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Pneumococcal Meningitis Induces Apoptosis in Recently Postmitotic Immature Neurons in the Dentate Gyrus of Neonatal Rats

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Key Words
Pneumococcal meningitis · Apoptosis · Dentate gyrus · Immature neurons

Abstract
Bacterial meningitis is associated with high rates of morbidity and mortality, despite advances in antibiotic therapy. Meningitis caused by *Streptococcus pneumoniae* is associated with a particularly high incidence of neurological sequelae including deficits resulting from damage to the hippocampus. Previous studies have documented that in neonatal rats with experimental pneumococcal meningitis, cells in the subgranular layer of the dentate gyrus undergo apoptosis. The aim of the present study was to define in more detail the nature of the dying cells in the dentate gyrus. Using bromodeoxyuridine labeling at different times before infection combined with immunocytochemistry, we identified the vulnerable cells as those which underwent mitosis 6–10 days before infection. A majority of these cells are of neuronal lineage. Thus, immature neuronal cells several days after the last cell division are preferentially triggered into apoptosis during pneumococcal meningitis. The loss of these cells may contribute to the long-lasting impairment of hippocampal function identified in animal models and in humans after bacterial meningitis.

D. Grandgirard and Y.-D. Bifrare contributed equally to this work.

Introduction

Bacterial meningitis, particularly when caused by *Streptococcus pneumoniae*, is associated with substantial mortality and severe morbidity, affecting up to 50% of survivors [1, 2]. Permanent neurological sequelae include hearing impairment, mental retardation and learning difficulties, sensory-motor deficits, seizure disorders and cerebral palsy. Children after meningitis are more than twice as likely to require special educational assistance. Even 12 years after the disease, deficits in intellectual, academic and executive abilities were detected in survivors [3–5]. Histopathological studies in human autopsies showed apoptotic cell death in the dentate gyrus, a region of the brain associated with learning and memory acquisition [6]. Furthermore, patients surviving bacterial meningitis presented unilateral or bilateral hippocampal atrophy by magnetic resonance [7]. In experimental models of pneumococcal meningitis, apoptotic cell death can be observed in the inner layer of the dentate gyrus [8–10].

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The extent of apoptosis was positively correlated with deficits in spatial memory [9, 11].

Together with the olfactory bulb and the subventricular zone, the dentate gyrus is characterized by the continuous production of new neurons into adulthood. As a consequence, neuronal cells of different degrees of maturity are found in the dentate gyrus. Located at the interface between the hilus and the granule cell layer, stem-cell-like cells with self-renewing properties divide to produce progenitor cells. These latter possess a restricted capacity to further replicate and are committed to differentiate after a few rounds of division. Once postmitotic, the immature neurons migrate a short distance into the granule cell layer, where they either integrate into the neuronal network through the formation of synaptic connections or are eliminated by apoptosis (reviewed in Ming and Song [12] and Abrous et al. [13]).

Different factors can influence the production or the survival of newly formed cells in the dentate gyrus [12]. A stimulating environment or physical activity [14–17] promote the proliferation and survival of new cells. In contrast, stress [18, 19] or age [20, 21] lead to reduced proliferation and an associated decline in cognition, learning and memory [22, 23]. Pathological situations, like ischemia, seizures or inflammation also increase proliferation and survival of neuronal cells [12, 13]. Depending on the cytokines and growth factors expressed in response to infections of the brain, the innate immune response can also inhibit neurogenesis [24, 25].

The aim of this study was to define in more detail the nature of the cells in the dentate gyrus which undergo apoptosis in experimental pneumococcal meningitis. The characterization of these cells will be of interest in understanding the selective vulnerability of the hippocampus to meningitis and other forms of brain injury.

Materials and Methods

Infecting Organisms

S. pneumoniae (serotype 3) isolated from a patient with bacterial meningitis was grown on blood agar plates, cultured overnight in 10 ml of brain heart infusion medium, diluted in fresh medium, and grown for 6 h to logarithmic phase. The culture broth was centrifuged for 10 min at 5,000 *g*, the pellet resuspended in sterile saline to the desired density, and used for intracisternal injection. The accuracy of the inoculum size was routinely confirmed by quantitative cultures.

Model of Meningitis

We used an established model of infant rat meningitis [9, 26, 27]. To determine whether the developmental stage of the animals influences the extent of neuronal injury in the dentate gyrus, nursing Wistar rat pups (Charles River, Germany) were infected on postnatal day 6, 11 or 16, by intracisternal injection of 10 μl saline containing $2.9 \pm 0.4 \times 10^6$ CFU/ml *S. pneumoniae*. Eighteen hours later, animals were weighed and assessed clinically as described previously [27]. To document meningitis, 5 μl of cerebrospinal fluid was obtained by puncture of the cisterna magna and cultured quantitatively on sheep blood agar plates. Antibiotic therapy was initiated at 18 h after infection with ceftriaxone (Rocephine, Roche Pharma; 100 mg/kg body weight s.c.).

Bromodeoxyuridine Labeling

For the identification of proliferating cells, animals received bromodeoxyuridine (BrdU, Sigma), a thymidine analog which incorporates into newly synthesized DNA. The following BrdU labeling paradigms were used. Scheme I: to label as many proliferating cells as possible, infant rats (n = 14) received 50 mg/kg BrdU intraperitoneally daily for 8 days preceding the day of infection. Scheme II: to identify distinct stages in neuronal development, infant rats received a single dose of 50 mg/kg BrdU intraperitoneally 8 (n = 4), 6 (n = 12), 4 (n = 10) or 2 (n = 8) days before infection. Animals were infected on postnatal day 11 in schemes I and II. Scheme III: to expand the observation to an older cell population without performing intrauterine labeling, infant rats received a single dose of 50 mg/kg BrdU intraperitoneally 14 (n = 6), 10 (n = 6), 8 (n = 4), 6 (n = 6), 4 (n = 6) or 2 (n = 2) days before infection, which was induced on postnatal day 16. All animals were sacrificed 24 h after the infection by intraperitoneal injection of a lethal dose of pentobarbital (Nembutal®, Abbott Laboratories, North Chicago, Ill., USA) and were perfused with phosphate-buffered saline (PBS).

Histopathological Evaluation of Apoptosis

For histopathological examination, rats infected on postnatal days 6, 11 and 16 (n = 9 per age group) were killed 24 h after infection. The baseline level of apoptosis was determined in uninfected animals for each age group (n = 3 per age group). After perfusion with 4% paraformaldehyde in PBS, brains were fixed in 4% paraformaldehyde in PBS and cryoprotected in 18% sucrose. Four sections of the dorsal brain region were mounted on polylysine-coated slides and stained for Nissl bodies with cresyl violet. Quantitative assessment of apoptosis was performed as previously described [9, 28–30]. Briefly, cells with morphological features of apoptosis (condensed, fragmented dark nuclei and apoptotic bodies) were counted in 3 visual fields (magnification ×400) in each of the 4 blades of the dentate gyrus (2 per hemisphere), and the following scoring system was applied for the count in each blade: 0–5 cells = 0; 6–20 cells = 1, and >20 cells = 2. The mean score per rat was calculated from all sections evaluated, and the median score per age group was determined.

Immunohistology

For immunofluorescence, animals were perfused with PBS and brains were embedded in paraffin after fixation in a solution of methanol and acetic acid 95:5. The fixative was omitted from the perfusion solution as immunoassaying for active caspase-3 yields better results in our hands when performed without the use of a fixative solution containing aldehyde. Five-micrometer sections were cut, mounted on Superfrost® plus glass slides (Menzel GmbH, Braunschweig, Germany), deparaffinized and rehydrat-

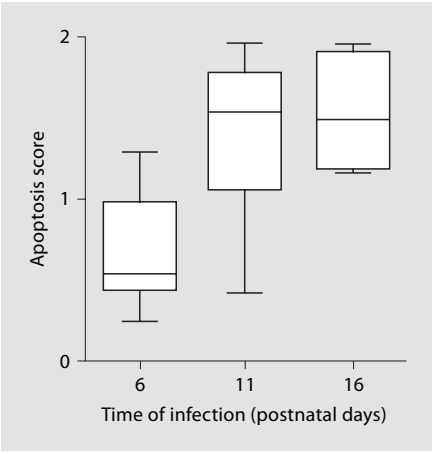

Fig. 1. Extent of apoptosis in the dentate gyrus of animals infected on postnatal day 6, 11 or 16, respectively. Animals were sacrificed 24 h after infection. In each age group, sham-infected animals were included. All sham-infected animals showed an apoptosis score of 0.

ed. After rinsing with PBS, sections were incubated overnight at 4°C with the following primary antibodies diluted in antibody buffer (0.5% bovine serum albumin in PBS and 0.5 M Tris buffer, pH 7.6, in 0.88% NaCl 1:1): antiactivated caspase-3 rabbit polyclonal antibody, CM1 (1:1,000; BD Biosciences); anti-BrdU mouse monoclonal antibody (1:50; Dako, Carpinteria, Denmark), and anti-NeuN monoclonal antibody MAB377 (Chemicon International, Inc., Temecula, Calif., USA). Following 3 washes in PBS, sections were incubated at room temperature for 1 h with the following secondary antibodies: goat antirabbit Alexa-488-conjugated antibody (1:1,000; Molecular Probe, Leiden, Netherlands) for caspase-3 and goat antimouse Cy3-conjugated antibody (1:1,000; Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) for BrdU or NeuN. Double labeling for Map2 was performed on floating sections cut from frozen tissue at 40 μm using a cryostat. Anti-MAP2 mouse monoclonal (clone AP20) was obtained from Sigma and used at 1:500 dilutions. The remainder of the staining protocol was similar except that PBS with 10% lamb serum and 0.1% Triton X-100 was used for diluting the antibodies and for washes. Cells positive for MAP2 and active caspase-3 were quantitatively assessed in animals sacrificed 24 h after infection (n = 3) and 3 sections were evaluated in each animal. For Prox1 staining, a rabbit polyclonal antiserum was used [31]. After endogenous peroxidase quenching, slides were incubated in the primary antibody solution (1:5,000 in 0.1 M Tris, 0.1% Triton X-100, 10% goat serum) overnight at 4°C. After incubation with a secondary goat antirabbit antibody coupled with peroxidase, positive cells were revealed by incubating the slides with DAB and counterstaining them with hematoxylin.

For BrdU staining, a DNA denaturing step was necessary prior to the application of the primary antibodies. Sections were incubated for 60 min at 70°C in a solution of 95% formamide. Slides were slowly cooled down, and washed 3 times in PBS before further processed as described above.

Statistical Methods

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, Calif., USA). Median apoptosis scores were compared using the Mann-Whitney U test. Overall differences between the different labeling groups were calculated with one-way ANOVA, and the post hoc test with Tukey multiple comparisons.

Results

Influence of Age at the Time of Infection on Hippocampal Apoptosis in Pneumococcal Meningitis

Proliferating activity in the dentate gyrus of postnatal rats significantly increases from postnatal day 9 to postnatal day 12 and then gradually decreases [32]. In light of this, we compared the extent of apoptosis in the dentate gyrus in animals infected on postnatal day 6, 11 or 16, respectively. Twenty-four hours after infection, there was no difference in the number of cells undergoing apoptosis between 11- and 16-day-old rats. However, there were significantly fewer apoptotic cells in 6-day-old than in 11- or 16-day-old rats [median 0.54 (0.44–0.98) vs. 1.54 (1.07–1.79), p = 0.02, for postnatal day 6 vs. postnatal day 11; fig. 1]. In uninfected animals, the number of apoptotic cells/visual field was <5 (score 0) in all age groups assessed.

Correlation between Recent Cell Division and Apoptosis

Apoptotic cells were detected by immunofluorescence using an antibody against the active form of caspase-3 [8]. Caspase-3-positive cells were located prominently at the border of the granule cell layer and the hilus, the subgranular zone, which is the neurogenic niche in the dentate gyrus (fig. 2). This niche contains several classes of cells that can be distinguished by markers; specifically, the rapidly dividing neural precursor cells that incorporate BrdU efficiently and are labeled with Nestin, a neural precursor marker, and immature neurons that label with several neuronal markers but no longer incorporate BrdU since they have become postmitotic [33]. In our case, we wished to find whether the dying cells underwent cell division at any point postnatally or whether they were embryonically born granule cells. To determine this, animals were pulsed with a daily BrdU dose for 8 days prior to infection. Of the cells stained positive for activated caspase-3, 58.6 ± 13.2% were also positive for BrdU. The remaining 40% of caspase-3-positive cells are most likely cells that divided more than 8 days prior to infection and cells that were not captured during cell division by the

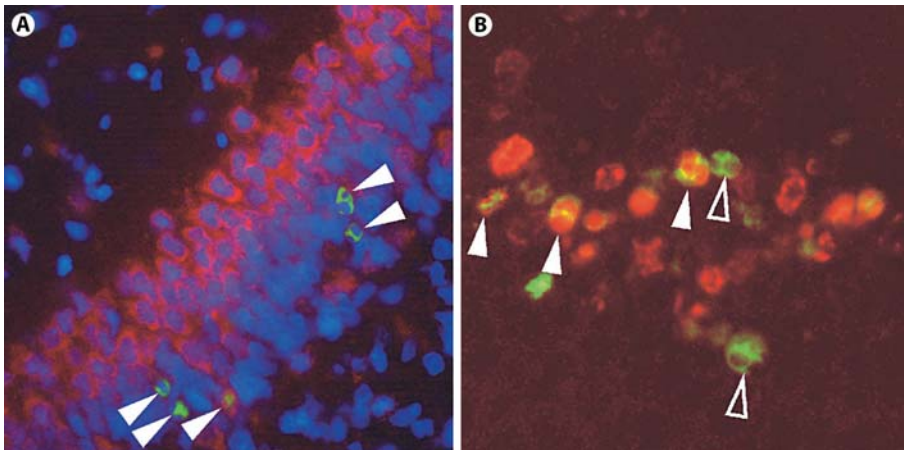

Fig. 2. A Representative immunofluorescence staining section of the dentate gyrus from an infected animal 24 h after infection. Apoptotic cells (antiactive caspase-3, green) are visible in the inner layer of the dentate gyrus, and generally do not colocalize with NeuN (red), a marker for mature neurons found in the outer half of the dentate granule cell layer at this age. **B** Immunofluorescence staining of the dentate gyrus from an infected animal treated with BrdU for 8 consecutive days prior to infection. Approx. 60% of cells positive for active caspase-3 (green) stained also positive for BrdU (red; closed arrowheads). The remaining caspase-3-positive cells did not show costaining with BrdU (open arrowheads) likely representing cells that divided before the start of BrdU treatment and cells that were not captured during cell division with the current labeling scheme.

current labeling scheme. Thus, more than half of the cells undergoing apoptosis showed evidence of at least one cell division during the labeling period of 8 days prior to meningitis. Since the BrdU injections were only once daily, we believe this indicates that all, or the vast majority, of dying cells were dividing in the postnatal period.

In order to examine whether the cells undergoing programmed cell death were precursors, still dividing just before meningitis, or immature neurons, we decided to prelabel the animals with BrdU to determine the 'age' of the dying cells by determining when they last divided. If the dying cells were actively dividing precursors, then most would be labeled with BrdU given at time points very close to meningitis, but if the dying cells were immature neurons, they should have undergone their last mitotic division at some prior point. BrdU was given once a day either on day 8, 6, 4, or 2 before infection (scheme II). The percentage of caspase-3-positive cells which integrated BrdU significantly decreased with shortening periods between BrdU labeling and infection. The highest percentage of colocalization was found for cells which divided 6–8 days before infection. In contrast, cells that divided 2–4 days before infection were less likely to be undergoing apoptotic cell death (fig. 3).

In order to get a better idea of the exact temporal window of vulnerable cells, we decided to extend the window of labeling towards longer intervals prior to infection by

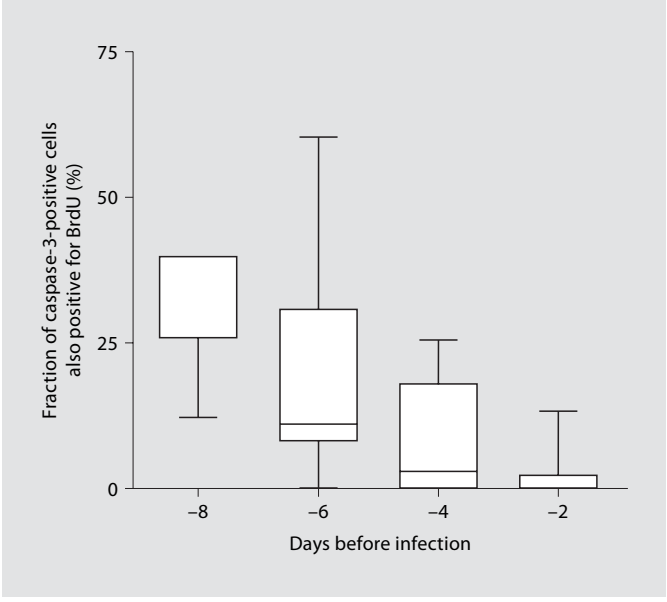

Fig. 3. Colocalization of active caspase-3 and BrdU in the dentate gyrus of animals infected on postnatal day 11. Animals received a single dose of BrdU on different days before infection and were killed 24 h after infection. The percentage of caspase-3-positive cells also positive for BrdU is determined. Overall difference between groups: $p = 0.0017$ (ANOVA). Statistically significant differences between individual groups: −8 vs. −4 ($p < 0.5$), −8 vs. −2 ($p < 0.01$) and −6 vs. −2 ($p < 0.05$).

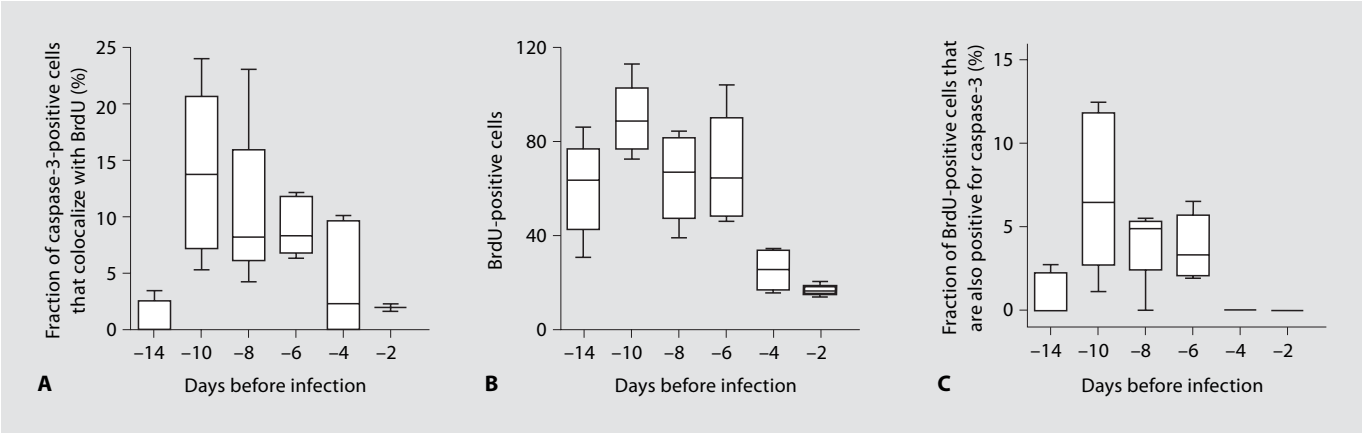

Fig. 4. Colocalization of active caspase-3 and BrdU in the dentate gyrus of animals infected on postnatal day 16. Animals received a single dose of BrdU at different times before the infection and killed 24 h after infection. **A** Percentage of caspase-3-positive cells also positive for BrdU. Overall difference between groups: $p = 0.009$ (ANOVA). Statistically significant differences between individual groups: –14 vs. –10 ($p < 0.001$), –4 vs. –8 ($p < 0.05$) and –10 vs. –4. **B** Absolute number of BrdU-positive cells found in the dentate gyrus. Overall difference between group: $p < 0.0001$ (ANOVA). Statistically significant differences between individual groups: –14 vs. –4 ($p < 0.05$), –14 vs. –2 ($p < 0.01$), –10 vs. –4 ($p < 0.001$), –10 vs. –2 ($p < 0.001$), –8 vs. –4 ($p < 0.05$), –8 vs. –2 ($p < 0.01$), –6 vs. –4 ($p < 0.01$) and –6 vs. –2 ($p < 0.001$). **C** Fraction of the recently divided cell pools (from the different labeling periods before infection) that undergo apoptosis. Overall difference between groups: $p < 0.0001$ (ANOVA). Statistically significant differences between individual groups: –14 vs. –10 ($p < 0.001$), –10 vs. –4 ($p < 0.001$) and –10 vs. –2 ($p < 0.001$).

inducing meningitis on postnatal day 16 instead of day 11. Dividing progenitor cells were labeled with BrdU once a day on 14, 10, 8, 6, 4 or 2 days prior to meningitis (scheme III). The majority of cells dying by apoptosis divided 6–10 days prior to infection. Similar to rats infected on day 11, cells that were still mitotic 2–4 days before infection were less affected (fig. 4A). Cells that were mitotic more than 10 days before infection were again less affected by apoptosis (fig. 4A). Thus, the most sensitive cells in response to meningitis were those that were dividing 6–10 days prior to meningitis.

The analysis presented thus far demonstrates that a larger percentage of the dying cells were dividing about 1 week prior to meningitis than just a few days prior to meningitis. This finding may be consistent either with a model implying that postmitotic cells that underwent their final division in that earlier temporal window are more sensitive. However, since some of the BrdU-labeled cells may not have been undergoing their final division, but rather have continued to divide for one or two more divisions still detectable with BrdU, it is possible that our data to this point are really compatible with the fact that there are more cells in the dentate gyrus containing BrdU label from earlier ages than later ages. Indeed, as shown in figure 4B, more BrdU-positive cells were found in the dentate gyrus when labeling was performed earlier (6–10 days before infection) compared to labeling 2–4 days prior to infection. This may be due to a fraction of cells continuing on to a second or third mitosis after the integration of BrdU, therefore increasing the number of positive cells. However, this is also consistent with the fact that the rate of dentate granule neuron production is at its height in the first postnatal week, thus leading to many more cells going through their terminal division at that time.

To address the possibility that an artifact of the higher numbers of BrdU-positive cells in animals labeled at earlier time points contributed to our data, we determined the percentage of the BrdU-positive cell population undergoing apoptosis at the different labeling times. Indeed, we found that the highest percentage of caspase-3-positive cells was found among cells that integrated BrdU 6–10 days before infection (fig. 4C), confirming the idea that the critical time period during which vulnerable cells undergo cell division is approximately 6–10 days prior to meningitis, independent of additional cell divisions prior to the infection.

Apoptotic Cells in the Subgranular Zone Are Immature Dentate Granule Neurons

Apoptotic cells were localized in the subgranular zone (inner layer), the region known to sustain continuous neurogenesis (fig. 5). In this region, there were nu-

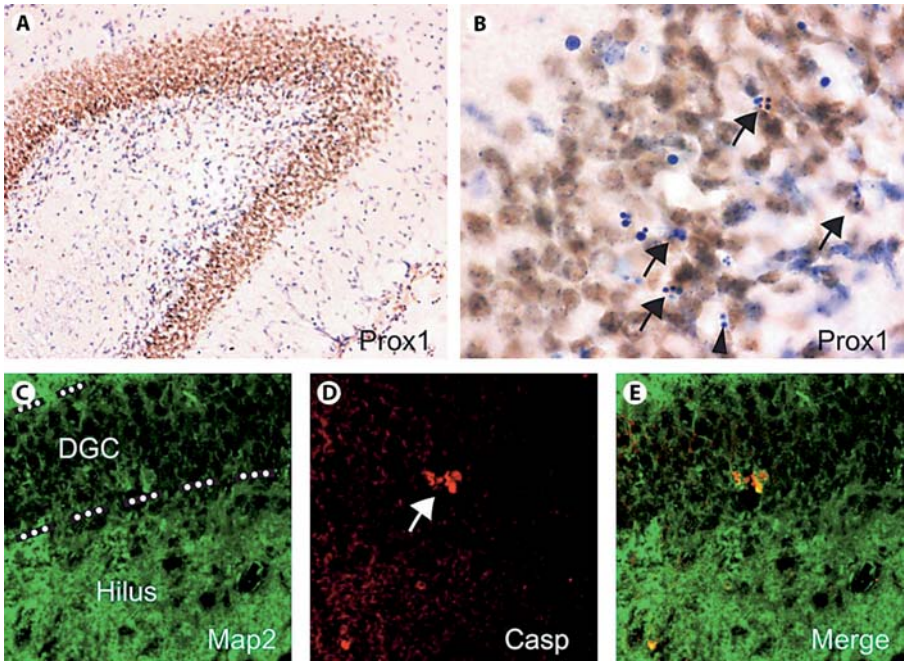

Fig. 5. Dying cells express dentate granule cells and neuron-specific markers. **A**, **B** Prox1 antibody staining demonstrates that at this postnatal age the dentate gyrus is a compacted layer with only small numbers of granule cells still being produced in the hilus. At higher power, numerous Prox1-positive cells with condensed nuclei are clearly visible (arrows). Several cells with condensed chromatin that are not labeled are likely to be cells that are dead and have lost expression of other cellular markers. **C–E** Double labeling with Map2 and Caspase demonstrates two cells undergoing apoptosis that are also labeled with the neuronal marker Map2 (arrows) and are located in the subgranular zone. The dashed lines mark the boundaries of the dentate granule cell layer (DGC).

merous cells with condensed chromatin that also labeled with Prox1, a specific marker of dentate granule neurons (fig. 5) [31]. Many of the caspase-3-labeled cells were also stained with antibodies against MAP2, which begins to be expressed several days after the last neuronal cell division (fig. 5). Colocalization studies in infected animals (n = 3) showed that 82% of the caspase-3-positive cells stained also positive for MAP2. In contrast, caspase-3 did not colocalize with NeuN, a marker for more mature dentate granule neurons (fig. 2). Thus, in agreement with the labeling experiments, the analysis of immunocytochemical markers indicates that postmitotic, immature rather than fully differentiated neurons are selectively vulnerable to undergo apoptosis in bacterial meningitis.

Discussion

Mental retardation and learning deficits are long-lasting neurological sequelae found in survivors of pneumococcal meningitis. In experimental animal models, damage to the dentate gyrus has been linked to deficits in spatial memory using the Morris water maze test [9, 11]. In the infant rat model of pneumococcal meningitis, a subset of cells located in the inner layer of the dentate gyrus was shown to undergo apoptosis during the acute phase of the disease [34]. Similar observations have been reported from human autopsies [6]. Identifying the nature of cells with selective vulnerability during meningitis may help to understand the mechanisms of brain damage in this disease. In the present study, we document that the vulnerable cells undergo cell division approximately 6–10 days prior to meningitis.

In the dentate gyrus, different cell types of neural lineage proliferate: putative stem-cell-like cells divide to give rise to transient amplifying progenitor cells; these progenitors differentiate into immature neurons, migrating a short distance into the granule cell layer where they finally integrate into the neuronal network through the development of synaptic connections [12, 13]. Using markers for the dividing precursor cells, like Nestin (the intermediate filament protein expressed by neural stem cells and dividing precursors) and acute BrdU labeling, we were unable to show colocalization with active caspase-3, or only in a small fraction of the apoptotic cells. These negative results must be interpreted with caution, since during apoptosis, the cytoarchitecture and cell membranes are profoundly affected so that the epitopes for the antibodies may have been altered and their recognition hampered in apoptotic cells. Despite this caveat, the assumption that the vulnerable cells are postmitotic immature neurons is supported by the finding that dying cells were colabeled with both Prox1 and Map2. The lack

of staining for NeuN, on the other hand, implies that the cells are not fully mature neurons.

The selective vulnerability to apoptosis of certain cells is likely the result of a change in the cellular microenvironment and characteristics specific for the developmental stage of the affected cells. A number of factors have been proposed to act as molecular triggers for apoptosis in bacterial meningitis. Based on studies in animal models, components of the host inflammatory response including cytokines, metalloproteinases, but also pneumococcal products, e.g. pneumolysin and hydrogen peroxide, may contribute to the cell death [35]. Furthermore, an excessive release of glutamate and the generation of reactive oxygen species have been associated with the occurrence of hippocampal apoptosis [36, 37].

In addition to proapoptotic stimuli, a relative lack of antiapoptotic factors and a transient selective vulnerability of cells must be considered as the basis for apoptotic neuronal cell death. Since the apoptotic cell death following meningitis is restricted largely to the dentate gyrus [8], the combination of cellular and environmental factors inducing apoptosis appears to be unique in this structure of the brain during bacterial meningitis.

In the adult rodent dentate gyrus, the vast majority of newly born cells are postmitotic and partially differentiated within 3–7 days [38]. About 50% of these cells die within 1 week of their generation [39]. Although the situation may be different in younger animals, it is tempting to speculate that bacterial meningitis interferes with neuronal differentiation or selectively affects the signaling for critical neuronal survival factors in immature neurons. We previously found sporadic occurrence of single-cell apoptosis in the dentate gyrus in uninfected 12-day-old rats [11]. Neurotrophins, a family of neurotrophic factors, control neuronal cell death and play an important role in neuronal differentiation and survival [40–42]. Brain-derived neurotrophic factor (BDNF) is of particular interest, since its receptor (trkB) is highly expressed in the hippocampus [43]. Administration of exogenous BDNF in experimental pneumococcal meningitis in neonatal rats prevented hippocampal apoptosis [28]. Following acute meningitis, the endogenous production of BDNF increases and has been proposed to contribute to neurogenesis [44, 45]. Other neurotrophic factors such as nerve growth factor, the expression of which is decreased 30 h after infection in an adult mouse model of pneumococcal meningitis [44], may also play a role.

Intrinsic properties of the immature postmitotic cells that could render them more sensitive to noxious stimuli associated with bacterial meningitis may be related to the maturation process these cells are undergoing. For example, biochemical pathways associated with maturation of neurons may play a role in cell death pathways. The expression of inhibitors of apoptosis (Bcl-X_L, XIAP) can increase with the stage of maturity. Bcl-X_L expression is upregulated in immature neurons as they migrate away from the mitotically active ventricular zone and remains highly expressed in mature neurons in the adult brain [46, 47]. It has been shown that the potential of the intrinsic apoptotic pathway is progressively reduced during neuronal maturation and that such repression was associated with a downregulation of Apaf-1 and caspase-3 gene expression [48]. Similarly, NMDA receptor activation, which occurs together with maturation of neurons, upregulates the expression of XIAP, while downregulating that of caspase-3 [49]. Finally, electrophysiological properties of young neurons render those more readily excitable [50], a feature probably attributable to the activity of low-threshold calcium channels [51]. This could render them more sensitive to excitotoxic stimuli.

In conclusion, we have shown that in this model of pneumococcal meningitis, recently divided, yet immature postmitotic cells in the inner layer of the dentate gyrus undergo apoptosis. The majority of these cells divided 6–10 days before the infection. Further studies are necessary to explore the consequences of this form of cell death for the development and function of the hippocampus and the associated neurofunctional deficits after bacterial meningitis.

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