RESEARCH ARTICLE

Entorhinal Cortex Lesion in the Mouse Induces Transsynaptic Death of Perforant Path Target Neurons

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Entorhinal cortex lesion (ECL) is a well described model of anterograde axonal degeneration, subsequent sprouting and reactive synaptogenesis in the hippocampus. Here, we show that such lesions induce transsynaptic degeneration of the target cells of the lesions pathway in the dentate gyrus. Peaking between 24 and 36 hours postlesion, dying neurons were labeled with DeOlmos silver-staining and antisera against activated caspase 3 (CCP32), a downstream inductor of programmed cell death. Within caspase 3-positive neurons, fragmented nuclei were co-localized using Hoechst 33342 staining. Chromatin condensation and nuclear fragmentation were also evident in semithin sections and at the ultrastructural level, where virtually all caspase 3-positive neurons showed these hallmarks of apoptosis. There is a well-described upregulation of the apoptosis-inducing CD95/L system within the CNS after trauma, yet a comparison of caspase 3-staining patterns between CD95 (lpr)- and CD95L (gld)-deficient with non-deficient mice (C57/bl6) provided no evidence for CD95L-mediated neuronal cell death in this setting. However, inhibition of NMDA receptors with MK-801 completely suppressed caspase 3 activation, pointing to glutamate neurotoxicity as the upstream inducer of the observed cell death. Thus, these data show that axonal injury in the CNS does not only damage the axotomized neurons themselves, but can also lethally affect their target cells, apparently by activating glutamate-mediated intracellular pathways of programmed cell death.

Brain Pathol 2004;14:249-257.

INTRODUCTION

Acute degeneration of fiber tracts within the central nervous system (CNS), eg, induced by traumatic brain injury (TBI) or spinal cord injury (SCI), induces anterograde (Wallerian) degeneration of the severed axons and often causes retrograde degeneration of the axotomized neurons depending upon several factors, including the proximity of the lesion to the cell body. In addition, there is also widespread secondary degeneration of neurons that were not directly injured, leading to additional neurological deficits (66). For example, cortical percussion induces delayed, secondary loss of neurons in the thalamus 2 weeks after the injury (21). Among the suspected causes of this secondary damage are ischemia-reperfusion, excitotoxicity, fluid-electrolyte disturbances, oxidative radicals, inflammatory/immunological processes, or a combination thereof (77).

Many TBI experiments employ fluid percussion or a solid impactor on the exposed dura, or a free-falling weight onto the closed skull. These models reproduce the damage found in human TBI patients to various extents (47); however, the neuroanatomic connectivity of vulnerable neurons in relation to the site of injury often remains ill-defined. Due to the complex damage, it is difficult to establish in these models whether individual dying cells were directly (mechanically) injured or undergoing secondary degeneration. This problem can be overcome by using the entorhinal cortex lesion model (ECL) (51). ECL induces a well-defined, acute degeneration of the glutamatergic perforant path, which arises in layers 2 and 3 of the entorhinal cortex and terminates at the distal segments of granule cell dendrites in the middle and outer molecular layers of the dentate gyrus. Due to this layer-specific termination, ECL allows the changes induced in the zones of axonal degeneration to be studied. Therefore, ECL has become a well-established model of investigating layer-specific sprouting (23, 24, 32, 51), transsynaptic dendritic alterations (56, 57) inflammatory responses (3, 6, 33, 40, 41), and retrograde neurodegeneration (7).

Programmed cell death is an often hypothesized cause of secondary degeneration after neurotrauma. Studies concerning the underlying mechanisms of this degeneration have shown that the apoptosis-inducing ligand CD95L (FasL) is upregulated after CNS injury, and that neurons can become susceptible to CD95L-induced apoptosis after brain lesions (4, 8, 28, 29, 52, 64). When CD95L (FasL) binds with the receptor CD95 (Fas), an intracellular pathway can be triggered, leading to the eventual activation of caspase 3. Once caspase 3 is activated, it begins cleaving proteins necessary to cell homeostasis, finally ending in programmed cell death (10). Caspase 3 has been found in neurons after TBI (9) and SCI (18) and seems to better correlate to dying neurons than TUNEL assays do (45). There are 2 strains of the C57 Black/6 (bl6) mice carrying naturally occurring mutations leading to deficient CD95 (lpr/bl6) and CD95L (gld/bl6) proteins. Therefore, these strains have been used to study the role of CD95L-mediated apoptosis under a variety of pathological conditions (55). If the CD95/CD95L pathway is critical for a given form of degeneration, a difference in the amount of dying cells and/or caspase 3 activation would be expected.

Another, CD95L-independent, pathway of neuronal programmed cell death is initiated by calcium-overload during excitotoxicity, leading to mitochondrial cytochrome c release and caspase activation

(14, 17, 49, 50, 73, 82). Such excitotoxic cell death leads to morphologic aspects of both necrosis and apoptosis; dying neurons observed under the electron microscope exhibit swollen mitochondria, the appearance of vacuoles in the endoplasmic reticulum, and the condensation and clumping of chromatin (14, 26, 30, 39, 59). The most common receptors involved in excitatory synaptic transmission in the central nervous system (CNS) are the AMPA and NMDA receptors, which are both activated primarily by glutamate. Overstimulation of neurons through the uncontrolled release of glutamate leads to the aforementioned intracellular calcium overload resulting in excitotoxicity.

In fact, by repetitively stimulating the glutamatergic perforant path, Sloviter et al (68) induced cell death of various neuronal populations in the hippocampus. Following ECL, hippocampal neurodegeneration has not yet been systematically studied. However, the upregulation of the "early gene" c-fos in the post synaptic granule cells after ECL can be blocked by MK-801, a non-competitive NMDA-receptor antagonist (56) suggesting that the lesion results in excessive glutamate levels. Thus, we speculated that lesioning a fiber tract within the CNS—besides retrograde degeneration of the axotomized cells (7)—may also lethally effect its target neurons due to glutamate-mediated secondary neuronal cell loss.

MATERIALS AND METHODS

Animals. A total of 12 gld, 8 lpr, and 70 C57/bl6 adult male (>6 weeks) mice were sacrificed. They were housed under standard laboratory conditions with free access to food and water. Gld/bl6 and lpr/bl6 mice were purchased from Jackson Labs (Bar Harbor, Me) and bred in the local animal facility. Gld and lpr mice are bl6, ie, the same strain as C57/bl6, which have spontaneously occurring point mutations in their CD95L and CD95 encoding genes respectively, resulting in the production of non-functioning protein (19, 31, 54, 55). Precautions were taken to minimize pain and discomfort to the animals in accordance with the animal experiment regulations of the state of Berlin.

Entorhinal cortex lesion. Stereotaxic surgery was performed under deep rompun/ketamin anesthesia. The left medial entorhinal cortex was lesioned using a 2 mm broad stainless steel blade. The medial edge of the knife was adjusted to the following coordinates as measured from the λ: anterior-posterior: 0.4 mm; lateral: 1.2 mm; dorsoventral: down to the base of the skull. No electricity was applied to the animals during or after the ECL. As controls, one C57 mouse received a lesion in the cerebellum, and another mouse remained unlesioned. Because improperly placed lesions in the brainstem and cerebellum failed to produce programmed cell death in the hippocampus, mice with improperly placed lesions were removed from the study at this time.

MK-801-treatment. The NMDA receptor-specific, non-competitive antagonist (+)-MK-801 (Biotrend, Cologne, Germany) was dissolved in 0.1 M phosphate buffer (PB). The solution was injected intraperitonially 30 minutes before lesioning, and every 12 hours thereafter, ie, at 12 and 24 hpl (hours post lesion) until sacrifice at 36 hpl, the time at which we found the highest number of neurons immune positive for activated caspase 3 in untreated animals. Four C57 mice received a solution with a concentration of 1 mg/kg body weight, and eight C57 mice received a solution with a concentration of 2 mg/kg body weight. The former dose is sufficient to prevent lesion-induced c-fos expression and loss of dendritic arborization after ECL (56). In addition, because MK-801 has a variety of adverse side-effects (13, 48, 60) MK-801 was given to 2 mice that were not lesioned in order to see if MK-801 on its own acutely affects neurons. To determine whether MK-801 prevents acute degeneration or merely prolongs neuronal survival time after an irreversible lethal insult ultimately ending in apoptosis, 4 groups of 5 mice each received 2 mg/kg body weight MK-801 at the time of lesioning then at 12 and 24 hpl. Group 1 was sacrificed at 36 hpl; group 2 at 48 hpl, group 3 at 60, and group 4 at 72 hpl.

Immunocytochemistry. In an initial survey of the effects of ECL, at least two C57 mice were sacrificed every 12 hours post lesion, beginning at 12 hpl and continuing to 96 hpl. The mice were deeply anesthetized with an overdose of rompun/ketamin and transcardially perfused with 100 ml of 0.9% NaCl, followed by 200 ml of 4% paraformaldehyde in 0.1 M PB (pH 7.4). The brains were then removed and allowed to postfix for several hours before they were cut into 50-µm horizontal or frontal slices on a vibratome.

Free-floating sections were blocked for endogenous peroxidase by incubation with 3% H_20_2 for 5 minutes. The slices were then washed 3 times in PB and incubated for 10 minutes in PB containing 10% goat serum in order to block non-specific binding of antibodies. Polyclonal antibodies specific for activated caspase 3 (1:1000; R+D Systems, Wiesbaden, Germany) and parvalbumin (1:5000; Swant Bellinzona, Switzerland) were diluted in a buffer containing 0.5% Triton and 1% normal goat serum. Sections were incubated in this antibody solution overnight (ca. 12 hours) at 4°C. After the sections were washed 3 times, they were incubated at room temperature for 90 minutes in a solution of biotinylated goat-anti-rabbit antibody 1: 250 (Vector Burlingame, Calif), 0.5% Triton-X 100, and 1% normal goat serum. After washing, the slices were incubated in an avidin-biotin complex (ABC-Elite, Vector) for 45 minutes, then developed using 3,3´-diaminobenzidine (DAB) as chromogen.

Hoechst staining. To visualize the nuclei of activated caspase 3-positive cells, Hoechst 33342 (Molecular Probes, Eugene, Ore) counterstaining was performed. Thirty-six-hpl sections previously stained for activated caspase 3 with DAB were incubated for 20 minutes on the microscope slide with Hoechst 33342 (10 mg/L) in PB. The slides were then rinsed 3 times with PB and coverslipped.

DeOlmos cupric silver staining. Using a protocol first established by DeOlmos and Ingram (25), slices from lesioned mice sacrificed at 24, 36, and 48 hpl were stained. Briefly, brains were post-fixed for 4 days after perfusion, cut into 50-µm slices, then incubated in a silver-cupric solution in the dark. Forty-eight hours later, the slices were developed in a citric acid solution, bleached with potassium ferricyanide, and stabilized with sodium thiosulfate. Degenerating cells had a distinct dark appearance due to the silver impregnation.

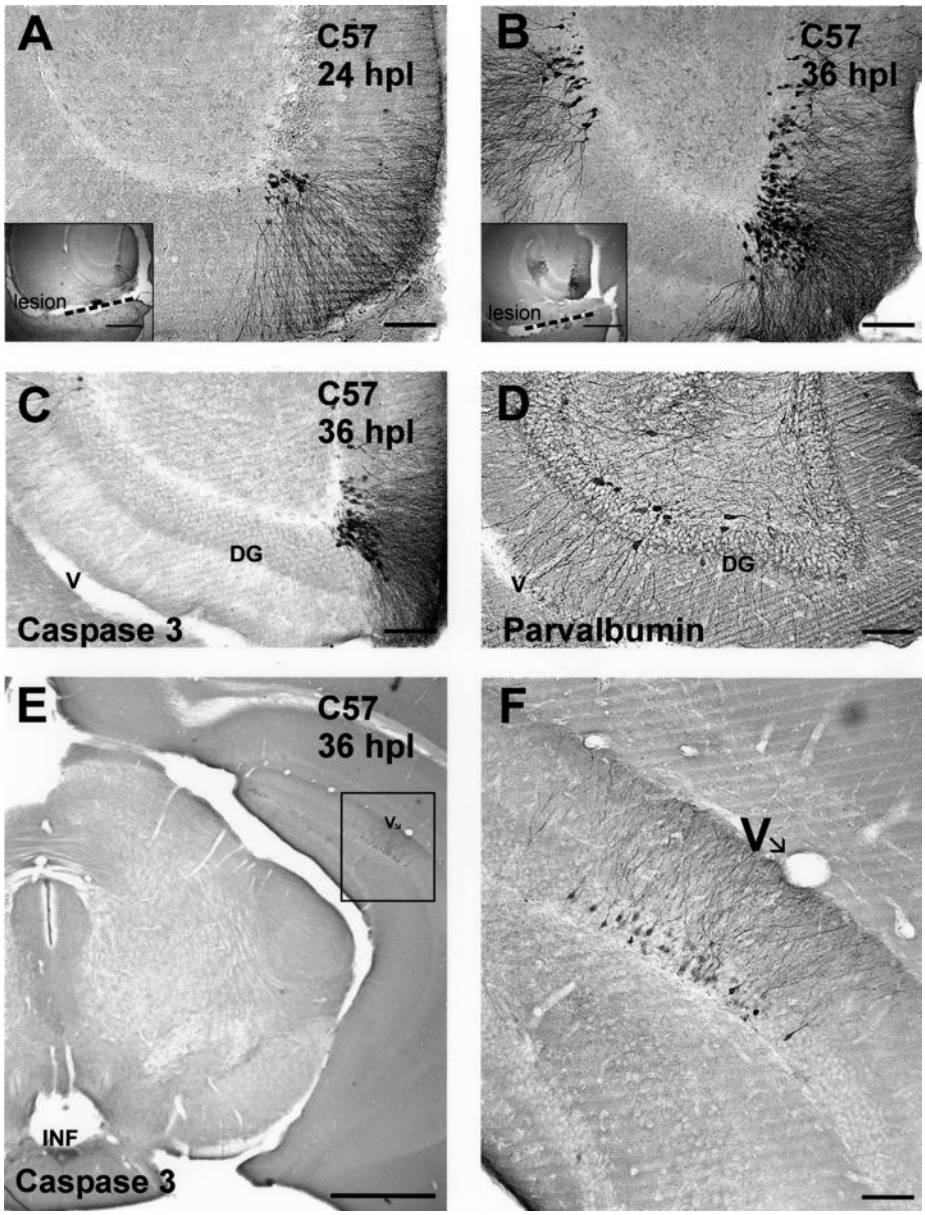

Figure 1. A-B. Horizontal sections of the dentate gyrus at 24 (**A**) and 36 (**B**) hpl (magnification: 100×, scale bar: 80 µm) immunohistochemically stained for activated caspase 3 using DAB as a chromogen. The inserts show the placement of the respective lesion (magnification: 40×, scale bar: 500 µm, dotted lines indicate the lesion site). There is an evident increase in the amount of capsase 3 positive cells at 36 hpl when compared to 24 hpl. Note the grouping of the cells in clusters, a consistent observation. **C-D**. "Mirror technique": the staining of subsequent cross sections against caspase 3 (**C**) and parvalbumin (**D**) revealed no overlap, implying 2 distinct populations of neurons. DG = dentate gyrus; V = blood vessel (magnification: 100×, scale bar: 80 µm). **E-F**. Caspase 3 activity can be detected throughout the longitudinal axis of the hippocampus. These figures show caspase 3 positive neurons approximately 3.5 mm rostral of the entorhinal cortex lesion. The frame in **E** (magnification: 40×, scale bar: 1000 µm) is shown at a higher magnification in **F** (magnification: 100×, scale bar: 100 µm). V = blood vessel; INF = Infundibulum.

Light and electron microscopy. Light microscopy was performed on an Olympus BX-50 microscope (Olympus, Hamburg, Germany). Pictures were taken with a Magnafire digital camera (Intas, Goettingen, Germany). Electron microscopy was performed on a Zeiss EM 900 (Jena, Germany). For ultrastructural analysis, the perfusion solution contained 4% paraformaldehyde plus 1% glutaraldehyde. The tissue was immunostained as described above, but the dilution buffer did not contain Triton. The tissue was then embedded in epon and ultrathin sections were cut on an ultratome. In addition, semithin sections from mouse hippocampi obtained 36 hpl were cut and counterstained with toluidine blue to detect morphological hallmarks of apoptosis/necrosis at the light microscopic level.

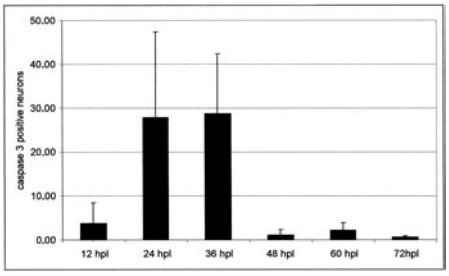

Figure 2. *Time course of transsynaptic apoptosis.* The number of degenerating caspase 3-positive neurons is shown in twelve-hour intervals. There is a clear peak at 24 to 36 hpl. At earlier and later times, only single caspase 3-positive cells could be found.

Quantification of apoptotic neurons. In order to determine the difference, if any, between wild-type (C57) mice and their CD95/L (lpr/gld)-deficient counterparts, the rate of apoptosis, the effect of MK-801, and the number of caspase 3-positive cells was quantified. Several (6-8) horizontal sections from each animal in the respective group were selected using the ventricles and basal ganglia as landmarks to indicate the level of the section. This insured that no discrepancies would arise due to possible differences in the number of affected cells in different levels of the hippocampus. The affected hippocampus of each section was photographed as above, printed and encoded, then given to a blinded counter familiar with CNS histology. The resultant raw scores were then decoded and the mean and standard deviation determined using Microsoft Excel. A t-test was performed in order to determine the statistical significance.

RESULTS

Slices were initially obtained from C57 mice at the following times: 12, 24, 36, 48, 60, 72, and 96 hpl. Caspase 3 positive neurons were detected from 24 to 48 hpl with a clear peak at 24 to 36 hpl (Figures 1A, B, 2). Staining sections from the mouse with the sham lesion in the cerebellum and the unlesioned mouse also failed to reveal caspase 3-positive cells in the hippocampus. At the peak time, the staining pattern varied from zero up to 185 caspase 3-positive neurons in the dentate gyrus of any individual section. In most sections, the immune positive cells built groups of 25 to 40 neurons consistently, but not ex-

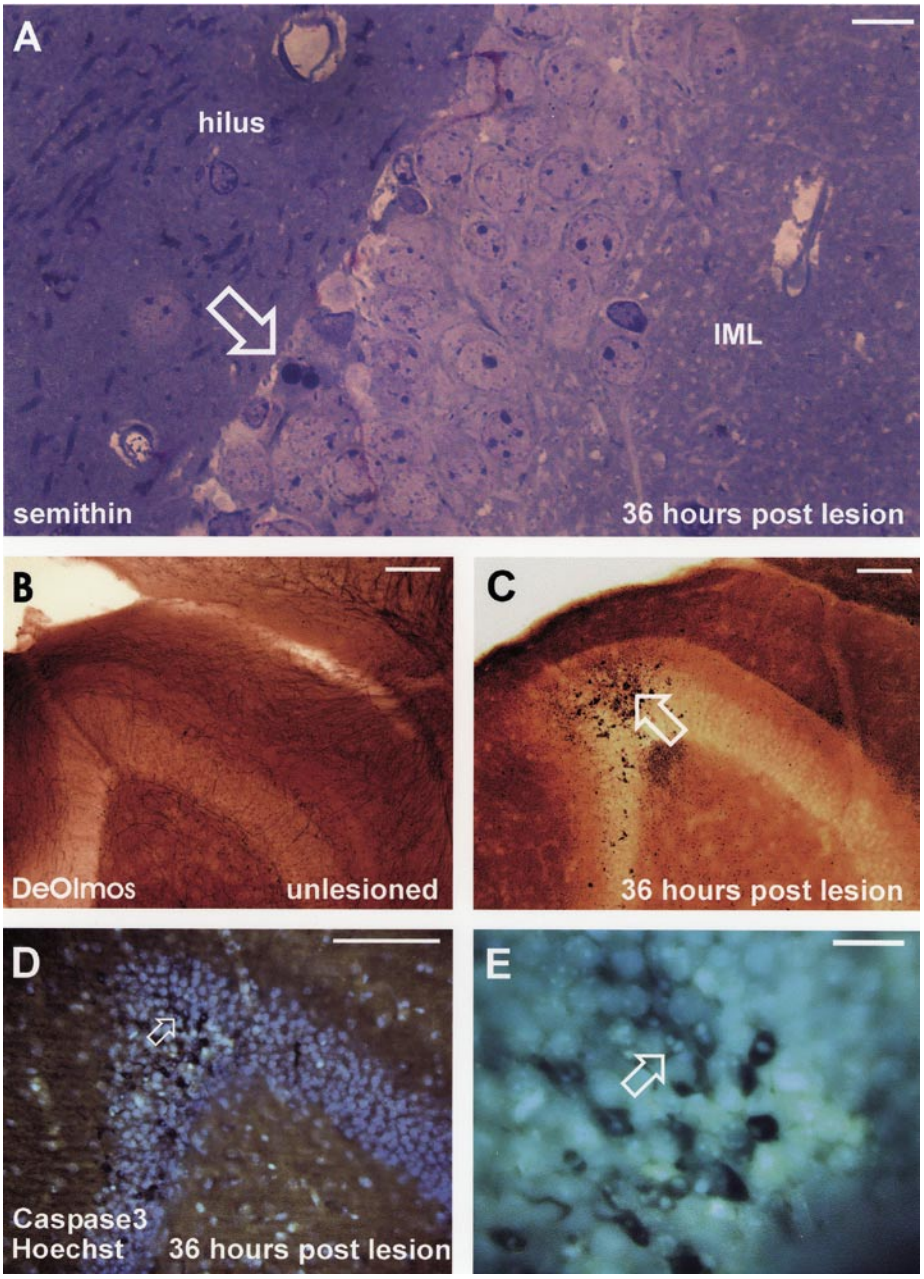

Figure 3. A. Semithin slices stained with toludine blue showed neurons with condensed chomatin and a fragmented nucleus (open arrow), while the surrounding neurons appear to be intact. IML = inner molecular layer (magnification: 1000×, scale bar: 10 µm). **B-C.** DeOlmos silver stainings show the entorhinal fibers reaching their target area in the hippocampus. No degenerating cells are observed in unlesioned animals (**B**). At 36 hpl after ECL (**C**) the distribution of degenerating neurons (open arrow) is similar to that found in sections stained for caspase 3 (magnification: 100×, scale bar: 80 µm). **D-E.** Counterstaining of caspase 3-positive cells with Hoechst 33342 at 36 hpl revealed condensed chromatin and fragmented nuclei (open arrow) within caspase 3-positive neurons, especially when compared to neighboring, unaffected granule cells with diffuse chromatin (magnification: 400×, scale bar: 150 µm). **E** shows the same neurons in picture **D** at a higher magnification (1000×, scale bar: 20 µm).

clusively, located in the crest of the dentate gyrus (Figure 1B, C). Clusters of neurons could be found in both blades of the hippocampus, from dorsal to ventral, with no identifiable variation due to latitude. Caspase 3-positive neurons were exclusively located within the granule cell layer, but not in the CA1 region, which also receives glutamatergic input from the entorhinal cortex. The morphology of the caspase 3-positive cells in the dentate gyrus resembled that of granule cells. In fact, comparing the distribution of parvalbumin-positive GABAergic interneurons and caspase 3-positive cells showed no overlap (Figure 1C, D). To exclude that the observed restriction of cell death to the granule cell layer is due to their topographic vicinity to the lesion site, frontal sections of the rostral pole of the hippocampus were stained. In these frontal slices a similar distribution of caspase 3-positive neurons were found throughout the longitudinal axis of the hippocampus (Figure 1E, F).

Although it is clear that caspase 3-induced programmed cell death plays a major role in the brain's morphogenesis (46), there are conflicting opinions over the specificity of caspase 3 in detecting neuronal apoptosis (65, see discussion). Caspase 3 immunostaining was therefore correlated with other methods of detecting and classifying the observed neuronal cell death. Morphological criteria for programmed cell death include: condensed chromatin, a fragmented cell nucleus, and finally, the formation of apoptotic bodies (43). Semithin slices stained with toludine blue revealed cells with clearly condensed DNA and fragmented nuclei (Figure 3A). For screening purposes, DeOlmos silver staining, an established method of detecting neurodegeneration (25), was employed. This technique also revealed degenerated cell fragments in the dentate granule cell layer of animals peaking at 36 hpl, but not in unlesioned controls (Figure 3B, C). The distribution of these cells resembled that observed in sections stained for activated caspase 3 (compare Figure 1B and Figure 3C). In order to co-localize condensed chromatin and fragmented nuclei within caspase 3 immunolabeled cells, previously immunostained slices were counterstained with the fluorescent, DNA-binding dye, Hoechst 33342. This combination unequivocally revealed that caspase 3-positive neurons contained fragmented nuclei (Figure 3D, E).

Using electron microscopy, condensed chromatin was observed in neurons that exhibited predominantly cytoplasmic activated caspase 3 (Figure 4A). Also observable were neurons exhibiting cytoplasmic and nuclear caspase 3, most likely representing a later phase of cell death (Figure 4B). In both stages, the neurons contained round, swollen mitochondria typical of both excitotoxicity (14, 39) and apoptosis (69) (Figure 4A, B). As anticipated from light microscopy, neurons were densely filled with activated caspase 3 DAB-precipitate (14). Thus, immune staining for

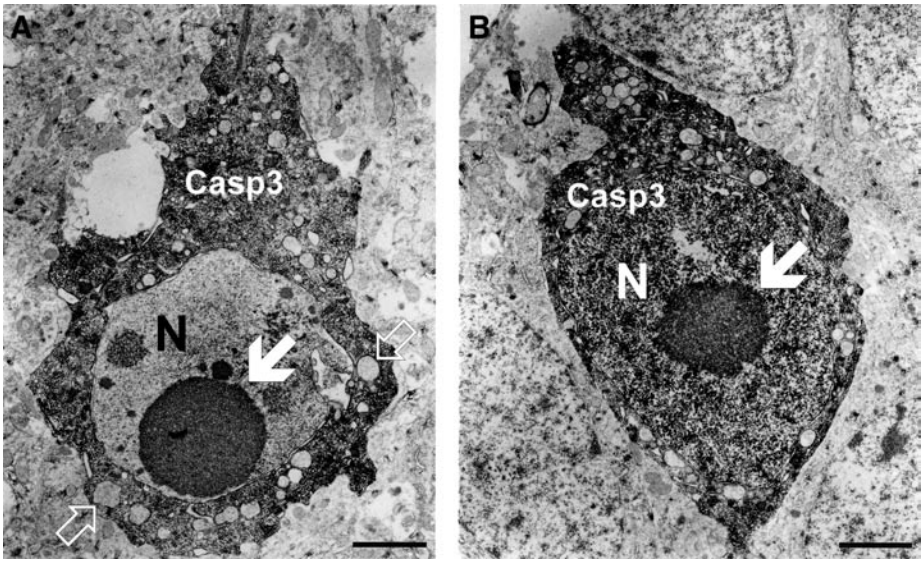

Figure 4. A-B. Electron microscopy of DAB stained slices from mice sacrificed at 36 hpl reveals neurons with caspase 3 immunoprecipitate, condensed chromatin (white arrows), and round swollen mitochondria (white open arrows). **A** shows a neuron in which the nucleus is still free of immunoprecipitate, whereas in **B** the immunoprecipitate is evenly distributed throughout the neuron, including the nucleus. N = nucleus. (magnification: 7000×; scale bar: 2 µm).

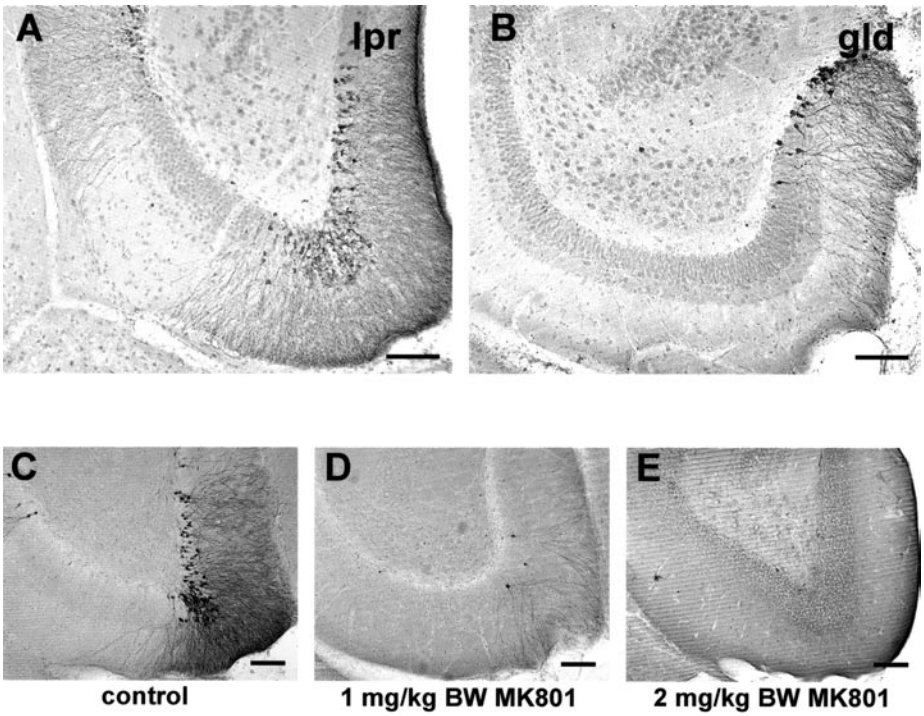

Figure 5. A-B. Both the CD95 deficient lpr (**A**) and CD95L deficient gld (**B**) mouse exhibit caspase 3-positive neurons 36 hours after lesioning, ruling out a crucial role for the CD95/CD95L system in the observed caspase 3 activation (magnification: 100×, scale bars: 80 µm). **C-E**. Application of MK-801 (one mg/kg body weight) to wild-type (C57/bl6) mice at the time of ECL and every 12 hours thereafter clearly reduces the amount of caspase 3-positive neurons 36 hpl when compared to untreated mice (**C** versus **D**), implying an excitotoxic cause of cell death. This protective effect is almost complete at a dosage of 2 mg/kg body weight (**E**) (magnification: 100×, scale bars: 80 µm). BW = body weight.

activated caspase 3 after ECL indeed labels dying neurons (9, 18, 49, 53), which ultrastructurally show morphologic characteristics of both apoptotic and excitotoxic degeneration.

To determine if the observed neurodegeneration was CD95/CD95L-mediated, gld (CD95L-deficient) and lpr (CD95-deficient) mice received entorhinal lesions and were sacrificed thereafter at 12, 24, 36, 48, 72 hpl. As previously mentioned, these

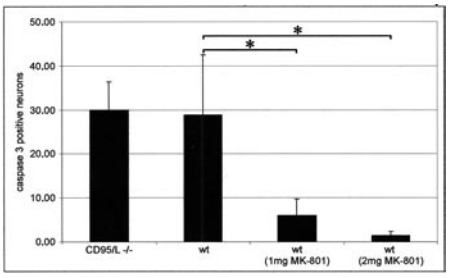

Figure 6. *Effect of CD95/L deficiency and MK-801 at 36 hpl*. There was neither an observable nor a statistical difference in the numbers of caspase 3-positive neurons between CD95/L-deficient animals and wild-type (wt) C57 mice, ruling out the CD95/L system as playing an important role in the observed neurodegeneration. Treatment with MK-801 significantly reduced the number of caspase 3-positive neurons ($p<0.01$) at dosages of one and 2 mg/kg body weight.

mice are based on the Black/6 (bl6) strain, ie, the same strain as the C57 mice used in this study. Caspase 3-positive neurons were also detected in these strains in comparable numbers and at similar locations and times, ie, most positive neurons were detected at 36 hpl (Figures 5A, B, 6). Thus, the CD95/CD95L-system is either not crucially involved in the observed degeneration or can be substituted by alternate mechanisms.

To examine whether the neuronal degeneration after ECL is mediated via NMDA receptors, mice were treated intrapertonially with (+)- MK-801 30 minutes before, and every 12 hours thereafter until sacrifice. At a dose of one mg/kg body weight MK-801 per injection this treatment clearly reduced the number of caspase 3-positive neurons such that only individual cells could be found in some sections at 36 hours (Figure 5D). At a dose of 2 mg/kg body weight per injection, this protective effect was almost complete and only single cells were found, while most sections were devoid of neurons exhibiting activated caspase 3 (Figures 5C-E, 6). Thus, blockade of NMDA receptors protects from transsynaptic neurodegeneration after ECL. To determine whether this protective effect is permanent or the cell death is merely delayed, 4 groups of 5 animals each were treated as above with 2 mg/kg body weight of MK-801 and sacrificed at 36, 48, 60, and 72 hpl. Individual dying neurons could be observed at 36 and 48 hpl, but there was no delay in neuronal cell death, ie, no clusters of caspase 3-positive neurons could be found in the hippocampus at later times (Figure 7). Thus, it appears that there is a limited time frame of susceptibility to

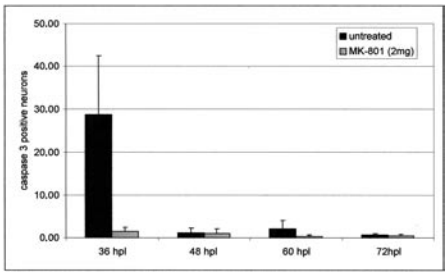

Figure 7. *Effect of MK-801 treatment over time.* The black bars show untreated, wild-type (wt) (C57) mice and the gray bars show wt mice treated with 2 mg/kg MK-801. MK-801 was applied at the time of lesioning and at 12 and 24 hours post lesion (hpl). This treatment did not delay the onset of transsynaptic apoptosis (Figure 2), but diminished this neurodegeneration.

excitotoxic apoptosis following lesion and that antagonizing NMDA receptors during this time has long-lasting neuroprotective effects.

DISCUSSION

Besides degeneration of directly damaged pericarya and axotomized neurons, widespread secondary neurodegeneration occurs in the CNS after trauma. To better understand why particular neurons are prone to die after mechanical injury, whereas others -often in closer proximity to the lesion site- survive, one approach is to define the neuroanatomic relationship of dying cells, not only to the lesion site itself, but also to the directly degenerating structures. Using the ECL-model, which allows clear-cut differentiation of anterograde, retrograde, and transsynaptic changes induced by axonal injury, we describe that a subpopulation of target cells die in response to degeneration of their input, regardless of their proximity to the lesion site (Figure 1 E, F). The principle of acute transsynaptic degeneration may be applicable to other forms of injury to the CNS.

The observed CD95/CD95L-independent activation of caspase 3 and subsequent cell death occurs within 48 hours after lesion. This time pattern clearly differs from previously described target cell degeneration in the medial mammillary nucleus after fimbria-fornix transsection in rats, which takes place weeks after lesioning (35), and from degeneration of CA 3 neurons, which is detectable several month after ECL in monkeys (61). Since the acute induction of caspase 3 and cell death described herein can be significantly reduced with MK-801, it seems that the acute release of glutamate from injured fibers induces a form of excitotoxic cell death.

The use of MK-801 is not without complication because of its well known side effects, which include hypothermia (13), hypertonus and tachycardia (48), psychosis and neuronal death (60). We nevertheless decided to apply this compound, since permeability of the blood-brain barrier of more specific NMDA receptor antagonists such as AP-5 and CPP is unclear, and their half life is short when compared to MK-801 (16), rendering treatment over several days difficult. With MK-801, we had previously established a well-tolerated dosage regime for successfully protecting denervated dendrites from degeneration following ECL in adult rats (56). Thus, since our aim was not to provide a therapeutic approach, we weight-adjusted this regime to adult mice in order to test whether the observed transsynaptic apoptosis was excitotoxic. The clear reduction in neurodegeneration following MK-801 treatment together with the morphology of dying neurons argue for this possibilty, although we cannot rule out that the neuroprotection by MK-801 is a result of hypothermia rather than NMDA blockade.

Indeed, glutamate-mediated excitotoxicity has been suggested as an important mechanism of secondary degeneration in response to CNS trauma (12, 57) and several studies have linked the activation of caspase 3 after brain and spinal cord injury to exitoxicity (9, 14, 18, 49, 63, 73, 81). Swollen mitochondria, which were prominent in dying neurons, are typical of excitotoxicity and apoptosis (39, 50, 59, 79). The release of cytochrome c from damaged mitochondria allows the formation of a caspase 3 activating complex consisting of cytochrome c, APAF-1 and caspase 9 (78, 81). This post-mitochondrial activation of caspase 3, in turn, can complete the amplification loop by processing cytoplasmic pro-caspase 8 (72, 80). The presented data supports such a link between axonal injury and excitotoxic caspase-3 activation to be relevant for secondary degeneration in vivo. Future studies, in which the individual intracellular signals are blocked after ECL, may help to further unravel how NMDA signaling leads to programmed cell death in vivo.

Neurodegeneration after ECL affected a subset of granule cells in the dentate gyrus, while the vast majority of the neighboring population did not exhibit signs of degeneration. Within the investigated time frame (12-96 hpl), degeneration of CA3 pyramidal cells, which are also innervated by the perforant path, could not be detected. The susceptibility of a given neuron may depend on features such as glutamatergic synaptic density, differential expression of glutamate receptor subtypes and calcium-binding proteins, or lack of inhibition by interneurons. One possibility is that interneurons undergo rapid apoptosis or cease to function following lesioning. Theoretically, the failure of caspase 3 and parvalbumin to co-localize could be due to a rapid catabolism of this protein and a failure to replace it by the damaged and nonfunctioning interneurons. The death of the interneurons, or their ceasing to function, may explain why most dying neurons were found in groups rather than being equally distributed within the granule cell layer. In fact, both direct (ie, excessive glutamatergic stimulation) and indirect (ie, insufficient GABAergic inhibition) mechanisms of excitotoxicity participate in the pathological cascade in the course of disorders such as Alzheimer disease, chronic alcoholism, and fetal alcohol syndrome (27, 37, 38, 60). However, at present we are unable to detect degeneration of parvalbumin-positive interneurons and to explain the localization of the degenerating cells in groups throughout the hippocampus. The identification of the exact mechanisms responsible for differential susceptibility of target neurons to degenerate following deafferentiation may be further explored using the ECL model.

Upon identification of NMDA excitation as the upstream signal inducing caspase 3 activation, the morphological aspects of the degenerating neurons were studied. Glutamate toxicity was reflected by swollen neurons, while caspase 3-positive neurons at the light and ultrastructural level revealed chromatin condensation and nuclear fragmentation as hallmarks of apoptosis. Importantly, "programmed cell death" and "apoptosis" are not synonymous. Programmed cell death requires gene expression and induction of intracellular suicide pathways leading to self-digestion of proteins and cleavage of DNA. The morphology of such dying cells do not always fulfill the hallmarks of apoptosis originally described in hepatocytes (43, 44), but can

exhibit necrotic or untypical morphologies (69, 75). Such "mixed morphologies" have been reported for several forms of neurodegeneration (20, 22, 70). Thus, necrotic morphologies of dying neurons described under various conditions (20, 30) do not allow the conclusion that their death was non-programmed. From a therapeutical point of view, it is moreover important to consider that blocking apoptosis signals can produce predominantly necrotic events instead of preventing the death of cells itself (75). Since apoptosis evokes little or no inflammation, and even can suppress immune responses (71), such inhibition may enhance immune responses with uncertain outcome in regard to the final damage (5, 62, 67). However, blocking programmed cell death can reduce neuronal loss under a variety of pathological conditions (2, 11, 34, 67, 76, 77). It will now be interesting to study whether caspase inhibition blocks this excitotoxic cell death, or how such treatment modifies an inevitable degeneration.

The obvious cell loss in the DG has not been preceded by reports of lower cell counts of neuronal density in the DG after ECL. One explanation may be the replacement of the dying neurons. In fact, there is evidence for neurogenesis after ECL (15) and also for integration of newly generated neurons into the hippocampal circuit (74). Hippocampal neural precursors have been described within the hilus at the border of the DG (42). These precursors proliferate throughout life and seem to be capable of replacing cells lost after mechanical and excitotoxic lesion (36) and after occlusive ischemia. Interestingly, this post-ischemia proliferation of precursor cells can be inhibited by MK-801 (1). If precursor cells are activated by the excitotoxic death of their neighbors, or directly sense toxic glutamate levels via NMDA receptors, ECL may become an important tool for the in vivo study of transneuronal degeneration and stem cell activation mechanisms.

ACKNOWLEDGMENTS

The authors would like to thank Prof Hrissanthi Ikonomidou for her assistance in performing the silver staining. We also gratefully acknowledge the excellent technical assistance of Gisela Duwe, Dore Wachenschwanz, Sabine Lewandoski, and Jacqueline Mahlo. A.D.K. was a recipient of a research scholarship from the Graduiertenkolleg (GRK 238). This study was supported by the DFG (Be 2272/1-2, SFB 269, He 150/2-1) and the Forschungskommission der Charité.

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