningitis (58 ± 20 colonies/35-mm plates, n = 9) versus from HC uninfected control animals (142 ± 42 colonies/35-mm plates, n = 7) (p < 0.0001) (Fig. 4B).

Death Triggers Induce Expression of Proapoptotic and Antiapoptotic Proteins

To investigate the molecular mechanisms influencing the vulnerability of the cells during the apoptotic process, HC extracts were assessed for expression of antiapoptotic and proapoptotic proteins during differentiation by Western blots. During maturation, stronger signals for the proapoptotic protein Bax were found at Days 1 and 21 (Fig. 5). Bax expression was enhanced after application of TNF at 1 day and after exposure to BC at all days evaluated. A stronger signal for Bax expression versus controls was observed during GFD at Days 7 and 14 but not at Days 1 and 21. Levels of the proapoptotic protein Bim decreased with the maturation process in control cells. Although they also declined with maturation, there were higher levels of Bim after TNF and BC treatments versus those in controls at all time points. There were continuously elevated levels of Bim after GFD. In control cells, maximal expression of the antiapoptotic protein Mcl-1 was observed at Day 1 with a steady decline until Day 21. For all death stimuli used, there was a similar pattern of Mcl-1 expression during differentiation.

DISCUSSION

The results of this study demonstrate that bacterial meningitis injures the neurogenic niche of the HC dentate gyrus.
We identified factors that are relevant in bacterial meningitis, namely, GFD, TNF, and BC, as triggers of apoptosis in stem and progenitor cells of the subgranular zone of the dentate gyrus. Using the collagen-based neurosphere assay (which allows the identification and enumeration of proliferative neurogenic cells [27]), we demonstrated a decrease of cells with self-renewal capacity in the hippocampus in experimental pneumococcal meningitis in infant rats. These results suggest that immature (DcX-positive) rather than fully differentiated neurons are selectively vulnerable to undergo apoptosis in bacterial meningitis and are consistent with our previous observations (28–30).

In bacterial meningitis, the pathogens are present in the CSF, but they are generally not in direct contact with the HC. Therapy for BM with β-lactam antibiotics induces a rapid bacteriolysis. The brisk increase of accrued BC in the CSF triggers an exacerbation of the host inflammatory reaction that critically contributes to brain damage (31). To mimic this situation in vitro, we incubated living pneumococci with antibiotics to cause bacterial lysis and produce BC. After

**FIGURE 5.** Immunoblot analysis of Mcl-1, Bax, and Bim expression in hippocampal cells at different stages of differentiation after challenge with death triggers and in unchallenged controls. During the maturation process, higher levels of Bax expression were found at Days 1 and 21. There was a slight increase in Bax expression versus control during growth factor deprivation (GFD) at Days 7 and 14 but not at Days 1 and 21. Bim expression in controls remained low during the differentiation. Growth factor deprivation increased the level of Bim at 7 days, which remained at the same level until 21 days versus controls. Mcl-1 expression decreased during the differentiation process in controls and after GFD. Bax was expressed at 1 day and declined at 7 to 21 days after tumor necrosis factor (TNF) and was continuously expressed throughout all differentiation stages after bacterial component (BC) treatment. Although declining with the maturation process, higher levels of Bim were detected after TNF and BC treatments when compared with control for all time points. Mcl-1 was expressed in early cellular differentiation stages after TNF (1 day) and BCs (1–7 days); β-actin was used to verify equal protein loading. Histograms illustrate the relative ratios versus β-actin. Blots and histograms are representative from 2 or more independent experiments.
exposure to BC, HC showed rapid cell death, including cell shrinking and condensation of nuclei, but caspase-3 activation was not detected and the caspase inhibitor z-VAD-FMK did not block apoptosis. Bacterial component–induced cell death, in this paradigm, is therefore predominantly independent of caspase 3 and affects all stages of differentiation including stem cells, immature neurons, and mature neurons. These observations are in line with our previous in vivo results showing that caspase 3–independent injury did not affect immature neurons selectively but rather clusters of cells including mature neurons relocating AIF to their pyknotic cellular nuclei (32). Moreover, in the present in vitro model, the regenerative capacity of HC was reduced by exposure to BC and TNF, suggesting a common mechanism regulating cell death induced by BC and TNF.

Growth factor deprivation enhanced caspase 3–dependent apoptosis with a maximum effect at 7 to 14 days of differentiation. Growth factors, including neurotrophins such as BDNF, enhance differentiation, induce proliferation, control cell death, and promote the survival of neurons during development. Brain-derived neurotrophic factor is of particular interest because its receptor, TrkB, is highly expressed in the hippocampus (33). In a previous study, we demonstrated that BDNF attenuates caspase 3–dependent HC apoptosis in infant rats with bacterial meningitis (24). Downregulation of nerve growth factor has been demonstrated 30 hours after infection in an adult mouse model of pneumococcal meningitis (34). Here the increased apoptosis after GFD at 7 to 14 days of differentiation coincided with an enhanced cellular expression of the proapoptotic protein Bim. These findings are in agreement with studies showing that GFD activates the intrinsic apoptosis pathway leading to increased expression of proapoptotic proteins such as Bim and Bax that activate caspase enzymes via the mitochondrial pathway (35, 36).

Tumor necrosis factor exposure induces caspase 3–dependent apoptosis starting at early stages of differentiation, similar to what has been shown in a previous study where application of 10 ng/mL of TNF increased apoptosis in adult neural stem cells (37). In an experimental model of pneumococcal meningitis, inhibition of a TNF-converting enzyme (TACE, ADAM17) downregulated the CSF concentration of TNF, attenuated apoptosis in the dentate gyrus of the hippocampus, and preserved spatial learning and memory function (9). Tumor necrosis factor is one of the extrinsic death signals that induce apoptosis directly through activation of caspase 8 and 3 or via caspase-mediated translocation of Bid and the release of cytochrome c (38, 39). During differentiation in vitro, the percentage of stem and progenitor cells decreased as they developed to immature and subsequently mature neurons. At the protein level, we demonstrated that on Day 1, when Nestin-positive cells predominate, Mcl-1 was highly expressed and declined steadily thereafter. It has been shown that loss of Mcl-1 in neuronal progenitor cells results in apoptosis of Nestin-positive cells, whereas in mature neurons, a similar loss of Mcl-1 did not induce cell death. Mcl-1 has, therefore, been identified as a key regulator of neural precursor cell survival during the transition from progenitor cells to postmitotic neurons (40). Furthermore, increased neuronal sensitivity to seizures has been observed in Mcl-1 heterozygous mice (41). Our results suggest that TNF and BC induce apoptosis in neurogenic cells at an early stage of differentiation, which include stem cells. In contrast, stem and early progenitor cells are protected from apoptosis under GFD. This finding is consistent with results from the neurosphere assay, in which the number of colonies obtained after GFD did not differ from control cultures. The observed lower sensitivity of stem cells toward GFD-induced apoptosis might be attributed to high expression levels of Mcl-1. Mcl-1 has been shown to sequester Bim (42), and its downregulation has been shown to be required for Bax activation (43).

Tumor necrosis factor and BC induce apoptosis in stem and progenitor cells through a pathway not regulated by Mcl-1. Because Bax knockout animals show increased numbers of neurons in the CNS, Bax is thought to play a role in neuronal programmed cell death (44). Here, Bax expression was upregulated by TNF and BC at Day 1 of differentiation, indicating that TNF– and BC-induced apoptosis depends on Bax. Similar to Bax, Bim expression was also enhanced by both death triggers in early differentiation. This may suggest that Bim cooperates in TNF- and BC-mediated apoptosis in HC stem and progenitor cells. In line with these results, it has recently been shown that Bim is essential for the activation of Bax and BAK-dependent cell death programs (45).

Our present investigations on the role of apoptosis regulators are limited. A number of factors (not yet investigated) could also influence the fate of the cells after death triggers in vitro. Apart from the factors explored in the present study, upregulation of antiapoptotic proteins (e.g. BelXL [46, 47] or XIAP [48]) in mature neurons may explain the observation that mature neurons were not affected by caspase 3–dependent apoptosis. A decline in the expression of proapoptotic factors (caspases, Apaf-1, Puma) may also desensitize the neurons (49). Conversely, noxious stimuli resulting in the elevation of other BH3-only proteins (Bim, Puma) could mediate the vulnerability of HC neurons, as similarly observed in vivo during proteasome inhibition-mediated apoptosis (50). Finally, posttranslational modifications not investigated in the present study may also account for the observed selective vulnerability. In our model of pediatric pneumococcal meningitis, the number of cells with colony-forming ability (stem and progenitor cells) decreased in the infant rats that survived pneumococcal meningitis. This finding was validated in an in vitro model of HC differentiation. Tumor necrosis factor and GFD induced caspase 3–dependent apoptosis, whereas BC induced caspase 3–independent death of cells in the neurogenic niche. Our data indicate that stem cells and immature neurons are vulnerable to undergo apoptosis, whereas mature neurons are more resistant to the death stimuli evaluated. Our results suggest that HC injury in bacterial meningitis targets stem and progenitor cells. The impaired neurogenesis in the hippocampus may help to understand why neurologic deficits persist after childhood bacterial meningitis. The pathogenic significance of BC, TNF, and GFD in the development of HC injury demonstrated herein identifies these death triggers as therapeutic targets. In consequence, interventions that minimize the releases of BCs (31), down-modulate...
TNF concentration in CSF (9), and increase BDNF (24) represent promising avenues in the search for therapeutic strategies to attenuate damage to the neurogenic niche of the hippocampus in bacterial meningitis.

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