Neuronal Deletion of Caspase 8 Protects against Brain Injury in Mouse Models of Controlled Cortical Impact and Kainic Acid-Induced Excitotoxicity

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Abstract

Background: Acute brain injury is an important health problem. Given the critical position of caspase 8 at the crossroads of cell death pathways, we generated a new viable mouse line (Ncasp8+/−), in which the gene encoding caspase 8 was selectively deleted in neurons by cre-lox system.

Methodology/Principal Findings: Caspase 8 deletion reduced rates of neuronal cell death in primary neuronal cultures and in whole brain organotypic coronal slice cultures prepared from 4 and 8 month old mice and cultivated up to 14 days in vitro. Treatments of cultures with recombinant murine TNFα (100 ng/ml) or TRAIL (250 ng/mL) plus cyclohexamide significantly protected neurons against cell death induced by these apoptosis-inducing ligands. A protective role of caspase 8 deletion in vivo was also demonstrated using a controlled cortical impact (CCI) model of traumatic brain injury (TBI) and seizure-induced brain injury caused by kainic acid (KA). Morphometric analyses were performed using digital imaging in conjunction with image analysis algorithms. By employing virtual images of hundreds of brain sections, we were able to perform quantitative morphometry of histological and immunohistochemical staining data in an unbiased manner. In the TBI model, homozygous deletion of caspase 8 resulted in reduced lesion volumes, improved post-injury motor performance, superior learning and memory retention, decreased apoptosis, diminished proteolytic processing of caspases and caspase substrates, and less neuronal degeneration, compared to wild type, homozygous cre, and caspase 8-floxed control mice. In the KA model, Ncasp8+/− mice demonstrated superior survival, reduced seizure severity, less apoptosis, and reduced caspase 3 processing. Uninjured aged knockout mice showed improved learning and memory, implicating a possible role for caspase 8 in cognitive decline with aging.

Conclusions: Neuron-specific deletion of caspase 8 reduces brain damage and improves post-traumatic functional outcomes, suggesting an important role for this caspase in pathophysiology of acute brain trauma.

Introduction

The loss of neurons underlies the pathophysiology of numerous neurological diseases. Understanding the key mediators of the neuronal cell death is critical to identification of potential targets for therapeutic intervention [1]. Caspases are highly conserved aspartate-specific cysteine proteases that have both apoptotic and non-apoptotic functions (reviewed in [2]).

Caspase 8 is the apical protease in the extrinsic apoptotic pathway activated at the plasma membrane by various TNF-family death receptors [3]. Stimulation of these receptors by pro-apoptotic ligands causes receptor clustering followed by the recruitment of FADD (Fas-associated death domain protein), which in turn binds Death Effector Domains (DEDs) in the proform of caspase 8, a protease that then cleaves and activates downstream effector caspases responsible for apoptosis [4]. In some types of cells, caspase 8 indirectly activates downstream caspases by cleaving the BH3-only protein Bid [5], leading to activation of the Apaf-1/caspase 9 apoptosome [6]. In some scenarios, caspase 8 activation can also occur downstream of the
mitochondrial apoptotic pathway [7]. Moreover, an alternative pathway involving caspase 8 activation is associated with endoplasmic reticulum (ER) stress [6,9,10,11]. Thus, caspase 8 bridges several death mechanisms, including extrinsic and intrinsic apoptosis pathways, ER stress, and possibly autophagic pathways (reviewed in [12]) [13].

A role for caspase 8 in neuronal cell death has been suggested by evidence of proteolytic processing and increases in caspase 8 protease activity in the settings of ischemia [14,15] and seizures [16,17]. However, attempts to genetically determine the function of caspase 8 in the brain have been hampered by the observation that caspase 8 gene knockout in mice is embryonic lethal [18]. As an alternative, transgenic mice have been created that over-express the endogenous caspase 8 inhibitor, c-FLIP, indirectly suggesting that caspase 8 is a critical mediator of glucose deprivation-induced neuronal cell death [19]. However, c-FLIP has additional functions besides suppression of caspase 8, including induction of NF-κB family transcription factors (reviewed in [20]), making interpretation of these results difficult. To date, ablation of caspase 8 expression in the brain of living mice has not been described.

Given the critical position of caspase 8 at the crossroads of cell death pathways, we sought to determine the contribution of this caspase to neuronal injury in vivo by the generation and characterization of a new viable mouse line (Neasp8flfl), in which caspase 8 has been selectively deleted in neurons. Our findings demonstrate a protective role of caspase 8 deletion in vivo using a controlled cortical impact (CCI) model of traumatic brain injury (TBI) and seizure-induced brain injury caused by kainic acid (KA), as well as in vitro employing cultures of embryonal primary cortical neurons and adult brain coronal slices.

Materials and Methods

Experimental protocols involving vertebrate animals were approved by the Institutional Animal Care and Use Committee (#07-043, 07-049, 10-087, and 10-089) of the Sanford-Burnham Medical Research Institute and comply with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Generation of mouse line with neuronal ablation of caspase 8

The generation and characterization of the CRE3 transgenic line were summarized elsewhere [21,22]. T-cell-lineage-specific casp8 mutant mice with deletion of exons 3 and 4 of caspase 8 (casp8fl3-4; LckCre mice) [23] were backcrossed for nine generations onto the FVB/N background to generate casp8fl/fl mice without cre. For deletion of the caspase 8 (casp8) gene specifically in neurons, homozygous casp8-floxed mice (casp8fl/fl) were bred with pan-neuronal CRE3 homozygous transgenic mouse (FVB/N background) to obtain heterozygous founders (casp8fl/cre). To generate homozygous congenic casp8fl/cre CRE3 mice, the heterozygous founders were intercrossed for nine generations; the Casp8fl/Cre3 homozygous line (henceforth named Neasp8flfl) was subsequently used in most experimental procedures. Genotyping was undertaken by PCR using primers detecting cre and casp8 floxed alleles. A 950-bp band was detected by forward and reverse primers (5’-GGCTGGATTACCGGCTGAGTCAAAGCA-3’ and 5’ GTGGCCAGATGGGCGGCAACGTC-3’, respectively; 320-bp, 650- and 200-bp bands for unrecombined floxed casp8 alleles, wild-type (WT) casp8 mouse gene, as well as recombinated floxed casp8, respectively, were obtained using forward and reverse primers (5’-GCG CCT CCT CAG TAC TGT CAC CTG T-3’ and 5’-CCA GGA AAA GAT TTA TGT CTA GC-3’).

Primary neuronal culture (PNC), treatment and viability assay

Primary cortical neurons were derived from embryos (E16.5) of CRE3 and Neasp8flfl homozygous mice and cultured as previously described [24]. Briefly, cortices were dissected under a microscope in ice-cold Mg2+- and Ca2+-free Hank’s Balanced Salt Solution (HBSS, Invitrogen, Carlsbad, CA, USA), and trypsinized (0.25% trypsin in HBSS) at 37°C for 15 min. The tissue was mildly triturated 5–10 times and filtered through a 40 μm cell strainer (BD Biosciences, San Diego, CA, USA). The isolated cells were counted by Trypan exclusion in a Neubauer chamber, then seeded at a density of 75,000 cells/cm² on poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA)–coated cover slips in 24 well plates and cultured in Neurobasal medium supplemented with B27 (Invitrogen). After 3 or 14 days in culture, neurons were treated with recombinant murine TNFα (100 ng/ml; PeproTech, Rocky Hill, NJ, USA) and cycloheximide (CHX) (1 μg/ml; MP Biomedicals, Solon, OH, USA) for 4 h or staurosporine (1 μM for 4 h and 50 nM overnight). To determine the proportion of viable neurons, cells were fixed, then stained with Hoechst dye to visualize nuclei and immunostained with an antibody to neuron-specific nuclear protein (NeuN) (Chemicon, Ramona, CA, USA; MAB377) followed by application of Alexa Fluor 594 conjugated anti-mouse antibody (Invitrogen). The ratio of neurons with NeuN immunoreactivity that showed Hoechst staining profile characteristic of viable cells to the total cell number (number of Hoechst-positive nuclei) [25,26] was determined by counting cells on ten random fluorescent images generated by microscopy using an ×20 objective. In addition, to define the proportion of apoptotic/digenenerating neurons in the 14-day-old culture, a polystyrene sensitive indicator for viability and apoptosis (pSIVA) (CytoGLO™ pSIVA-IANBD Apoptosis/Viability Microscopy Set Imgenex IMG-6701K), diaalyzed into PBS, was applied to the PNC at a concentration of 10 μg/ml TC medium. In this test, fluorescently tagged annexin binds to externalized phosphatidylserine exposed on apoptotic cell membranes allowing for monitoring changes that occur at different stages of apoptosis in living cells [27]. After 15 min incubation, the cells were fixed and immunostained using MAP2 antibody. The ratio of pSIVA-positive neurons with colocalized MAP2 immunopositive reactions to total number of MAP2 positive cells was determined after incubation with Alexa Fluor 594 conjugated anti-mouse antibody, as described above. Using EVOS fluorescence microscope (Advanced Microscopy Group, Bothell, WA), 15 random digital images of cells were taken at 20x magnification from 3 wells per experiment for manual cell counting.

Brain organotypic coronal slice cultures (BOCSC)

Brain slices were prepared and cultured using a simple method for organotypic cultures of nervous tissue [29], slightly modified. Briefly, 4 and 8 month old mice, representing all three homozygous lines (3 mice/line), were anesthetized, and transcardial perfusion-flush was performed with PBS containing protease inhibitors (Sigma). After 2–3 minutes of perfusion, the mice were quickly decapitated, the brains promptly removed, and submerged in pre-cooled and 95% oxygenated ACSF (artificial cerebrospinal buffer). Subsequently, both hemispheres were separated from cerebellum and the brain stem, mounted on the vibrotome stage with a supportive agarose block to obtain ~500–600 μm thick coronal sections of both hemispheres using a Leica VT1200S vibrotome. The slices were plated on organotypic inserts (Milllicell 30 mm Organotypic cm PTFE 0.4 μm; Millipore/Chemicon) and maintained for 72 h in 6-well plates on the surface of Neurobasal A medium supplemented with B27 and GlutaMax under standard conditions.
incubation conditions (5% CO₂, 95% air). Representative slices at bregma levels from −1.22 to 3.08 were maintained in culture for 72 hours, and then treated with TRAIL (Calbiochem, La Jolla, CA, USA) at 250 ng/mL for 4 h. Following treatment, the slices were fixed in Z-fix solution and processed into paraffin blocks. Additional brain slices derived from CRE3 and Nsamp−/− lines (6 mice/line) were cultured for 14 days for further processing. Histological and immunocytochemical results were generated using 5 μm thick serial paraffin sections numbered from 5 to 50, counting from the bottom aspect that faces the mesh and culture medium. The surface layer was removed by trimming before serial sections were collected. The sections were stained either with H&E or Masson’s trichrome or subjected to TUNEL assay or immunostaining using antibodies specific for cleaved caspase 3 (Cell Signaling Technology, Danvers, MA, USA; cat.#9661 and ImageX, San Diego, CA, USA; cat.#5699), MAP2 (clone HM-2), NeuN (clone A60 Millipore Chemicon, cat.# MAB377), β3-tubulin (Abcam; ab18207), GFAP (Abcam, Cambridge, UK; ab7260), S100 (DakoCytomation, Carpinteria, CA, USA; cat.# Z0311), and Bax 1 (Wako Chemicals USA, Richmond, VA; Code No. 019-19741).

Controlled Cortical Impact (CCI) model of Traumatic Brain Injury

Mice (12 to 28 weeks of age; weight 24–38 g) of wild-type, CRE3, casp8fl/fl, and casp8fl/fl/cre genotypes were anesthetized with 4% isoflurane (Aerrane; Baxter, UK) in 70% N₂O and 30% O₂ and positioned in a stereotaxic frame. Using a head restraint, a 5-mm craniotomy was made using a portable drill and a trephine over the bregma levels from 1.22 to 3.08 were maintained in culture for 14 days for further processing. Histological and immunocytochemical results were generated using 5 μm thick serial paraffin sections numbered from 5 to 50, counting from the bottom aspect that faces the mesh and culture medium. The surface layer was removed by trimming before serial sections were collected. The sections were stained either with H&E or Masson’s trichrome or subjected to TUNEL assay or immunostaining using antibodies specific for cleaved caspase 3 (Cell Signaling Technology, Danvers, MA, USA; cat.#9661 and ImageX, San Diego, CA, USA; cat.#5699), MAP2 (clone HM-2), NeuN (clone A60 Millipore Chemicon, cat.# MAB377), β3-tubulin (Abcam; ab18207), GFAP (Abcam, Cambridge, UK; ab7260), S100 (DakoCytomation, Carpinteria, CA, USA; cat.# Z0311), and Bax 1 (Wako Chemicals USA, Richmond, VA; Code No. 019-19741).

Motor function assessment by the wire grip test

Vestibulomotor function was assessed using a wire-grip test ([31,32]). Mice (n = 8–12/group) were placed on a metal wire (45 cm long) suspended 45 cm above a foam mat between 2 vertical bars. Mice were allowed to cross the wire for 60 s. The latency that a mouse remained on the wire within a 60 s interval was measured, and wire grip scores were quantified using a 5-point scale [31,33]. Mice that were unable to remain on the wire for less than 30 s were given a score of zero. A score of one point was given if the mouse failed to hold on to the wire with both sets of fore paws and hind paws together; two points were given if the mice held on to the wire with both fore paws and hind paws but not the tail; three points were given if the mouse used its tail along with both fore paws and both hind paws; four points were given if the mouse moved along the wire on all four paws plus tail; and five points were given if mice that scored four points also ambulated down one of the posts used to support the wire. The wire grip test was performed in triplicate and an average value calculated for each mouse on each day of testing. For the statistical analysis, cumulative scores were produced from all three trials. The test was performed before CCI and repeated weekly after CCI through the entire duration of the experiment (21 days). An additional cohort of aged mice with no brain injury (described above) was also subjected to this test.

Sensorimotor function evaluation using beam walking test

Beam walking is a motor skill and balance test. Prior to CCI, each mouse was trained to run across a 29.5 cm long x 1.2 cm wide square wooden beam placed 33 cm above a table to reach its home cage located at the far end of the beam. Training was followed by a repeat test (probe 1) performed at one week before the CCI procedure, which was repeated 7 days (probe 2) and 21 days (probe 3) after the procedure. Performance was measured as...
the number of foot slips while traversing the length of the beam three times. Progressive impairment correlates with increased numbers of foot slips during crossing. The test was also performed on the separate cohort of aged uninjured mice (described above).

**Hindlimb flexion for testing paresis and asymmetry**

The hindlimb flexion was elicited by gently lifting the animal by its tail and holding it 0.5 meters above a table [34]. The absence of asymmetry/paresis was scored as zero (no neurological abnormalities – “no”). The presence of asymmetry/paresis was scored as one (“yes”). The test was performed 1 week before CCI (probe 1) and 7 and 21 days after the procedure (probes 2 and 3, respectively).

**Tissue preparation and staining**

Mice were anesthetized with Avertin (BD Franklin Lakes, NY, USA) and transcardially perfused with phosphate-buffered saline (PBS) pH 7.4, followed by zinc-buffered formalin (Z-fix; Anatech, Inc., Battle Creek, MI, USA). After decapitation, the whole head was fixed in the Z-fix solution. Following skull removal and 3–5 days of brain post-fixation, each brain was cut in 1.5 mm thick coronal slices fitting in one cassette to be processed and embedded into a single paraffin block. Thus, all sections from each coronal slice were stained on the same slide ensuring identical staining conditions. In addition to H&E, brain sections were stained with Masson’s trichrome (American Master*Tech Scientific, Inc.; Lodi, CA, USA); Dewaxed tissue sections (4.0–5.0 μm) were immunostained as reported previously [35] using an antibody to mouse IgG (DakoCyotmation), rabbit polyclonal antibodies to GFAP (Abcam); βI (Zak Chemicals USA); phospho-Tau (Thr231) (Thermo Fisher Scientific, Lafayette, CO, USA); cat.#OPA1-05156), phospho-Tau (T181) (Abcam ab38505), cleaved caspase 3 (Cell Signaling Technology and Imgenex), and phospho-c-Jun (Cell Signaling Technology, cat.#9164), as well as mouse monoclonal antibodies to NeuN (clone A60; Millipore Chemicon), and microtubule-associated protein (MAP2) (clone HM-2; Sigma-Aldrich), and rat monoclonal antibody to Ly-6G (BD Pharmingen; cat.#351459). Application of the primary antibody was followed by incubation with goat anti-mouse or goat anti-rabbit polymer-based EnVision-HRP-enzyme conjugate (DakoCyotmation). Diaminobenzidine (DAB; DakoCyotmation) and SG-Vector (Vector Lab, Inc.; Burlingame, CA, USA) chromogens were applied, yielding brown and black colors, respectively.

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) assay**

The detection of nuclei with fragmented DNA by TUNEL was assessed volume of lesion and lesion cavity. Lesion volume was calculated as previously reported in detail by our group [35]. Briefly, all slides were scanned at an absolute magnification of 400× [resolution of 0.25 μm/pixel (100,000 pix/in.)] using the Aperio ScanScope CS system (Aperio Technologies). The acquired digital images representing whole tissue sections were analyzed applying the Spectrum Analysis algorithm package and ImageScope analysis software (version 9; Aperio Technologies) to quantify IHC and histochemical stainings. These algorithms make use of a color deconvolution method [38] to separate stains. Algorithm parameters were set to achieve concordance with manual scoring on a number of high-power fields, including intensity thresholds for positivity and parameters that control cell segmentation using the nuclear algorithm. Colorimetric intensity measurements were applied to Masson’s trichrome histochemical stain to obtain quantitative characteristics of brain tissue damage, as well as percentage and total number of degenerating neurons in the annotated area of interest [35]. In addition, NeuN and MAP2 immunohistochemistry were performed for assessment of neuronal cell preservation. Automated morphometric analyses were accomplished for entire regions of interest and not limited to a few selected fields.

**Neuropathological scoring of brain injury**

Brain injury was evaluated by an observer masked to the study groups using a neuropathological scoring system: grade 0 - no observable injury; grade 1 - restricted cortical impact area with small petechial bleedings and variable degree of acute ischemic neuronal degeneration but no cortical tissue loss; grade 2 - necrotic lesion core and variable tissue loss of the injured cortex with penumbra demonstrating neuronal degeneration limited to cortex only; grade 3 - sporadic larger hematomas and ischemic, necrotic, spongiotic changes crossing the corpus callosum and involving ipsilateral hippocampal sectors CA1–CA3; and grade 4 - confluent impact core encompassing large cortical area, entire hippocampus and deeper thalamic structures in the upper quadrant of the ipsilateral hemisphere. The score was given to each brain coronal section containing a pathological focus, and the total score was a sum of all scores for the slices recorded for each animal.

**Immunoblotting**

Immunoblotting methods were used to analyze proteolytic processing of caspases and their substrates in the brains of control and knockout mice after CCI [39]. Extracts from cortex and basal ganglia ipsi- and contralateral to the injury site, derived from Ncasp8−/−, control CRE3 and casp8fl/fl, (n = 4–5 per group) were prepared, and 30 μg of protein per lane was analyzed by SDS-PAGE/immunoblotting, probing blots with rabbit polyclonal antibodies to caspase 8 (Imgenex IMG#5703, #5704; Cell Signaling cat.#4927), and caspase 3 (Imgenex IMG-5699), as well as mouse monoclonal antibody to cleaved PARP (Aep214; 19F4; Cell Signaling #9546S). Antibody to β-actin (Sigma-Aldrich) served as a loading control. Detection was accomplished...
Induction of status epilepticus

Mice were injected intraperitoneally (i.p.) with kainic acid (KA) solution (K-0250, monohydrate, Sigma) at 25 or 35 mg/kg (n = 8–11 per group). Eight control and 11 Ncasp8<sup>-/-</sup> mice were used in this study. Animals were monitored during status epilepticus to determine duration and severity of seizure activity and the neurological status in post-seizure period.

Seizure scoring

KA-induced seizures were scored every 15 min for 2 h according to a described procedure [40] with minor modifications [41]. Seizure activity was scored as follows: 1 - arrest of motion; 2 - myoclonic jerks of the head and neck with brief twitching movements; 3 - unilateral clonic activity; 4 - bilateral forelimb tonic and clonic activity; 5 - generalized tonic-clonic activity with loss of postural tone; 6 - death due to continuous convulsions.

Statistical Analysis

Data were analyzed using the STATISTICA software package (StatSoft; Tulsa, OK, USA). Statistical analyses of pairs of control vs knockout mice or specimens were performed using a one-way Student’s test. A one-way analysis of variance (ANOVA) method was used to determine the significance of differences of multiple point data sets. Box and whisker or column mean±SEM plots were applied to visualize the data in a graphic form. Mean values are plotted as a middle point at each time assessed after brain trauma or KA treatment, while the whiskers reflect ± standard error of mean (SEM). Chi-square test was performed to test whether the frequencies of neurological deficits, observed in the hindlimb flexion test, differed significantly from the expected ones. P values<0.05 were reported as statistically significant.

Results

Generation and characterization of mice with neuron-specific ablation of caspase 8 expression

To establish mice with neuron-specific caspase 8 gene ablation, we bred casp8<sup>-/-</sup>-floxed (casp8<sup>fl/fl</sup>) mice with transgenic pan-neuronal CRE3 mice that express the cre protein exclusively in neurons [21]. Consecutive breeding of heterozygotes resulted in establishment of neuron-specific casp8<sup>-/-</sup> knockout mice, henceforth termed Ncasp8<sup>-/-</sup> mice. Genotypes were confirmed by PCR (Figure 1A). Genomic DNA was analyzed from various tissues to determine whether CRE3 induced recombination of the floxed-casp8<sup>-/-</sup> gene in a tissue-specific manner (Figure 1B). PCR primers were designed to differentiate unrecombined and recombined floxed-casp8<sup>-/-</sup>, as well as the wild-type murine casp8<sup>-/-</sup> gene. Specimens from brain (cortex, thalamus/basal ganglia, brain stem) but not from other organs of the Ncasp8<sup>-/-</sup> mice and not from CRE3 (casp8<sup>fl/fl</sup>/cre<sup>cre</sup>) mice showed the expected PCR product of 200 bp indicative of brain-specific recombination (Figure 1B). Immunoblot analysis revealed decreased pro-caspase 8 levels in lysates from various regions of brain, including cortex (C), basal ganglia (BG), and cerebellum (CB) of the Ncasp8<sup>-/-</sup> mice compared to those from CRE3 (casp8<sup>fl/fl</sup>/cre<sup>cre</sup>) or casp8<sup>-/-</sup>-floxed (casp8<sup>fl/fl</sup>/cre<sup>cre</sup>) controls (Figure 1C), confirming that our genetic manipulations resulted in reduced caspase 8 expression in brain. Because the cre transgene employed is expressed only in neurons, gial expression of caspase 8 is expected to remain [42], possibly accounting for the residual pro-caspase 8 protein detected. Using immunohistochemistry (IHC), brains derived from control mice (Figure 1D–G) exhibited clear caspase 8 immunostaining in neurons and fibrous astroglia, whereas negligible caspase 8 staining was observed in neurons in brains from the Ncasp8<sup>-/-</sup> animals (Figure 1H–K). No gross or histological brain malformations were detected in the Ncasp8<sup>-/-</sup> mice.

Casp8<sup>-/-</sup>-ablating cultured embryonic neurons show resistance to TNFα-induced cell death

To functionally verify the effects of caspase 8 deletion in murine neurons, cultured embryonic cortical neurons from the CRE3 and Ncasp8<sup>-/-</sup> homozygous mice were maintained in vitro for 3 and 14 days prior to further experimentation. Comparison of CRE3 and Ncasp8<sup>-/-</sup> neurons after 14 days of culture revealed significant differences in their phenotype and survival rates (Figure 2A, B; Figure 3C1–D2). In contrast to sparsely distributed, clustered control (CRE3) neurons (Figure 3C1, C2), cultures derived from Ncasp8<sup>-/-</sup> homozygous mice formed dense networks of highly branched interconnected neurons (Figure 3D1, D2). Double staining for MAP2 (red) and pSIVA (green) [27] confirmed this observation, revealing decreased proportion of spontaneously degenerating neurons in the Ncasp8<sup>-/-</sup> culture compared to the CRE3 control (Figure 2B, Figure 3E1–F4). After 3 and 14 days in culture, cell death was induced using either TNFα plus cyclohexamide, an agonist of the death extrinsic apoptosis pathway (death receptor), or staurosporine, a broad-spectrum protein kinase inhibitor that induces the intrinsic (mitochondrial) pathway of cell death. Consistent with a prominent role for caspase 8 in the extrinsic pathway, casp8<sup>-/-</sup> gene deletion significantly protected immature and mature neurons against cell death induced by TNFα plus cyclohexamide (Figure 2A **p = 0.03, B ***p<0.0001; Figure 3A, B, I1–J4), as determined by the ratio of remaining (surviving) NeuN-positive neurons with Hoechst staining profile characteristic of viable cells, relative to total cell number (number of Hoechst-positive nuclei) (Figure 3A). Partial protection against staurosporine-induced cell death was observed only for mature Ncasp8<sup>-/-</sup> neurons (**p = 0.004) but not for the immature neuronal cells (Figure 2A). PSIVA staining of the mature neurons confirmed this observation, demonstrating a reduced proportion of apoptotic/degenerating neurons in the STS-treated and also in untreated and TNFα/CHX-treated Ncasp8<sup>-/-</sup> neurons compared to the controls (**p = 0.0001; *p = 0.004; ***p<0.0001) (Figure 2B; Figure 3E1–J4). This tendency was particularly apparent in the TNFα plus cycloheximide-treated neurons, where preserved neuronal morphology in the Ncasp8<sup>-/-</sup> cultures contrasted with the presence of devastating neuronal degeneration with loss of axonal and dendritic branches, and numerous apoptotic bodies in the CRE3 cultures (Figure 3I1–J4). We conclude that homozygous deletion of genes encoding caspase 8 in neurons significantly protects immature and mature neuronal cells against cell death induced via the extrinsic apoptosis pathway, thus functionally validating the genotypic and expression data above.

Neuronal deletion of casp8 protects neurons in an organotypic culture model

In addition to assessing the impact of caspase 8 deletion in explanted monolayer cultures of fetal neurons, we also evaluated brain coronal slices from adult Ncasp8<sup>-/-</sup> mice compared to control animals. Although brain slice cultures are well-established models for in vitro investigations, they have been typically prepared from early postnatal rodent brains, which show a high plasticity and resistance to mechanical trauma during the slice preparation, thus maintaining longer viability in culture (reviewed in [43]) [44].
In contrast, our brain slices are derived from 4–8 month old adult mice. Although preparation of slice cultures from older animals is more challenging, the model is more suitable to study pathology of the mature brain [45,46]. Unlike organotypic slices encompassing restricted brain regions, we have deliberately prepared whole brain slices to study cell death of neurons in mice with pan-neuronal expression of casp8<sup>fl/fl</sup> line, and mice with conditional deletion of casp8 in neurons.

The aim of the present study was to determine the preservation of neurons in mature brain slices maintained in vitro for 72 h and 14 days. First, we have analyzed “spontaneous” neuronal cell death following slice preparation in the 3-day-old culture. The insults associated with this technique, including tissue injury (trauma), loss of cerebral blood flow (with likely ischemia/hypoxia and hypoglycemia), and denervation (axotomy) are known to induce cell death. Microscopic analysis of paraffin-embedded brain slices by H&E and Masson’s trichrome staining revealed various degrees of cellular degenerative and edematous changes, particularly pronounced in the hippocampi and deep basal ganglia. The survival of neurons in brain slices, however, was maintained significantly better in the knockout compared to control mice. The percentages of surviving neurons in brain slices were evaluated in various regions of the brain by MAP2 and neuron-specific nuclear protein (NeuN) immunostaining. MAP2 protein localizes predominantly to the dendrites and perikaryon of intact neurons and is disrupted by neuronal damage [47]. As

![Figure 1. Generation of mouse line (Ncasp8<sup>−/−</sup>) with neuron-specific ablation of caspase8.](image-url)

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assessed by MAP2 or NeuN immunostaining, spontaneous loss of neurons in brain slices was highly pronounced in tissue sections derived from CRE3 or casp8fl/fl mice (Figure 2C, D; Figure 4A1–A2), whereas significantly more surviving neurons were present in brain slices from Ncasp82/2 animals (Figure 2C, D; Figure 4B1–B2; Table 1). The decline in MAP2 protein expression was associated with increased levels of cleaved caspase 3 (Figure 4A1, A2; A2 inset – high magnification micrograph of the primary motor cortex and hippocampus indicated by red and blue arrows).

Investigation of the entire coronal brain slices at 72 h provided information about regional vulnerability of neurons and the dynamics of spontaneous neuronal degeneration in organotypic culture. Immunostaining revealed a relatively early loss of MAP2 immunoreactivity, suggesting rapid vulnerability of the microtubular cytoskeleton, whereas more robust immunoreactive signals for NeuN were detected even in neurons with balloon degeneration (pointed to by the red arrows in Figure 4C1, C2). In normal mice (that contain intact caspase 8 genes), degeneration and death of neurons occurred during the first 24 h in the entire brain. In brain slices derived from Ncasp8−/− animals, the most susceptible regions were entorhinal cortex and hippocampus, whereas neurons in basal ganglia (particularly in the amygdala and the bed nucleus of the stria terminalis) maintained well-preserved morphology and MAP2 immunoreactivity for up to 3 days in culture (Figure 4B1–D3; green arrows). Also, the median preoptic nucleus, the dorsal part of caudate putamen, median and lateral habenular nuclei with adjacent parafascicular thalamic nucleus, and posterior thalamic nuclear group contained well-preserved neurons. In neocortex of the Ncasp8−/− slices, neurons in superficial cortical layers (I–II) were more affected (Figure 4D1; red arrows), whereas deeper cortical layers (III–VI) were less impacted by organotypic culture conditions (Figure 4D1; green arrows). Similar regional susceptibility had been suggested in prior reports for cultures of dissociated cortical layers [48].

Considering that at least part of neuronal cell death that occurs in the brain slice culture model is due to signals produced by tissue injury, we speculated that cytokines are elaborated locally in the sliced brain tissue, resulting in caspase 8 activation. As an example, we used the TNF-related apoptosis-inducing ligand (TRAIL) to evoke cell death in the cultured brain slices. TRAIL is known to transmit the apoptotic signals through death receptors that directly activate caspase 8 [49]. Interestingly, expression of TRAIL-binding death receptors is induced by the transcription factor CHOP during ER stress, a condition associated with oxidative stress and ischemia-reperfusion injury (reviewed in [50]). In the brain organotypic culture model, casp8 gene deletion significantly protected neurons against cell death induced by TRAIL, as estimated in the cortical region and basal ganglia. In contrast to control brain slices from CRE3 and casp8fl/fl mice where TRAIL reduced the prevalence of MAP2 and NeuN

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**Figure 2. Neuroprotective effects of caspase 8 gene ablation demonstrated for cultured primary neurons.** (A) The ratio of surviving NeuN-positive neurons that showed Hoechst staining profile characteristic of viable cells to the total cell number (number of Hoechst-positive nuclei) was determined after 3 and 14 days of culture for untreated (UNT), staurosporine (STS) and TNFα/CHX-treated primary cortical neurons derived from embryos (E16.5) of CRE3 and Ncasp8−/− homozygous mice. Cells were counted on ten random fluorescent images (x20). (B) The ratio of pSIVA-positive neurons with colocalized MAP2 expression to the total number of MAP2-positive cells was determined after 14 days of culture for UNT, STS- and TNFα/CHX-treated primary cortical neurons derived from embryos of CRE3 and Ncasp8−/− mice. Cells were counted on fifteen random fluorescent images (x20) from 3 experiments. (C–I) Bar graphs present average percentages of MAP2 (C), NeuN (D), cleaved caspase 3 (E), GFAP (G), S100 (H), Iba1 (I) immunopositive staining and mean percentage of TUNEL positive signaling (F) in cortices (left) and basal ganglia (right) of untreated and TRAIL-treated cultured coronal brain slices derived from CRE3, casp8+/− (Casp8 line) and Ncasp8−/− mice after 3 days of culture. Tables 1 and 2 provide the results of statistical analyses.

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Caspase 8 Contributes to Acute Brain Injury

| PNC 3D          | PNC 14D/pSIVA       |
|-----------------|---------------------|
| **CRE3 (TNFα&CHX)** | **CRE3 (UNT)**  |
| ![Image A]       | ![Image E1]         |
| ![Image B]       | ![Image G1]         |

| **CRE3 (STS)** | **CRE3 (TNFα&CHX)** |
|----------------|---------------------|
| ![Image I1]    | ![Image H1]         |

| **CRE3 (UNT) 14D** | **Ncasp8 Δ (UNT)** | **Ncasp8 Δ (STS)** | **Ncasp8 Δ (TNFα&CHX)** |
|--------------------|--------------------|--------------------|-------------------------|
| ![Image C1]        | ![Image F1]        | ![Image H1]        | ![Image J1]              |
| ![Image D1]        | ![Image F2]        | ![Image H2]        | ![Image J2]              |

| **Ncasp8 Δ (UNT) 14D** | **Ncasp8 Δ (UNT)** | **Ncasp8 Δ (STS)** | **Ncasp8 Δ (TNFα&CHX)** |
|------------------------|--------------------|--------------------|-------------------------|
| ![Image C2]            | ![Image F3]        | ![Image H3]        | ![Image J3]              |
| ![Image D2]            | ![Image F4]        | ![Image H4]        | ![Image J4]              |
neurons (Figure 4E, F), no significant difference was observed in the number of cells expressing neuronal markers following TRAIL treatment of Ncasp8\(^{-/-}\) slices (Figure 2C, D; Figure 4G, H; Table 1). Deletion of casp8\(^{-/-}\) offered partial protection against TRAIL-induced apoptosis in organotypic cultures, as demonstrated by significantly decreased percentages of cleaved caspase 3- and TUNEL-positive cells in the Ncasp8\(^{-/-}\) compared to CRE3 and casp8\(^{+/+}\) brain slices (Figure 2C, D, F; Table 1). Figure 4 (E-H) demonstrates representative images of a reciprocal pattern of TUNEL and MAP2 expression in brain slices derived from the control line and Ncasp8\(^{-/-}\) mice.

Not only did TRAIL induce apoptosis of caspase 8-containing neurons in organotypic brain slice cultures, but some types of glial cells also appeared to be vulnerable to treatment with this cytokine in the organotypic brain slice setting. In this regard, TRAIL treatment significantly decreased the number of GFAP-positive fibrous astroglia in all three genotype groups (Figure 2G), suggesting that these glial cells are sensitive to TRAIL and serving as a control to show the selective protection of neurons in Ncasp8\(^{-/-}\) slices (Figure 4K, L). Interestingly, the percentage of S100-positive protoplasmic astrocytes did not change following TRAIL treatment (Figure 2H; Figure 4I, J), implying that these glial cells are insensitive to TRAIL under the culture conditions tested (Table 1). Immunostaining for the microglial marker Iba1 increased after TRAIL treatment in brain slice culture derived from all 3 genotypes (Figure 2I), serving as a further indicator of the neuron-specific differences in the responses of Ncasp8\(^{-/-}\) slices to TRAIL treatment and a sign of viability of stromal cells in the brain in vitro. After 14 days of culture, untreated Ncasp8\(^{-/-}\) brain slices demonstrated significantly more surviving neurons compared to the controls as evidenced by higher levels of NeuN protein in the cerebral cortices (Table 2 p = 0.0004; Figure 4Q, T). In neocortex; Figure 4O – arrow, P, R in archicortex/hippocampus; in basal ganglia (Figure 4O) and higher prevalence of microtubule-associated class III β-tubulin in gray matter, particularly in basal ganglia of the Ncasp8\(^{-/-}\) brains (Table 2 p = 0.0009; Figure 4U, W). MAP2 protein was undetectable in the control slices and it was found only in neurupil but not in neuronal soma in the Ncasp8\(^{-/-}\) cultures. Activation of astro- and microglia was more pronounced in Ncasp8\(^{-/-}\) brain slices than in the controls, as demonstrated by higher percentage of GFAP (Table 2 p = 0.001 in basal ganglia; Figure 4X) and Iba1 (Table 2 p = 0.0004 in cortex; p = 0.0003 in basal ganglia) immunostainings. Levels of S100 protein, that visualizes protoplasmic astroglia, were comparable at different time points for all three lines, as presented for Ncasp8\(^{-/-}\) slices at day 3 and 14 in culture (Figure 4M and N, respectively).

Neuronal deletion of caspase 8 protects against traumatic brain injury and facilitates learning and memory retention in aged mice

To assess the effect of caspase 8 neuronal knockout on functional performance and histopathological outcome following brain injury, we subjected mice to TBI using a CCI model. Comparisons were made of Ncasp8\(^{-/-}\), CRE3 (cre/casp8\(^{+/+}\)), casp8\(^{+/+}\)-boxed mice (casp8\(^{+/+}\)), and wild-type littermates (casp8\(^{+/+}\)). All knockout (n = 135) and 152 of 160 (95%) control animals survived CCI procedure and selected groups were put through behavioral tests. Sensorimotor function was evaluated by beam walking and hindlimb flexion tests. All naive control and knockout mice learned to balance on the wooden beam (Figure 5A; probe 1). Although all mice were impaired in their performance after CCI, brain-injured Ncasp8\(^{-/-}\) mice performed significantly better at this task (probe 2, 3; *p = 0.0009; **p = 0.04), demonstrating fewer foot slips during crossing compared to their control counterparts. In the hindlimb flexion test, none of the tested mice showed movement asymmetry or paresis prior to the CCI procedure (probe 1). Following the brain trauma, 6 of 7 (86%) control vs 2 of 7 (29%) Ncasp8\(^{-/-}\) mice (probe 2; Chi-square p = 0.03), and 6 of 7 (86%) control vs 1 of 7 (14%) Ncasp8\(^{-/-}\) animals (probe 3; Chi-square p = 0.008) showed neurological deficits. To assess motor function, we used the wire grip test. Ncasp8\(^{-/-}\) mice performed better than the control cohort (Figure 5B), with statistically significant differences observed between groups at 7 days after CCI (probe 2 *p = 0.03). To determine the effect of neuronal caspase 8 ablation on spatial memory acquisition, mice were subjected to Morris water maze (MWM) test before and after CCI. Ncasp8\(^{-/-}\) mice demonstrated better learning performance to locate the hidden platform based on visual cues, as well as superior memory retention following the cued learning phase prior to CCI and significantly shorter latencies after CCI compared to the control animals (T3 *p = 0.005; probe 1 **p = 0.001; probe 2 ***p = 0.009) (Figure 5C).

Better performance of Ncasp8\(^{-/-}\) mice in the wire grip and MWM tests compared to control animals was evident also in a cohort of aged (18 month old) casp8\(^{+/+}\) n = 19, and Ncasp8\(^{-/-}\) n = 20) mice with no brain injury (Figure 5E *p = 0.01; Figure 5F *p = 0.04, respectively), although the wire grip test revealed a dramatic age-dependent decline in motoric performance (p<0.0001 – probes 1–3) in all lines. Behavior of aged mice in the beam walking test did not reveal statistically significant differences between the genotypes (Figure 5D).

Protective effects of neuronal caspase 8 deletion on brain histopathology after CCI

In the brain-injured Ncasp8\(^{-/-}\) mice, the average lesion volume was significantly reduced at 6 (*p = 0.03), 24 (**p = 0.002), and 48 h (**p = 0.003) after CCI compared to the control animals (Figure 5G). Whereas brain lesions of Ncasp8\(^{-/-}\) mice were consistently confined to the ipsilateral cortex (Figure 6A2, A4), in caspase 8-expressing control animals the lesions often extended beyond the cortex, reaching the white matter or even the hippocampus (Figure 6A1, A3). Control animals also exhibited greater average cavity volumes at later time points post injury (6 h *p = 0.02; 24 h **p = 0.03) (Figures 5H and 6A1, A3). In addition
Figure 4. Casp8 ablation provides neuroprotection in organotypic brain slice cultures. The coronal brain slices derived from the CRE3 control line and Ncasp8−/− homozygous mice were maintained in in vitro conditions for 3 (A1–M) and 14 days (N–X). The presented results were obtained from untreated (A1–D3, I, K, N–X) and TRAIL-treated (E–H, J, L) cultures. Immunohistochemical stainings are depicted in cortical and subcortical regions of CRE3 (A1, A2, E, F, Q, S, W) and Ncasp8−/− (B1–D3, G–P, R, T, U, X) brain slices. Examples of single marker (M–W) and double (A1–L, X) immunostainings are presented for S100 (M, N), NeuN (O–T), and β3-tubulin (U, W) as well as for double labeled MAP2 (DAB-brown) and cleaved caspase 3 (SG-black; A1–B2) or GFAP (DAB; D1–D3), TUNEL (DAB; E–H) with MAP2/NeuN antibody cocktail (5G) and S100 (DAB; I–J); IbA1 (DAB; C1–C2) or GFAP (DAB; D1–D3) with NeuN (5G) immunostaining. Scale bar indicates ~1000 μm on macro images and ~100 μm on
analysis of TUNEL assay and protein biomarkers in cortical and basal ganglia regions of untreated and TRAIL-treated coronal brain slices (3-day-old BOSC).

### Table 1. Analysis of TUNEL assay and protein biomarkers in cortical and basal ganglia regions of untreated and TRAIL-treated coronal brain slices (3-day-old BOSC).

| MOUSE LINES & BRAIN REGIONS | TUNEL vs TRAIL-Treated | c-casp8 vs TRAIL-Treated | MAP2 vs TRAIL-Treated | NeuN vs TRAIL-Treated | GFAP vs TRAIL-Treated | S100 vs TRAIL-Treated | lba1 vs TRAIL-Treated |
|-----------------------------|------------------------|--------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| CORTEX: Untreated vs TRAIL-Treated |                         |                          |                      |                      |                      |                      |                      |
| CRE3                        | 63 ± 2.9 vs 71 ± 3.0; p = 0.007 | 10 ± 0.4 vs 15 ± 1.2; p = 0.03 | 14 ± 1.7 vs 9 ± 1.1; p = 0.03 | 21 ± 2.0 vs 8 ± 0.6; p = 0.002 | 7 ± 1.0 vs 4 ± 0.4; p = 0.03 | 70 ± 0.4 vs 67 ± 2.8; p = 0.002 | 2 ± 0.3 vs 6 ± 1.04; p = 0.001 |
| casp8fl/fl vs TRAIL-Treated | 46 ± 2.3 vs 55 ± 2.9; p = 0.007 | 11 ± 1.1 vs 8 ± 0.7; p = 0.002 | 28 ± 2.3 vs 4 ± 0.3; p = 0.0001 | 12 ± 0.8 vs 7 ± 0.5; p = 0.0001 | 74 ± 1.8 vs 71 ± 2.1; p = 0.0001 | 17 ± 0.2 vs 5 ± 0.6; p = 0.0006 |
| Ncasp8−/− vs TRAIL-Treated | 37 ± 2.7 vs 41 ± 2.8; p = 0.02 | 11 ± 0.8 vs 11 ± 1.1; p = 0.0001 | 51 ± 2.3 vs 47 ± 1.9; p = 0.0001 | 27 ± 0.8 vs 10 ± 1.04; p = 0.0001 | 84 ± 2.4 vs 80 ± 1.1; p = 0.0001 | 5 ± 0.3 vs 9 ± 0.7; p = 0.0001 |
| BASAL GANGLIA: Untreated vs TRAIL-Treated |                         |                          |                      |                      |                      |                      |                      |
| CRE3                        | 67 ± 3.1 vs 73 ± 2.6; p = 0.0002 | 14 ± 1.2 vs 21 ± 1.5; p = 0.006 | 12 ± 2.3 vs 10 ± 1.1; p = 0.002 | 6 ± 0.8 vs 5 ± 0.3; p = 0.0001 | 13 ± 1.4 vs 5 ± 0.5; p = 0.0001 | 74 ± 2.3 vs 71 ± 1.6; p = 0.0001 | 17 ± 0.3 vs 2 ± 0.3; p = 0.004 |
| casp8fl/fl vs TRAIL-Treated | 58 ± 2.9 vs 65 ± 0.7; p = 0.0002 | 11 ± 1.1 vs 23 ± 1.4; p = 0.0001 | 12 ± 0.7 vs 2 ± 0.2; p = 0.0001 | 11 ± 0.6 vs 7 ± 0.1; p = 0.0001 | 78 ± 2.3 vs 73 ± 1.3; p = 0.0001 | 2 ± 0.3 vs 5 ± 0.6; p = 0.002 |
| Ncasp8−/− vs TRAIL-Treated | 43 ± 1.0 vs 46 ± 2.7; p = 0.002 | 9 ± 0.8 vs 13 ± 1.1; p = 0.0001 | 50 ± 1.7 vs 48 ± 1.4; p = 0.0001 | 23 ± 1.4 vs 20 ± 1.2; p = 0.0001 | 28 ± 1.6 vs 8 ± 0.6; p = 0.0001 | 82 ± 1.6 vs 78 ± 2.3; p = 0.0001 | 5 ± 0.3 vs 7 ± 0.6; p = 0.002 |
| CORTEX: TRAIL-Treated |                         |                          |                      |                      |                      |                      |                      |
| CRE3 vs Ncasp8−/− | 71 ± 3.0 vs 41 ± 2.8; p = 0.000007 | 15 ± 1.2 vs 11 ± 1.1; p = 0.006 (NS) | 9 ± 1.1 vs 49 ± 1.9; p = 0.0001 | 5 ± 0.3 vs 47 ± 1.9; p = 0.0001 | 4 ± 0.4 vs 10 ± 1.04; p = 0.0001 | 6 ± 0.4 vs 84 ± 2.4; p = 0.00004 | 2 ± 0.3 vs 5 ± 0.3; p = 0.00001 |
| casp8fl/fl vs TRAIL-Treated | 55 ± 2.9 vs 41 ± 2.8; p = 0.002 | 15 ± 1.4 vs 11 ± 1.1; p = 0.006 (NS) | 8 ± 0.7 vs 49 ± 1.9; p = 0.0001 | 4 ± 0.3 vs 47 ± 1.9; p = 0.0001 | 4 ± 0.4 vs 10 ± 1.04; p = 0.0047 | 74 ± 1.8 vs 80 ± 1.1; p = 0.0003 | 17 ± 0.2 vs 9 ± 0.7; p = 0.0002 |
| Ncasp8−/− vs TRAIL-Treated | 73 ± 2.6 vs 46 ± 2.7; p = 0.000008 | 21 ± 1.5 vs 13 ± 1.1; p = 0.003 | 10 ± 1.1 vs 48 ± 1.4; p = 0.0001 | 5 ± 0.3 vs 20 ± 1.2; p = 0.0001 | 5 ± 0.5 vs 8 ± 0.6; p = 0.0001 | 71 ± 1.6 vs 78 ± 2.3; p = 0.0001 | 2 ± 0.3 vs 7 ± 0.6; p = 0.0001 |
| BASAL GANGLIA: Untreated vs TRAIL-Treated |                         |                          |                      |                      |                      |                      |                      |
| CRE3 vs Ncasp8−/− | 67 ± 3.1 vs 43 ± 1.0; p = 0.000008 | 14 ± 1.2 vs 9 ± 0.8; p = 0.01 | 12 ± 2.3 vs 50 ± 1.7; p = 0.0001 | 6 ± 0.8 vs 23 ± 1.4; p = 0.0001 | 13 ± 1.4 vs 28 ± 1.6; p = 0.0001 | 74 ± 2.3 vs 82 ± 1.6; p = 0.0001 | 17 ± 0.3 vs 5 ± 0.3; p = 0.00001 |
| casp8fl/fl vs TRAIL-Treated | 58 ± 2.9 vs 43 ± 1.0; p = 0.001 | 11 ± 1.1 vs 9 ± 0.8; p = 0.0001 | 13 ± 1.7 vs 50 ± 1.7; p = 0.0001 | 12 ± 0.7 vs 23 ± 1.4; p = 0.0001 | 11 ± 0.6 vs 28 ± 1.6; p = 0.0001 | 78 ± 2.3 vs 82 ± 1.6; p = 0.0001 | 2 ± 0.3 vs 5 ± 0.3; p = 0.0001 |
| Ncasp8−/− vs TRAIL-Treated | 65 ± 0.7 vs 46 ± 2.7; p = 0.000008 | 23 ± 1.4 vs 13 ± 1.1; p = 0.0001 | 10 ± 0.7 vs 48 ± 1.4; p = 0.0001 | 2 ± 0.2 vs 20 ± 1.2; p = 0.0001 | 7 ± 0.1 vs 8 ± 0.6; p = 0.0001 | 73 ± 1.3 vs 78 ± 2.3; p = 0.0001 | 5 ± 0.6 vs 7 ± 0.6; p = 0.0001 |

Statistical analyses of control (CRE3, casp8fl/fl vs Ncasp8−/−) mouse specimens were performed using Student’s t-test. The resulting average percentages of positive immunopositive cells and standard error of mean (SEM) values are provided. P values < 0.05 are reported as statistically significant. “NS” stands for nonsignificant (p ≥ 0.05).

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high magnification images of the slices. Abbreviations: DG = Dental Gyrus; CA2; CA4 = sectors of hippocampus; TH = Thalamus; AMG = Amygdala; PC = Piniformis Cortex; WM = White Matter.
Table 2. Analysis of TUNEL assay and protein biomarkers in cortical and basal ganglia regions of untreated 14-day-old organotypic brain slice cultures (BOCSC).

| MOUSE LINES & TUNEL | c-casp3 | MAP2 | NeuN | β-tubulin | GFAP | S100 | Iba1 |
|---------------------|---------|------|------|-----------|------|-----|-----|
| **BRAIN REGIONS**   |         |      |      |           |      |     |     |
| **CORTEX:**         |         |      |      |           |      |     |     |
| CRE3 vs Ncasp8<sup>−</sup> |         |      |      |           |      |     |     |
| 72±2.4 vs 60±8.0; p NS | 41±6.6 vs 53±7.9; p NS | 16±1.7 vs 13±2.7; p NS | 32±1.4 vs 57±4.5; p = 0.0004 | 39±4.5 vs 44±3.3; p NS | 21±4.9 vs 25±2.1; p NS | 51±2.7 vs 49±1.6; p = 0.00004 |
| **BASAL GANGLIA:**  |         |      |      |           |      |     |     |
| CRE3 vs Ncasp8<sup>−</sup> |         |      |      |           |      |     |     |
| 68±1.8 vs 62±5.9; p NS | 29±5.5 vs 39±4.9; p NS | 15±1.4 vs 11±1.9; p NS | 16±3.9 vs 24±3.1; P NS | 27±1.6 vs 24±3.1; P NS | 15±1.7 vs 26±1.7; P NS | 53±5.8 vs 57±2.0; p = 0.00003 |

Statistical analyses of control CRE3 vs Ncasp8<sup>−</sup> mouse specimens were performed using Student’s t-test. The resulting average percentages of positive/immunopositive cells and standard error of mean (SEM) values are provided. P values < 0.05 are reported as statistically significant. "NS" stands for nonsignificant. (p = 0.05).

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Compared levels of this protein in the ipsi- and contralateral Ncasp8<sup>−</sup>/− regions was noted at 21 day following brain trauma (Figure 5M, N). The differences we observed in NeuN immunoreactivity may be indicative of a protective effect of neuronal caspase 8 deletion on survival of neurons, but it should be noted that loss of NeuN immunostaining theoretically could be attributed to depletion of NeuN protein and not necessarily to neuronal disintegration or death [53,54].

Microtubule-associated proteins (MAPs), such as MAP2 and tau, are known to be sensitive markers of neuronal injury. In our TBI study, loss of MAP2 immunostaining following CCI indicated an early vulnerability of the microtubular cytoskeleton to brain trauma not only in the contused but also to a lesser extent in the contralateral cortex (Figure 6F1–F4). A correlation was observed between loss of MAP2 protein and neuronal damage as revealed by Masson’s trichrome staining. Comparison was made when the density of cleaved caspase 3-positive cells was calculated (*p = 0.0001; **p < 0.0001; ***p = 0.05; ****p = 0.01) (Figure 6K2).

Apoptosis contributes to neuronal cell death. Caspase 3 is activated by most apoptotic stimuli, becoming proteolytically cleaved to generate immunodetectable neo-epitopes [56]. In our TBI experiments, the percentage of cells with cleaved caspase 3 immunopositivity was elevated at 2 h post CCI in the lesion and penumbra, declining at 21 day after brain trauma. Although showing a similar kinetics of caspase 3 expression, the percentage of cells with caspase 3-positive cells remained significantly lower in the lesion and penumbral region of Ncasp8<sup>−</sup>/− mice compared to control animals (Figure 6L; *p = 0.02). Similar observations were made when the density of cleaved caspase 3-positive cells was calculated (*p = 0.02; **p = 0.03) (Figure 6L2).

Caspase 8 cleaves and activates the effector caspase 3 to cleave PARP thus connecting this protease to the mitochondrial pathway for cell death (reviewed in [12]). By immunohistochemistry, we observed a depletion of the caspase 8 proform in the impact lesion samples from the control CRE3 and Casp8<sup>fl/fl</sup> but not Ncasp8<sup>−</sup>/− mice; caspase 8 proform was detected in contralateral hemispheres (CLH) of all experimental groups (Figure 6M). Strong bands corresponding to cleaved PARP were recognized in lesion samples from the control CRE3 and Casp8<sup>fl/fl</sup> but not Ncasp8<sup>−</sup>/− mice. Barely detectable cleaved PARP levels were noted in the contralateral hemisphere samples (Figure 6M).

The c-Jun N-terminal protein kinase (JNK) signaling pathway modulates the activity of several Bel-2 family proteins, promoting apoptosis [57,58]. At 2 days following CCI, the frequency or density of cells with nuclear phospho-c-Jun in the lesion and surrounding penumbra significantly increased in control (Figure 6N-R) but not in Ncasp8<sup>−</sup>/− (Figure 6S-X) mice (Figure 6Y,Z; Y: *p = 0.03; Z: *p = 0.02; ***p = 0.05). Nuclear immunoreactivity of phospho-c-Jun was barely present in the contralateral semihemisphere of control mice, thus confirming that the elevated nuclear expression of c-Jun is trauma-related.

**Assessment of the blood-brain barrier, reactive gliosis and inflammatory response in the post-traumatic brains**

Post-traumatic alterations in the blood-brain barrier (BBB) were determined immunohistochemically by assessing extravasation of mouse immunoglobulin G (mIgG). The immunostaining demonstrated greater extravasation of mIgG in impact lesion/penumbra...
immunostaining results are compared among the six experimental groups [control (Ctrl) and Ncaps8−/−] (n = 12) and Ncaps8−/− (n = 15) mice. Average latency to reach and climb the platform or enter the platform zone before and after CCI is shown (mean±SEM) (p < 0.05; **p < 0.01; ***p < 0.001). Beam walking (D) and wire grip (E) tests were conducted in uninjured 18 month old casp8−/− (Ctrl; n = 19) and Ncaps8−/− (n = 20) mice (3 testing sessions, spaced one week apart). P values result from Student’s t-test (*p < 0.05; **p < 0.01; ***p < 0.001). (F) WMM task was performed in the same aged cohort to test memory retention up to 60 days after completion of the learning sessions. Average latency to reach and climb the platform is shown (mean±SEM) (p = 0.04). (G–I) Histopathological outcomes were compared between the control (Ctrl) and Ncaps8−/− mice 2 h, 6 h, 24 h, 48 h, and 3 weeks after the trauma (8–10 mice per time point in each experimental group). Using Aperio scanning system, lesion (G) and cavity (H) volumes were determined by summing each lesion or cavity area multiplied by the distance between each coronal slice (mean±SEM). Plots depict average lesion volume (G; p < 0.03; **p < 0.002; ***p < 0.003), cavity volume (H; p < 0.02; *p < 0.03), and neuropathological scores (I; p < 0.02; **p < 0.02; ***p < 0.004). (J) Time course for density (n/mm2) of NeuN-immunopositive neurons in traumatic brain lesions is depicted for the control (Ctrl) and Ncaps8−/− mice. (K) The NeuN immunostaining results are compared among the six experimental groups [control (Ctrl) and Ncaps8−/− naive (no surgery −), sham (surgery "S"), and subjected to CCI ("C") mice] in impact lesions (CCI-treated) or corresponding regions (naive or sham-operated) at 21 day after procedure. Density (n/mm2) of NeuN-immunopositive neurons in impact lesions or matching ipsilateral regions (IL) and corresponding contralateral (CL) regions of the control (Ctrl) (L) and Ncaps8−/− (M) naive (no surgery −), sham (surgery "S"), and subjected to CCI ("C") mice is presented in a time-course manner (L, M) or at 21 day after procedure (N). J: *p < 0.02; **p < 0.01; ***p < 0.004; L: *p < 0.0005; **p < 0.001; ***p < 0.0008; M: *p < 0.0002; **p < 0.006; ***p < 0.002; ****p < 0.0007. (O–P) Percentages of MAP2 immunopositive neurons were compared in impact lesions or corresponding ipsilateral regions of the control (Ctrl) and Ncaps8−/− naive (no surgery −) sham (surgery "S"), and subjected to CCI ("C") mice (8–10 mice/time point/group) and presented as time-course data (O) at 5 time points post CCI (mean±SEM) (L; p = 0.002) and at 21 day after CCI (bar graph/P). (Q) Phospho-tau immunoreactivity was assessed in ipsilateral semispheres of the control and Ncaps8−/− mice 48 h and 3 weeks after CCI (n = 8–10 per group) (mean ± SEM p < 0.001).

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Discussion

Acute brain injury constitutes a leading cause of disability and death. Devising therapeutic strategies for ameliorating irreversible loss of neurological function from these disorders begins with identifying targets for drug discovery. In this study, we provide evidence that neuron-specific deletion of caspase 8 reduces brain damage and improves functional outcomes induced by CCI in mice, suggesting an important role for this caspase in the pathophysiology of brain trauma. The protective effect of neuronal ablation of caspase 8 was evident by reduced lesion and cavity volumes, the lower neuropathological scores, reduced frequencies of neuronal cell death, reduced numbers of cells with cleaved caspase 3, better learning performance, superior retention of spatial memory and improved sensorimotor function. Interestingly, better learning performance and superior memory retention were also observed in aged Ncaps8−/− mice with no brain injury. Alterations in CNS are known to underlie age-related impairment of motoric function. In the wire grip test, age-related decline in motoric performance is reduced in the casp8 knockout mice compared to the age-matched controls. Further studies will be necessary to examine the effects of neuronal depletion of caspase 8 on learning and motoric function and to provide clues as to the mechanisms by which neuronal deletion of caspase 8 affects cognitive and behavioral function.

Neuronal caspase 8 deficiency also protected against neuronal injury caused by KA-induced excitotoxicity and seizures. Excitotoxicity contributes to secondary damage and cell death following acute brain injury [63] and is caused by excessive release of excitatory amino acids such as glutamate and aspartate that stimulate various glutamate receptors [64]. Neuronal cell death in excitatory amino acids such as glutamate and aspartate that stimulate various glutamate receptors [64]. Neuronal cell death (particularly abundant in the CA3 region) was decreased by caspase 8 deletion, as assessed by both methods. Results of cleaved-caspase-3 immunostaining confirmed this observation (Figure 8I–K).

and ipsilateral semispheres of the control compared to the Ncaps8−/− mice (Figure 7A, D–K). The extravasation area decreased significantly by 21 days post CCI in the casp8 knockout but not in the control animals (*p = 0.02; **p = 0.04) (Figure 7A).

A comparison of microglia density between the experimental groups was accomplished by measuring the percentage of BAl positive cells in the annotated ipsilateral semispheres at different time points after CCI (Figure 7B). The dynamics of changes in microglia prevalence were similar in both experimental groups with higher density of activated microglia with hypertrophic soma and thick processes in the Ncaps8−/− mice, most pronounced at 21 day post CCI (Figure 7B (p = 0.02) and L–S). TBI causes active astrogliosis, which may cause glial scar formation and promote activation of microglia [59]. Increased frequencies of GFAP-immunopositive activated astroglia were observed in ipsilateral regions of both control and Ncaps8−/− mice compared to the contralateral semispheres, with no statistically significant difference between the control and Ncaps8−/− mice (not shown). Recent studies revealed that reactive astrocytes undergo substantial molecular changes that dictate their beneficial function for neuronal survival [60]. Histological examination of the leukocyte infiltration into brain hemispheres following CCI indicated a delayed influx of neutrophils (Ly6G+ cells) into the damaged area in mice lacking neuronal caspase 8 compared to controls (Figure 7C; *p = 0.03; **p = 0.02).

Caspase 8 deletion protects against seizure-induced brain injury caused by kainic acid (KA)

Systemic administration of excitotoxic glutamate receptor agonist KA induces seizures and subsequent neuronal damage [61,62]. We treated Ncaps8−/− and control mice with KA to investigate the role of caspase 8 in excitotoxicity-induced brain injury. Observation and scoring of convulsions revealed shorter mouse survival (Figure 8A) and greater severity of seizures (Figure 8B; *p = 0.008) in the control group compared to the Ncaps8−/− mice. To determine whether neuronal ablation of caspase 8 plays a protective role in KA-induced hippocampal cell death, the numbers of apoptotic and degenerating neurons were assessed by TUNEL assay (Figure 8C (p = 0.002), G, H) and Masson’s trichrome stain (Figure 8D (p = 0.005), E, F) in hippocampal regions. Neuronal cell death (particularly abundant in the CA3 region) was decreased by caspase 8 deletion, as assessed by both methods. Results of cleaved-caspase-3 immunostaining confirmed this observation (Figure 8I–K).
Caspase 8 Contributes to Acute Brain Injury
Figure 6. Assessment of lesion volume, cell death and immunoreactivity for MAP2, NeuN, and phospho-tau in control and Ncasp8−/− brains after CCI. (A1–A4) Masson’s trichrome-stained brain sections were digitalized using the Aperio scanning system. Digitized virtual slides were then analyzed by matching the brain tissue loss (cavitary lesion and lesion edges) (left) to intact brain tissue of the control (A1, A3) and Ncasp8−/− (A2, A4) mice. Scale bar = ~1000 µm on low and ~100 µm on high magnification images. Examples of nuclear NeuN (B–E) and the perikaryonal and dendritic MAP2 (F1–F4) immunostaining are presented in the coronal brain sections (F1, F2), cortical lesions and adjacent structures (B–E, F3, F4) of the control (B, C, F1, F3) and Ncasp8−/− (D, E, F2, F4) mice after CCI. Scale bar = ~1000 µm on low (F1, F2) and ~100 µm on higher magnification images (B–E, F3, F4). Examples of phospho-tau immunostaining in control (G, H) and Ncasp8−/− (I, J) brains are provided at low and high-power magnifications (scale bar = ~100 µm). Using Aperio software, percentage (K1, L1 and density (n/mm²) (K2, L2) of degenerating (K1, L1) and apoptotic (L1, L2) neurons was identified by Masson’s trichrome stain and cleaved caspase 3 immunostaining, respectively (mean ± SEM; n = 8–10 per group and per time point) in lesion areas of brains from CRES (white bar) and Ncasp8−/− (black bar) mice at 2 h, 6 h, 24 h, 48 h, and 3 weeks after trauma (K1: *p = <0.0001; **p = 0.0003; ***p = 0.02, K2: *p = 0.0001; **p = <0.0001; ***p = 0.05; ***p = 0.01; L1: *p = 0.02; L2: *p = 0.02; **p = 0.03). (M) SDS-PAGE immunoblot analysis of caspase 3 and cleaved PARP protein levels in mouse brain tissues. Brain samples from impact lesion and corresponding region in the contralateral hemisphere (CLH) of Ncasp8−/− mice (lanes 3, 6) were compared with those from CRES (lanes 1, 4) and casp8−/− (lanes 2, 5) animals. Lysates were normalized for total protein content (30 µg/lane) and analyzed by SDS-PAGE/immunoblotting using antibodies specific for caspase 3, cleaved PARP, or β-actin, using a multiple antigen detection method [39]. (N–X) Examples are provided of phospho-c-Jun immunostaining in ipsilateral semi-hemispheres of brains from the control (N–B) and Ncasp8−/− (S–X) mice (magnification scale bar = ~100 µm). Antibody detection was accomplished using polymer-based EnVision-HRP-enzyme conjugate (DakoCytomation) and diaminobenzidine (DAB) chromogen (brown); neurons were counterstained with hematoxylin (blue). Percentage (Y) and density (Z) of phospho-c-Jun immunopositive nuclei were determined in impact lesions from control (white bar; Ctrl) and Ncasp8−/− (black bar) animals at 6, 24, 48 h, and 21 days post CCI (mean ± SEM; n = 8–10 mice per group and per time point) (Y: *p = 0.03; Z: *p = 0.02; **p = 0.05).
Figure 7. Immunohistochemical assessment of mIgG extravasation, microglia activation, and neutrophil infiltration in post-traumatic mouse brains. (A) Percentage of mIgG positive immunostaining was determined at various times in lesion or corresponding ipsilateral areas of the control (Ctrl) and Ncasp8^{-/-} sham- or CCI-treated mice (8–10 mice per group) (mean±SEM; *p = 0.02; **p = 0.04). (B) Percentage of Iba1 immunopositive cells was compared in ipsilateral semihemispheres of the control vs Ncasp8^{-/-} sham- or CCI-treated mice (*p = 0.02). (C) Neutrophil density (n/mm²) was determined by Ly6G immunostaining and compared in ipsilateral (IL) and contralateral (CL) semihemispheres of the CRE3 (Ctrl) and Ncasp8^{-/-} mice at various times after CCI (*p = 0.03; **p = 0.02). Examples of digital (D, F, H, J) and mark-up (E, G, I, K) images of mIgG immunostaining are presented in brains from the control and Ncasp8^{-/-} mice 48 h (D–G) and 3 weeks (H–K) after CCI. Brown color (DAB) on digital images and red, orange and yellow pixels on mark-up images denote positive immunostaining (magnification scale bar = 100 μm). Digital (L, N, P, R) and mark-up (M, O, Q, S) images of the Iba1 (L–S) immunostainings are presented in the ipsilateral semihemispheres of the control (L, M, P, Q) and Ncasp8^{-/-} (N, O, R, S) mice after brain trauma. Magnification scale bar = 100 μm. Insets show 800–1,200 × magnification of activated microglia (P, R).

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Figure 8. Caspase 8 deficiency protects neurons in in vivo KA-induced seizure mouse model. (A) Mouse survival was monitored for 24 hours after exposure of control (n = 8) and Ncasp8−/− mice (n = 11) to KA. Non-linear x-axis corresponds to time following KA injection. (B) Seizure scores for control and Ncasp8−/− mice were determined every 15 min for 2 h and at longer intervals thereafter [40] [41]. Percentage of TUNEL-positive neurons (C) and density of degenerating neurons (Masson’s trichrome stain) (D) were assessed in the hippocampi of the control and Ncasp8−/− mice (mean ± SEM). P values result from Student’s t-test (B: *p = 0.008; C: *p = 0.002; D: *p = 0.005). Colorimetric intensity measurements were applied to Masson’s trichrome-stained control (E) and Ncasp8−/− (F) hippocampi to obtain density of degenerating neurons in the annotated area (mark-up images). Arrows indicate hippocampal CA3–CA4 sectors and the hilus (E) and the CA3 sector (F). Apoptotic neurons were visualized by TUNEL assay (G, H), and cleaved-caspase 3 immunostaining (I–K) as presented on virtual (I, K) and mark-up (G, H, J) images of the hippocampal regions of the control (G, I, J) and Ncasp8−/− (H, K) mice. Brown color (DAB) on digital images and red, orange and yellow pixels on mark-up images denote positive staining. The arrow indicates TUNEL positive neurons in the CA3–CA4 sector.

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caspase 8 using RNA interference (RNAi) in L929 cells [83,84], suggesting that the absence of caspase activity favors induction of autophagic cell death. Also lymphocytes in which caspase 8 was reduced by RNAi showed an increased tendency towards autophagy [83]. While associated with cell death, autophagy is often viewed as a survival mechanism during times of nutrient insufficiency and hypoxia (ATP depletion) (reviewed in [83]).

With respect to brain injury, autophagy was shown to be persistently activated after TBI, and was proposed to represent a protective mechanism [86]. Thus, if caspases oppose autophagy as previously suggested [87], then gene ablation of caspase 8 would be anticipated perhaps to promote autophagy, which might enhance neuronal survival in the setting of decreased ATP resulting from acute brain injury caused by TBI.

Following TBI and KA-induced seizures, we observed reduced apoptosis of neurons in brains of mice lacking neuronal caspase 8, as demonstrated by Masson’s trichrome and TUNEL stainings or cleaved caspase 3 immunostainings. Reduced lesion cavity volume in Ncasp8−/− mice subjected to CCI, as well as improved neuropathological scores, indicated that necrotic processes are less pronounced in brains from caspase 8 knockout mice compared to control animals, thus alleviating concerns about whether interfering with caspase 8 would promote increased brain damage by necrosis.

It is noteworthy that hundreds of measurements conducted in this study were done using image analysis algorithms. Unlike semiquantitative manual scoring, computer-based image analysis permits objective quantification on entire tissue sections or anatomical regions of interest.

In addition to in vitro models of brain injury, we have utilized in vivo models including primary cortical neuron cultures and organotypic brain slices to characterize effects of conditional deletion of caspase 8 on neuronal survival and death. Together, these results provide additional evidence of protective effect of neuronal deletion of caspase 8, corroborating the in vivo data. For undertaking these studies, we established an improved organotypic whole brain slice culture method using tissue from adult (4–8 month old) mice, wherein viable brain structures are maintained for up to 14 days, as evidenced by preserved morphological architecture, expression of neuronal markers (NeuN, β3-tubulin), and concomitant glial reactions. Gradually declining levels of MAP2 protein in brain slices indicate a time-dependent increase in neuronal vulnerability in these brain slice cultures and may be used to monitor viability of neurons in electrophysiological studies. Application of a polarity sensitive indicator for viability and apoptosis (pSIVA) facilitated characterization of neuronal cell death and survival in 14-day-old PNC. Thus, we developed a method of long-term whole brain slice cultures (BOSCS) using brain tissue derived from adult mice—a technique that has rarely been accomplished [88]. This model seems to be particularly advantageous in studies of genetically engineered mice.

In summary, the present findings establish a functionally important role in vivo for caspase 8 in neuronal cell death following acute brain injury, and encourage the development of therapeutics that target this protease as potential countermeasures for TBI and perhaps other causes of acute brain injury. It should be noted however that humans contain an additional caspase not produced in mice, namely caspase 10, that may be functionally redundant with caspase 8. Therefore, therapeutic strategies should also consider caspase 10, which is structurally and functionally highly similar to caspase 8 (reviewed in [89]), and thus likely to be sensitive to the same interventions that neutralize caspase 8.

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Author Contributions

Conceived and designed the experiments: SK MK JCR BH. Performed the experiments: MK ZY JR JY TK RL SB C-HS SK. Analyzed the data: MK ZY JR JY TK RL SB C-HS SK. Contributed reagents/materials/analysis tools: MW LS KH BH JCR SK. Wrote the paper: MK JCR SK.
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