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DISTINCT CASPASE PATHWAYS MEDIATE NECROSIS AND APOPTOSIS IN SUBPOPULATIONS OF HIPPOCAMPAL NEURONS AFTER STATUS EPILEPTICUS

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Summary

Status epilepticus in the immature brain induces neuronal injury in the hippocampal formation, but the mode and mechanism of death are poorly understood. Our laboratory have recently investigated the role of caspase-3, -8 and -9 in neuronal injury, using a lithium-pilocarpine model of status epilepticus in 2-week-old rat pups. Our results showed that dying neurons in the dentate gyrus and CA1-subiculum area do not share the same mechanism of death. In CA1-subiculum, caspase-8 upregulation preceded caspase-3 activation in morphologically necrotic neurons. The pan-caspase inhibitor Q-VD-OPH reduced CA1 damage, showing that caspases contribute to status epilepticus-induced necrosis. In the dentate gyrus, dying neurons were caspase-9 and -3 immunoreactive and morphologically apoptotic. It is not clear why the same seizures cause different types of cell death in neurons which are connected in series along the same hippocampal circuit, but the apoptotic dentate neurons express doublecortin, and do not express calbindin-D28k, suggesting that their immaturity may be a factor in producing an apoptotic mode of death.

Keywords

Pilocarpine; hippocampus; development; injury

Status epilepticus induces neuronal death in the immature brain

Experimental status epilepticus (SE) induces neuronal death in the developing brain (Thompson and Wasterlain, 1997, 1998; Sankar et al., 1998; Tan et al, 2002; Kubova et al, 2001). In our lithium-pilocarpine model of SE in 2-week-old rat pups, neuronal damage is extensive in the CA1 pyramidal cell layer and moderate in the inner granule cell layer of the dentate gyrus. Light and electron microscopic (EM) studies showed that these 2 neuronal populations die by different modes of death (Sankar et al, 1998; Niquet et al, 2007; Lopez-Meraz et al, in press). By hematoxylin-eosin staining, 24–72 h following SE, CA1-

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subiculum injured neurons are characterized by eosinophilic cytosol and pyknotic nucleus, feature suggestive of necrosis (Fig. 1C). By contrast, in the dentate gyrus from the same animals, eosinophilic cells displayed fragmented nuclei 7-72 h following SE. A semi-quantitative analysis by EM showed that necrosis is the widespread form of death in CA1-subiculum 6-72 h following SE (Niquet et al, 2007). They displayed early endoplasmic reticulum and mitochondrial swelling at 6 h (Fig. 1E), followed at 24 h by gross cytoplasmic and organelle swelling, loss of plasma membrane and a tigroid nucleus, a clearly necrotic morphology (Fig. 1F). By contrast, dentate gyrus injured neurons display an apoptotic morphology with large rounded chromatin clumps, loss of nuclear membrane and preservation of the plasma membrane (Fig. 2).

SE induces caspase-3 activation in morphologically necrotic CA1 neurons

Progress in understanding the mechanism of SE-induced CA1 necrosis has been made recently (Niquet et al, 2007; Lopez-Meraz et al, in press). Traditionally, necrosis is considered a passive form of death that does not require the activation of proteolytic cascades as caspases. However, our immunohistochemical studies suggest that the extrinsic pathway, through initiator caspase-8-dependent activation of “cell executioner” caspase-3, contributes to SE-induced neuronal necrosis (table 1). We reported a massive upregulation of initiator caspase-8 immunoreactivity (IR) in CA1-subiculum 7 h following SE (Fig. 2), at a time CA1-subiculum pyramids display organelle swelling, an early stage of necrosis (Niquet et al, 2007). This was followed by an increase of active caspase-3 (caspase-3a) IR 24-72 h following SE. Light and EM studies confirmed that most caspase-3a-positive CA1 neurons are morphologically necrotic. At all times studied (24-72 h following SE), caspase-3a staining is restricted to the nucleus. By double-labeling with the chromatin dye Hoechst 33342, most caspase-3a-positive neurons in CA1-subiculum have pyknotic nuclei (98.2 % at 24 h), a feature suggestive of necrosis (Fig. 1B & 2). A caspase activation assay on unfixed frozen sections confirmed the activation of caspase-3 in pyknotic nuclei from CA1-subiculum injured neurons. By EM, most caspase-3a-immunoreactive (IR) CA1-subiculum neurons (48 out of 50) had a necrotic morphology characterized by cytoplasmic and mitochondrial swelling, and pyknosis of the nucleus (Lopez-Meraz et al, in press). Notably, only a small but significant increase of initiator active caspase-9 IR was detected 72 h following SE, suggesting that the intrinsic pathway does not significantly contribute to SE-induced CA1-subiculum necrosis.

Is caspase activation during SE-induced neuronal necrosis an epiphenomenon?

These histological studies raised the possibilities that CA1 injured neurons die by a caspase-dependent, active form of necrosis. Alternatively, caspase activation may have been “intended” to trigger a classical form of apoptosis that has been prematurely aborted. If the latter is true, caspases are accidentally activated during necrosis and their blockade would not interfere with the final necrotic process. Pharmacological inhibition of caspase showed that it is not the case (Lopez-Meraz et al, in press). Pretreatment of animals with the pancaspase inhibitor Q-VD-OPH reduced neuronal injury in CA1-subiculum by 44% ($p < 0.01$; Student's t test vs vehicle), suggesting that caspases do contribute to neuronal necrosis. This is in agreement with our previous studies in primary neuronal cultures showing that caspase pathways contribute to excitotoxic neuronal necrosis (Niquet et al., 2003; Seo et al., 2009). We called this active form of death “programmed necrosis”. This may have important therapeutic implications. In the late stage of necrosis, the rupture of plasma membrane may lead to the release of cytoplasmic material and to an inflammatory response causing secondary necrosis. Treatment with a pan-caspase caspase inhibitor may block “programmed necrosis” and reduce late secondary necrosis.

SE activates distinct caspase pathways in CA1-subiculum and the dentate gyrus

While the extrinsic pathway may contribute to CA1-subiculum necrosis, our immunohistochemical studies suggest that the intrinsic pathway plays a significant role in SE-induced dentate gyrus apoptosis (Lopez-Meraz et al, in press). Nearly 80% and one-third of apoptotic neurons express caspase-3a 7 and 24 h following SE. Nearly 70% of them are active caspase-9-IR 7-72 h following SE. Caspase-8 IR is not significantly increased in the dentate gyrus after SE (table 1). By light microscopy, a majority of caspase-3a- or caspase-9a-IR cells have large round clumps of chromatin or display chromatin margination, both features are suggestive of apoptosis (Fig. 2).

Does the level of cell maturity determine the mode and mechanism of neuronal injury?

Our studies suggest that, in cells which are aligned in series as parts of the same excitatory hippocampal circuit, the same seizures induce neuronal death through different mechanisms in the postnatal brain. The reason for this difference is uncertain. Degree of cell maturity may be a factor. In the highly differentiated CA1-subiculum pyramidal neurons, upregulation of caspase-8, but not caspase-9 precedes caspase-3 activation and necrosis, the main form of SE-induced neuronal death in the adult brain (Fujikawa et al, 2000). In the dentate gyrus, caspase-9 and -3 activation, but not caspase-8, was observed in apoptotic neurons. While pyramidal neurons differentiate during fetal life, most dentate gyrus granule cells are generated post-natally and some cells remain immature throughout life (Gage et al., 1998). In our model of SE in the immature brain, eosinophilic, caspase-3a-IR neurons were localized to inner granule cell layer, which is the last layer in the dentate gyrus to mature. Accordingly, we found that all caspase-3a-IR neurons were doublecortin-IR, a feature of immature neurons (Fig. 2), and did not express calbindin-D28k, a marker of mature dentate gyrus granule cells (Lopez-Meraz et al, in press). Interestingly, the developing brain has been considered more susceptible to develop apoptosis than its adult counterpart after brain injury (Martin et al., 1998). This phenomenon is associated with an age-dependent downregulation of cell death factors involved in apoptosis, such as caspases (Shimohama et al., 2001; Yakovlev et al., 2001). From our study, we may speculate that the level of an individual's neuron maturation may be as important as the level of maturation of the whole organism in determining neuronal vulnerability to seizures.

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Figure 1.

(**A-C**) Images of caspase-3a (green) and NeuN (neuronal marker, red) immunoreactivity (**A**), Hoechst staining (chromatin dye, blue in **B**) and hematoxylin-eosin staining (**C**) in the subiculum 24 h following SE. Caspase-3a staining is mainly seen in pyknotic nuclei from acidophilic neurons (arrows). (**D-F**) Evolution of cell death in CA1 neurons in control (**D**) and experimental animals 6 h (**E**), and 24 h (**F**) following SE. Control neurons have healthy cigar-shaped mitochondria and intact nuclei (**D**). Mitochondrial swelling (arrowheads) and cytoplasmic vacuolization (arrow) are the first signs of cell damage 6 h following SE, suggesting early necrosis (**E**). Twenty-four h following SE (**F**), this neuron displays mild irregular chromatin fragmentation while cytoplasm, mitochondria and endoplasmic reticulum show severe swelling. Bars = 5 microns (**A-C**) and 25 microns (**D-F**). From Niquet et al (2007).

Figure 2.

Putative neuronal death pathways induced by SE in the immature hippocampus. Fluorescent images show caspase-3a (green), caspase-8 (red), caspase-9a (green) and DCX (red) immunoreactivity and Hoechst staining (chromatin dye, white) in CA1 and the dentate gyrus following SE. In CA1, images of caspase-8 and caspase-3 immunoreactivity were taken 7 and 24 h respectively. Images from dentate gyrus were taken 24 h following SE. At the bottom, electron microscopy micrographs obtained 24 h after SE show a CA1 necrotic neuron and dentate gyrus apoptotic neuron. In CA1, seizures induce early and widespread activation of caspase-8, which subsequently activates the effector caspase-3 and leads to “programmed necrosis”. In dentate gyrus, seizures activate caspase-9 and caspase-3, and trigger apoptosis in immature neurons.

Table 1

Neuronal injury and caspase expression in the immature hippocampus after SE.

Hippocampal area	Staining	Control	7 h after SE	24 h after SE	72 h after SE
CA1-subiculum area	H&E	0	0	++	++
	Caspase-8	0	+++	0	0
	Caspase-9a	0	0	0	0
	Caspase-3a	0	0	+	+
Dentate Gyrus	H&E	0	+	+	+
	Caspase-8	0	0	0	0
	Caspase-9a	0	+	+	+
	Caspase-3a	0	+	+	+

Scale: (0), 0-5 eosinophilic or caspase-IR cells per section; (+), 5-50 cells; (++) , 100-200 cells; (+++), >200 cells.