

Short communication

# Hippocampal neurons of mice deficient in DNA-dependent protein kinase exhibit increased vulnerability to DNA damage, oxidative stress and excitotoxicity

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## Abstract

DNA damage has been documented in neurodegenerative conditions ranging from Alzheimer's disease to stroke. DNA-dependent protein kinase (DNA-PK) is involved in V(D)J recombination and DNA double strand break repair, and may play a role in cell death induced by DNA damage. We now report that cultured hippocampal neurons from severe combined immunodeficient (scid) mice which lack DNA-PK activity are hypersensitive to apoptosis induced by exposure to topoisomerase inhibitors, amyloid beta peptide (A $\beta$ ) and glutamate. A similar increased vulnerability of hippocampal CA1 and CA3 neurons was observed in adult scid mice after kainate-induced seizures. Our results suggest that DNA-PK activity is important for neuron survival under conditions that may occur in neurological disorders. © 2001 Elsevier Science B.V. All rights reserved.

*Theme:* Development and regeneration

*Topic:* Neuronal death

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Increasing data suggest that neurons may die by a form of programmed cell death called apoptosis in many different neurodegenerative conditions [14,33,34]. Although a variety of initiating factors may contribute to such neuronal cell deaths, they appear to share a common biochemical cascade involving oxidative stress, mitochondrial dysfunction and activation of cysteine proteases of the caspase family [10,11,16,27,30]. DNA damage is a feature of neuron cell death that has been detected in Alzheimer's disease and stroke patients [1,29] and in experimental models of these disorders [18,33,34]. Whereas enzyme-mediated cleavage of DNA into nucleosome-size frag-

ments occurs in the late stages of apoptosis [34,47], DNA damage induced by free radicals or enzymatic modifications can be a trigger that initiates the cell death program [28,41].

The cell death cascade induced by DNA damage can involve activation of poly-ADP-ribose polymerase (PARP), the tumor suppressor protein p53, and caspase-2 [12,36,41]. Repair of DNA double strand breaks by nonhomologous end joining requires DNA-dependent protein kinase (DNA-PK), a nuclear serine/threonine kinase consisting of a 350–450 kDa catalytic subunit (DNA-PKcs) and DNA targeting heterodimer subunits (p76 and p86) called Ku70 and Ku80 autoantigen [2,3]. Cell lines deficient in one of the Ku subunits or DNA-PKcs show impaired DNA double strand break repair and are highly susceptible to ionizing radiation [17,26,35]. Mice lacking DNA-PKcs activity (scid mice) exhibit severe combined immunodeficiency which is associated with defects in

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DNA repair and V(D)J recombination [22,42,46]. Recent studies showed a correlation between reduced DNA-PKcs activity due to caspase cleavage or pharmacological inhibition and apoptosis in several types of non-neuronal cells, and a role for DNA-PK in preventing apoptosis was postulated [8,9,40]. In contrast, *in vitro* studies revealed that DNA-PK is able to phosphorylate many transcription factors associated with apoptosis, such as fos, jun, and myc, indicating a possible pro-apoptotic function of DNA-PK [2,7]. In addition, a selective regulation of p53-dependent apoptosis by DNA-PK was reported, although others found that DNA-PK was not required for p53 activation in response to DNA damage [15,20,21,25,43]. Thus, contradictory results exist on the role of DNA-PK in apoptosis suggesting a protective function in DNA-repair and antiapoptotic signaling on the one hand and activation of proapoptotic transcription factors by phosphorylation on the other hand.

The aim of the present study was to elucidate the role of DNA-PK in neuron cell death. Our studies comparing hippocampal cell cultures from scid and wild-type mice show that scid neurons deficient in DNA-PK activity exhibit increased sensitivity to apoptotic insults including DNA damaging agents, overactivation of glutamate receptors, and oxidative stress. *In vivo*, excitotoxin-induced degeneration of hippocampal CA1 and CA3 neurons was greatly accelerated in scid mice.

Mice homozygous for the severe combined immune deficiency spontaneous mutation (CBySnm.CB17-Prkdc<sup>scid</sup>/J) (scid) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The donor strain of these scid mice was C.Bka-Igh<sup>b</sup>/IcrSnm (CB-17) and the background strain was BALB/cBySnm. Data demonstrating the lack of DNA-PK activity in the scid mice and its critical involvement in the phenotypes of these mice have been reported previously [5,19]. BALB/cByJ 001026 mice were used as controls. The CB17 mice are identical to BALB/c mice in all background genes except for the immunoglobulin (Ig) loci and the scid mutation (the Ig loci in this strain comes from C57BL/6 mice). Primary hippocampal cell cultures were established from E14 scid or wild-type embryos by methods similar to those described previously [31,32]. Briefly, hippocampal cells were dissociated by mild trypsinization and trituration and then seeded into polyethyleneimine-coated 35 mm culture dishes. Experimental treatments were performed on 7–8-day-old cultures. Camptothecin and etoposide (Sigma) were prepared as 500× stocks in dimethylsulfoxide. Glutamate was prepared as a 200× stock in saline. Amyloid  $\beta$ -peptide 1-42 was purchased from Bachem, and was dissolved in sterile water at a concentration of 1 mM and allowed to incubate for 2 h at room temperature prior to addition to cultures. Neurons were subjected to withdrawal of growth factors by replacing the culture maintenance medium with Locke's buffer (mM: NaCl 154; KCl, 5.6; CaCl<sub>2</sub>, 2.3; MgCl<sub>2</sub>, 1.0; NaHCO<sub>3</sub>, 3.6; glucose, 10; Hepes buffer, 5;

pH 7.2). Methods for quantifying neurons with apoptotic nuclei in cells stained with Hoechst dye, and for quantifying neuron survival using morphological criteria, were identical to those employed in our previous studies [23,31].

DNA damage was induced in hippocampal neurons in dissociated cell cultures from scid or wild-type mice by exposure to camptothecin (topoisomerase-I inhibitor, 5  $\mu$ M) and etoposide (topoisomerase-II inhibitor, 2.5  $\mu$ M), or UV irradiation (1.5 min exposure). Previous studies have shown that each of these DNA-damaging agents induces p53-dependent neuronal apoptosis [13,44]. Each of the topoisomerase inhibitors induced a small 10% increase in the numbers of neurons with apoptotic nuclei in wild-type cultures after 24 h of exposure to camptothecin and etoposide. A significantly greater increase in neuronal apoptosis (30–40%) was detected in cultures derived from scid mice (Fig. 1a). Hippocampal neurons were also exposed to growth factor withdrawal, glutamate and A $\beta$  to test whether the lack of DNA-PK activity sensitized scid neurons to insults other than those that directly damage DNA. In the case of each insult, the number of neurons with apoptotic nuclei was increased in cultures from scid mice compared to cultures from wild-type mice (Fig. 1b). In separate experiments we quantified neuron survival by counting undamaged neurons prior to and 48 h after exposure of the cultures to A $\beta$ , glutamate and UV radiation. Significantly fewer neurons in the cultures from scid mice survived exposure to each of these insults compared to cultures from wild-type mice (Fig. 1c).

We next determined whether hippocampal neurons in scid mice are more vulnerable to excitotoxic damage *in vivo*. The methods for inducing excitotoxic damage to hippocampal neurons *in vivo* have been described previously [6]. Briefly, mice were anesthetized with chloral hydrate and xylazine (350 and 4 mg/kg), placed in a stereotaxic holder, and the skull was exposed along the midline. Kainic acid (KA, 0.25  $\mu$ g in a volume of 0.5  $\mu$ l) was injected unilaterally into the dorsal hippocampus (A/P –2.0, M/L +2.4, D/V –0.8 from bregma) [39]. All mice administered KA exhibited seizures within the first h after injection. Twenty-four hours following KA injection, mice were perfused transcardially with 4% paraformaldehyde. Coronal brain sections (30  $\mu$ M) were cut on a freezing microtome and stained with cresyl violet. Nissl-positive undamaged neurons were counted in three 40× microscope fields in the hippocampal regions CA1 and CA3. Counts were made without knowledge of mouse genotype or treatment history in four sections/brain.

Examination of brain sections from scid and wild-type mice indicated that loss of hippocampal CA3 and CA1 neurons was markedly enhanced in scid mice as compared to controls (Fig. 2a). Quantification of undamaged neurons revealed highly significant increases in neuronal death in regions CA3 and CA1 (Fig. 2). Thus, hippocampal neurons in mice lacking DNA-PK activity are hypersensitive to excitotoxic injury.

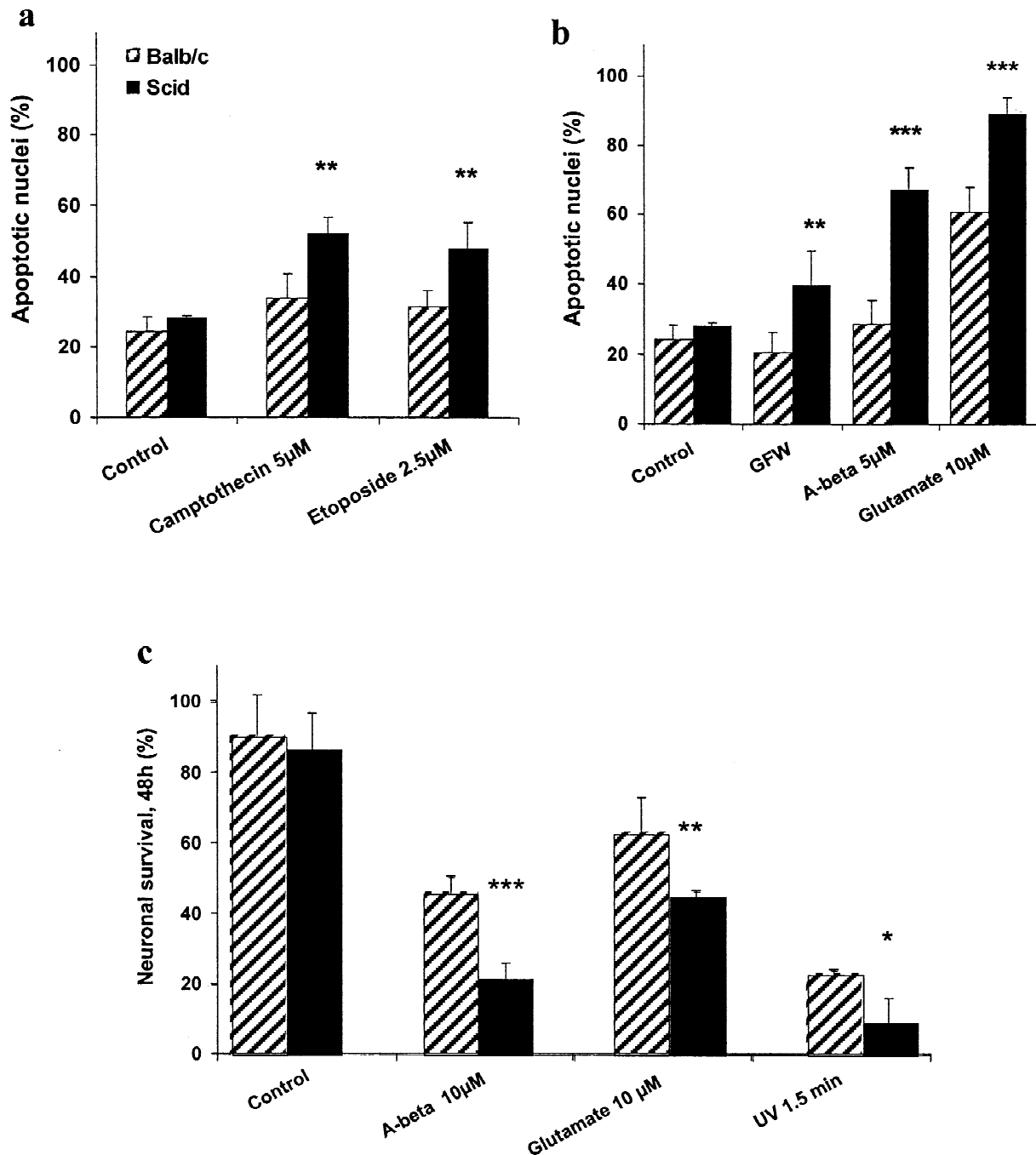


Fig. 1. Enhanced vulnerability of neurons lacking DNA-PK to death induced by an array of insults. (a and b) Embryonic hippocampal cultures from scid and wild-type mice were exposed to: (a) the topoisomerase inhibitors camptothecin (5  $\mu$ M) and etoposide (2.5  $\mu$ M), (b) to growth factor withdrawal (GFW), amyloid beta peptide (A-beta, 5  $\mu$ M) and glutamate (10  $\mu$ M). After 24 h cells were fixed in 4% paraformaldehyde and stained with the Hoechst 33342 dye to detect apoptotic nuclei. (c) In parallel experiments, neuronal survival was evaluated 48 h after treatment of hippocampal cultures with amyloid-beta peptide, glutamate, and UV irradiation. The percentages of neurons with apoptotic nuclei (a, b) and the percentage of neurons surviving (c) are given for each group (mean and S.D. of determinations made in 4–6 cultures). \* $P$ <0.05, \*\* $P$ <0.01, and \*\*\* $P$ <0.001 compared to wild-type cultures (ANOVA with Scheffé's posthoc tests).

The results obtained in the present study demonstrate that hippocampal neurons from scid mice, which lack DNA-PK activity, are highly susceptible to apoptosis and excitotoxicity. Neurons from scid mice are more vulnerable to a range of insults, including those that directly

damage DNA and those that may indirectly cause DNA damage, than are normal neurons. The increased vulnerability of scid neurons to these different insults is most likely due to lack of DNA-PK activity that results in defective DNA repair in cells of this mouse strain

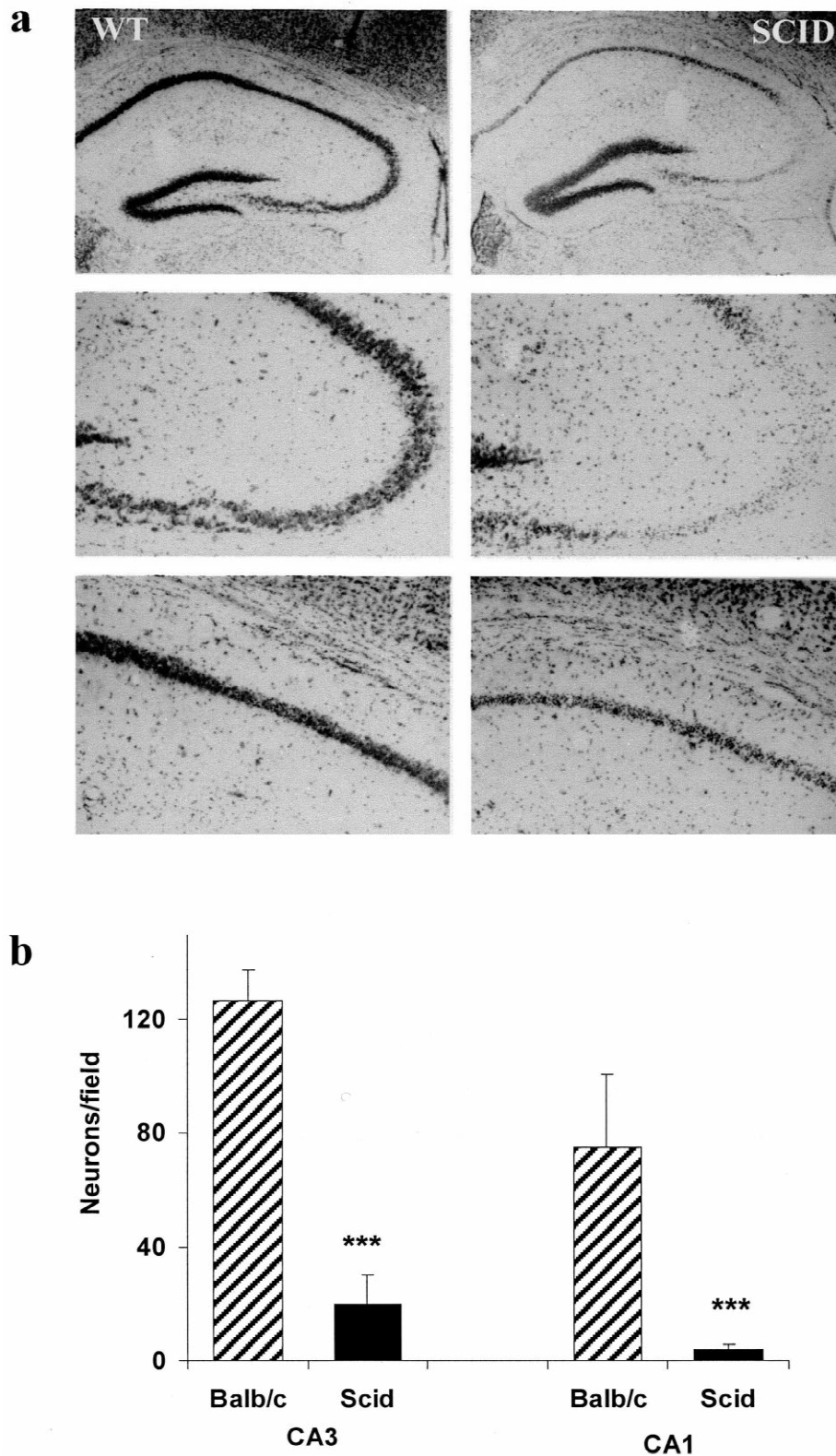


Fig. 2. Hippocampal neurons in mice lacking DNA-PK are hypersensitive to seizure-induced damage. (a) Cresyl violet-stained sections of hippocampi from scid and wild-type (WT) mice 24 h after administration of KA. The upper micrographs show low magnification view of the entire hippocampus, and the middle and lower panels show high magnification views of regions CA3 and CA1. Note that the damage in the CA3 and CA1 regions is markedly increased in scid mice. (b) Values for numbers of undamaged neurons in regions CA3 and CA1 of KA-treated wild-type Balb/c and scid mice (mean and S.D. of determinations made in 10 wild-type and 12 scid mice). \*\*\* $P < 0.001$  compared to the value for wild-type mice (Student's *t*-test).

[22,42,46]. Our findings are in line with previous reports suggesting a protective role of DNA-PK against DNA damage-induced apoptosis [38,45]. Studies of embryonic stem cells, fibroblasts and cell lines have shown that mutations in the Ku subunits or DNA PKcs that result in reduced DNA-PK activity also increase the vulnerability of cells to DNA-damaging conditions including  $\gamma$ -ionization, UV irradiation, and exposure to topoisomerase inhibitors [17,26,35,46]. In neuroblastoma cells exposed to staurosporine or UV irradiation enhanced apoptotic cell death was detected when DNA-PK activity was selectively inhibited [8].

In our studies of scid mice we detected an enhancement in cell death in our primary neuron cultures after exposure to A $\beta$ -mediated oxidative stress and glutamate-induced excitotoxicity, and in a mouse model of kainate induced seizures. These insults are usually not associated with an apoptotic cascade initiated by DNA strand breaks, suggesting that activation of DNA-PK plays a protective role against apoptosis that is exerted regardless of the starting point of the cell death cascade. However, initial reversible lesions of the DNA that trigger life/death signaling from the nucleus to the cytosol might be a common feature of apoptosis that has been overlooked in many experimental settings of neurodegeneration. Indeed, DNA damage has been documented in neurons in models of cerebral ischemia within 10–30 min after recirculation before the detection of internucleosomal fragmentation characteristic of programmed cell death [28]. In the same context we reported recently that homocysteine, a possible risk factor in stroke and Alzheimer's disease, induces neuronal apoptosis that involves DNA damage at an early time point, which is then followed by activation of PARP and p53 [24].

In the model of KA-induced excitotoxic neuronal death in vivo we detected a pronounced increase in hippocampal neuron cell death in scid mice lacking DNA-PK, suggesting that DNA repair is crucial for neuronal survival after the excitotoxic insult. Our observation correlates well with data obtained in a model of spinal cord ischemia in rabbits showing that enhanced DNA-PK expression and Ku80 DNA-binding activity is associated with recovery of tissue and function after mild ischemia, while severe ischemia results in DNA-PK cleavage and irreversible ischemic damage [38]. Increased DNA-PK activity after a mild insult most likely reflects a repair mechanism, as DNA-PKcs is inactivated by caspase cleavage after severe ischemia resulting in irreversible damage of tissue [8,38–40]. The specific signals that increase levels or activity of DNA-PK are not known, but may involve PARP and/or nitric oxide [37,45]. It is therefore likely that neurons that survive a potentially lethal insult that involves DNA damage are able to repair the damage. In addition to being involved in DNA-repair, activated DNA-PK may also play a role in transcription processes to prevent apoptotic cell death, for example by activation of the transcription factor NF- $\kappa$ B [4].

Our results suggest that DNA repair is critical in neurons for their survival under conditions of cellular stress, and that DNA-PK may mediate cytoprotective responses of neurons under conditions of injury or disease.

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