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Enforced DNA Repair Enzymes Rescue Neurons from Apoptosis Induced by Target Deprivation and Axotomy in Mouse Models of Neurodegeneration

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Abstract

It is unknown whether DNA damage accumulation is an upstream instigator or secondary effect of the cell death process in different populations of adult postmitotic neurons in the central nervous system. In two different mouse models of injury-induced neurodegeneration characterized by relatively synchronous accumulation of mitochondria, oxidative stress, and DNA damage prior to neuronal apoptosis, we enforced the expression of human 8-oxoguanine DNA glycosylase (hOGG1) and human apurinic-pyrimidinic endonuclease/Ref1 (hAPE) using recombinant adenoviruses (Ad). Thalamic lateral geniculate neurons and lumbar spinal cord motor neurons were transduced by Ad-hOGG1 and Ad-hAPE injections into the occipital cortex and skeletal muscle, respectively, prior to their target deprivation- and axotomy-induced retrograde apoptosis. Enforced expression of hOGG1 and hAPE in thalamus and spinal cord was confirmed by western blotting and immunohistochemistry. In injured populations of neurons in thalamus and spinal cord, a DNA damage response (DDR) was registered, as shown by localization of phospho-activated p53, Rad17, and replication protein A-32 immunoreactivities, and this DDR was attenuated more effectively by enforced hAPE expression than by hOGG1 expression. Enforced expression of hOGG1 and hAPE significantly protected thalamic neurons and motor neurons from retrograde apoptosis induced by target deprivation and axotomy. We conclude a DDR response is engaged pre-apoptotically in different types of injured mature CNS neurons and that DNA repair enzymes can regulate the survival of retrogradely dying neurons, suggesting that DNA damage and activation of DDR are upstream mechanisms for this form of adult neurodegeneration in vivo, thus identifying DNA repair as a therapeutic target for neuroprotection.

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Keywords

Neuronal apoptosis; motor neuron; spinal cord; DNA damage; ALS

INTRODUCTION

The genome is inherently unstable and vulnerable due to its primary structure and mechanisms of operation and is also subjected constitutively to endogenous genotoxic insults such as reactive oxygen species (ROS) and assimilated environmental cytotoxins (Lindahl, 1993; Harman, 1981). While these properties of DNA provide a substrate for natural selection and biological evolution for the species in germline cells (Vijg, 2014), they also can be an Achilles heel to somatic cells, even non-cycling cells such as neurons. DNA damage is a suspected trigger for neuronal death in a many neurological disorders occurring throughout life (Caldecott, 2004; Martin, 2008; Jeppesen et al., 2011; Hedge et al., 2012; Pan et al., 2014). DNA damage has been implicated in the mechanisms of neurodegeneration in childhood neurological disorders, age-related diseases, and adult cerebral ischemia and traumatic brain injury. In the developing brain, migrating newborn neurons can sustain oxidative DNA damage and, if not repaired, undergo apoptosis (Narasimhaiah et al., 2005). Mutations in polynucleotide kinase 3'-phosphatase (*PNKP*) cause microcephaly, seizures, and intellectual disability but not structural brain abnormalities, apparent neurodegeneration, or neurological symptoms such as ataxia (Shen et al., 2010). Other human disorders link more definitely DNA damage as a driving mechanism for neurodegeneration. Cleaver recognized first a link between defective repair of DNA damage, cancer, and neurologic disease in children with xeroderma pigmentosum (XP) (Cleaver, 1968). The neurological phenotype of XP patients includes ataxia, microcephaly, deafness, learning disability, peripheral neuropathy with loss of large sensory axons and dorsal root ganglion cells, cerebellar/cerebral atrophy, and primary neuronal degeneration (Kraemer et al., 2007). Mutations in genes involved in the DNA nucleotide excision repair (NER) pathway cause most forms of XP (Kraemer et al., 2007). Other human diseases associated with abnormalities in NER genes and neurodegeneration are Cockayne syndrome and trichothiodystrophy (Kraemer et al., 2007). A notable childhood disease that strengthens the concept of a link between defective DNA repair and neurodegeneration is ataxia-telangiectasia (AT) (Taylor et al., 1975). These patients develop prominent neurological symptoms, including ataxia, dysarthria, dyssynergia, and oculomotor apraxia, and neuropathology confined primarily to myelin and the cerebellum, notably the Purkinje and granular neurons (Aguilar et al., 1968). AT is caused by mutations in the *ataxia telangiectasia mutated* gene (*ATM*). The ATM protein is a protein kinase that functions in DNA damage response (DDR) and DNA-double strand break (DSB) repair (Taylor et al., 1975; Savitsky et al., 1995). More recently, gene mutations in *aprataxin* (*APTX*) and *tyrosyl-DNA phosphodiesterase 1* (*TDP1*) have been identified that cause ataxia oculomotor apraxia 1 (AOA1) and spinocerebellar ataxia with axon neuropathy (SCAN1), respectively (Date et al., 2001; Moreira et al., 2001; Takashima et al., 2002). Both APTX and TDP1 proteins function in DNA single-strand break (SSB) processing and repair (Yang et al., 1996; Clements et al., 2004). SCAN1 is very rare and milder clinically than AOA1; the latter has prominent degeneration of cerebellar Purkinje neurons (Tada et al., 2010). Despite

accruing evidence indicating faulty DNA repair as an upstream mechanism of neurodegeneration (Caldecott, 2004; Martin, 2008; Jeppesen et al., 2011; Hedge et al., 2012; Pan et al., 2014), it remains puzzling that the neurodegeneration found in AT, AOA1, and SCAN1 is so limited within the nervous system, being confined mostly to the cerebellum. Moreover, in some human DNA-DSB repair disorders like Nijmegen breakage syndrome, caused by mutation of the *NBS1* gene, developmental hypoplasia of the corpus callosum and frontal lobes is found but not neurodegeneration (Reynolds and Stewart, 2013). It therefore seems plausible that inadequate DNA repair and DNA damage accumulation may not be sufficient primary triggers for neurodegeneration in most terminally differentiated neurons.

In rodent models of axotomy and target deprivation and in transgenic mouse models of amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) expressing human mutant superoxide dismutase-1 and α -synuclein, respectively, we have found increased oxidative stress and the accumulation of 8-hydroxy-2-deoxyguanosine (OHdG), DNA-SSB, and DNA-DSBs in neurons very early in the process of their neurodegeneration (Martin et al., 1999; Liu and Martin, 2002; Martin and Liu, 2002; Martin et al., 2003; Martin et al., 2006; Martin et al., 2007; Martin et al., 2014). Some of these animal models also show that the neuronal degeneration is a p53- and bax-regulated process (Martin et al., 2001; Martin and Liu, 2002; Martin et al., 2005). This work supports the premise that DNA damage might induce apoptotic neuronal cell death; yet, critically, the initiating or sufficient and engaging role for DNA damage in this process for neurodegeneration in the mature CNS is still equivocal. In the present study, we tested the hypothesis, using two different adult mouse models that have distinctly different populations of neurons vulnerable to cell death, that DNA damage is an upstream mechanism for neuronal apoptosis. We enforced conditionally by recombinant adenoviruses the expression of DNA repair enzymes, human 8-oxoguanine DNA glycosylase (hOGG1) and human apurinic-pyrimidinic endonuclease (hAPE) in vulnerable neurons prior to their injury. Our results demonstrate directly that hOGG1 and hAPE protect neurons from apoptosis *in vivo*, thereby supporting the premise that insufficient DNA repair and DNA damage accumulation are causes of neurodegeneration in different types of terminally differentiated adult CNS neurons and showing that DNA repair is a target for neuroprotection in the adult brain and spinal cord.

MATERIALS & METHODS

Construction of replication-incompetent adenoviral vectors for *in vivo* delivery of hOGG1 and hAPE genes

Plasmids containing hOGG1 gene (Hollenbach et al., 1999) and hAPE gene (Barzilay et al., 1995) were kindly provided by Dr. Pablo Radicella (Commissariat à l'Energie Atomique, Fontenay aux Roses, France) and Dr. Ian Hickson, University of Oxford, UK), respectively. These gene constructs encode functional proteins with DNA repair activity in cells and cell-free systems. The plasmids were used as templates to generate blunt-end PCR products encoding the open reading frames of hOGG1 and hAPE. Transcription of the genes was constitutively driven by the human cytomegalovirus immediate early promoter. Each protein product was flanked by the 14 amino acid-long V5 epitope. The stop codon was omitted in the reverse primer to accommodate an in-frame C-terminal V5 tag (Southern, et al., 1991) in

the final adenoviral vector. The amplified cDNA sequences were cloned into the pENTRTMTOPO[®] vector (Invitrogen, CA) by directional TOPO[®] cloning. The target genes in the resulting entry clones were then transferred to the pAd/CMV/V5-DESTTM destination vector (Invitrogen, CA) in a recombination reaction catalyzed by the Clonase II enzyme mix (Invitrogen, CA). Clonase II contains components of the bacteriophage lambda recombination system that allow strand exchange between DNA sequences flanked by specific attachment (att) sites (Landy, 1989, Ptashne, 1992). After the reaction, the target genes, previously flanked by att sites in the entry clones were now positioned between att sites within the pAd/CMV/V5-DESTTM destination vector. The identity of all vectors and clones was confirmed by restriction enzyme digestion and DNA sequencing.

The pAd/CMV/V5-DESTTM destination vector contained essential genetic elements that allow packaging of viral particles. However, the E1 region necessary for viral replication (Graham et al. 1977, Krougliak and Graham, 1995) was deleted from the vector. Adenovirus generated using the pAd/CMV/V5-DESTTM destination vector can only replicate in mammalian cells expressing E1 proteins. In order to propagate the virus, the linearized destination vectors were transfected into the human embryonic kidney cell line 293A (Invitrogen, CA) using lipofectamine (Invitrogen, CA). With an integrated copy of E1 in its genome, 293A cells provided the E1 proteins in trans. The transfection generated a crude adenoviral stock for further amplification in the same cell line. High titer adenoviral stocks were purified by ultracentrifugation in a cesium chloride gradient. Viral infectivity was determined using the QuickTiterTM Adenovirus Titer Immunoassay Kit (Cell Biolabs, CA). The immunocytochemical assay used an antibody against the hexon structural protein in the adenoviral capsid to detect infected 293A cells. Wildtype adenovirus contamination was detected by PCR as described (Zhang, et al, 1995).

Animals

We used adult male wildtype C57BL/6 mice and B6.Cg-Tg (Hlxb9-gfp)^{1Tmj/j} transgenic mice expressing eGFP driven by the mouse Hlxb9 (Hb9) promoter (Wichterle et al., 2002) originally obtained from Jackson Laboratories (Bar Harbor, Maine). Adult Hb9-eGFP mice have spinal motor neurons highly expressing eGFP (Chang and Martin, 2011; Martin, 2011; Chestnut et al., 2011) that can allow the unequivocal identification of motor neurons expressing human DNA repair genes in vivo. The institutional Animal Care and Use Committee approved the animal protocols.

Adenovirus transduction in vivo

The replication incompetent recombinant adenovirus containing hOOG1-V5 and hAPE-V5 were used for in vivo transduction of mouse brain and spinal cord neurons. Under deep anesthesia isoflurane:oxygen:nitrous oxide (1:33:66), a unilateral right posterior craniotomy was made with negligible damage to the underlying cerebral cortex. A Hamilton microsyringe was used to deliver a total of 10 μ l of Ad-hOOG1 (3.8×10^{11} ifu/ml), Ad-hAPE (3×10^{12} ifu/ml), or Ad-eGFP (Vector Biolabs, 1×10^{10} ifu/ml) into the occipital cortex at five different injection sites. This was done so virus would be assimilated by dLGN neuron synapses and retrogradely transported to neuronal cell bodies in thalamus (Martin et al., 2011). To transduce lumbar spinal cord motor neurons, 10 μ l of Ad-hOOG1 (3.8×10^{11}

ifu/ml), Ad-hAPE (3×10^{12} ifu/ml), or Ad-GFP (1×10^{10} ifu/ml) was injected unilaterally into the right gastrocnemius at 5 different sites along the length of the muscle. After 3-4 days, the mice were killed for histological or biochemical studies or were subjected to the lesioning paradigms.

Verification of gene transduction in vivo

Mice conditionally transduced by cerebral cortical or hind-leg muscle injection of Ad-hOGG1, Ad-APE1, and Ad-GFP were deeply anesthetized and killed by perfusion-fixation through the heart with phosphate-buffered saline (PBS) and then 4% paraformaldehyde or by rapid decapitation. For histology, the tissues were allowed to fix *in situ* overnight before removal of the brain and spinal cord and were cryoprotected in 20% glycerol and then serial sectioned (40 μ m) on a sliding microtome. Sections were stored in antifreeze buffer at -20°C . Immunohistochemistry was used on brain and spinal cord sections to detect V5 epitope or GFP with monoclonal antibodies (Invitrogen, CA) and an immunoperoxidase detection method using diaminobenzidine (DAB) as chromogen or an immunofluorescence or direct fluorescence method. For western blotting, fresh lumbar spinal cord segments were harvested and microdissected under a stereomicroscope into ipsilateral (transduced) and contralateral (non-transduced) spinal columns and used for tissue lysates. Samples were subjected to SDS-PAGE, transferred to nitrocellulose membrane, stained with Ponceau S to assess protein transfer and to scan for protein loading, rinsed, and immunoblotted using V5 monoclonal antibody, rabbit polyclonal antibody to hOGG1 (Novus Biologicals, Littleton, CO), and mouse monoclonal antibody to hAPE (Affinity Bioreagents, Golden, CO). Recombinant hOGG1 (Trevigen, Gaithersburg, MD) was used as a positive for western blotting. Secondary antibodies were horseradish peroxidase-conjugated (Biorad). Western blots were developed on X-ray film using enhanced chemiluminescence. Immunoblots were scanned and analyzed for optical densitometry of OGG1 and APE immunoreactivities using ImageJ software (NIH). Intensities of immunoreactivity were normalized to comparably-sized bands of protein detected by Ponceau S staining of nitrocellulose membranes. Immunoblotting experiments were reproduced at least in triplicate.

Adult mouse models of neurodegeneration

All surgical procedures were performed under deep anesthesia using a mixture of isoflurane:oxygen:nitrous oxide (1:33:66). A unilateral occipital cortex ablation model was used to produce retrograde neurodegeneration in the dorsal lateral geniculate nucleus (dLGN) of thalamus by distal axotomy and target deprivation. The neurons die by apoptosis over 7-days (Al-Abdulla and Martin 1998; Martin et al., 2002; Martin et al., 2003; Natale et al., 2002; Martin et al., 2011). Cortical ablations were done on adult C57BL/6J and Hb9-eGFP mice. Mouse cohort sizes were 10/genotype. The validation and reproducibility of this model of remote retrograde neuronal apoptosis in mouse brain have been described (Al-Abdulla and Martin 1998; Martin et al., 2002; Martin et al., 2003; Natale et al., 2002; Martin et al., 2011). A unilateral sciatic nerve avulsion served as the model for inducing retrograde apoptosis of motor neurons in vivo. The validation and reproducibility of this model of motor neuron degeneration in mouse has been described (Martin and Liu, 2002; Martin et al., 2005).

Evaluation of neuroprotection and DDR

Mice with cortical ablation and sciatic nerve avulsion lesions were killed at 4, 7, 8 or 18 days postlesion. They were deeply anesthetized and killed by perfusion-fixation through the heart with PBS followed by 4% paraformaldehyde. After cryopreservation, symmetrical transverse serial sections (40 μ m) through the diencephalon and lumbar spinal cord were cut using a sliding microtome and were stored individually in 96-well plates. Sections were selected with a random start and then systematically sampled (every 9th section) to generate a subsample of sections from each mouse that were mounted on glass slides and stained with cresyl violet for neuronal counting. Thalamic neuronal counts in the ipsilateral and contralateral dLGN were made at 1000x magnification. Motor neuron counts in L4-L6 were made at 400x magnification. The stereological optical dissector method was used as described (Al-Abdulla and Martin, 1998; Martin et al., 1999; Martin et al., 2005; Martin et al., 2011). dLGN neurons and motor neurons without apoptotic structural changes were counted using strict morphological criteria. These criteria included a round, open, pale nucleus (not condensed and darkly stained), globular Nissl staining of the cytoplasm, and a diameter of ~10-15 μ m (dLGN) or ~20-35 μ m (motor neurons). With these criteria, astrocytes, oligodendrocytes, and microglia were excluded from the counts of thalamic neurons and motor neurons.

The localization patterns of phospho-p53, -Rad17, and -RPA32 were examined in thalamus and spinal cord of lesioned mice with and without DNA repair gene transduction by light microscopy. DDR proteins were detected using an immunoperoxidase method with DAB as chromogen so that the localizations of these proteins were seen as brown staining in tissue sections. Phosphorylated p53 was detected with an affinity-purified rabbit polyclonal antibody to serine-15 phosphorylated p53 (R&D Systems). While this antibody recognizes human p53 only when it is phosphorylated at Ser-15, it detects the comparable phosphorylated Ser-18 site in mouse cells but does not recognize unphosphorylated mouse p53 (R&D Systems). Phosphorylated Rad17 was detected with a rabbit antibody to human phosphoserine-645 (serine-646 in mouse) Rad17 (Cell Signaling Technology). Phosphorylated RPA32 was detected with a rabbit antibody to human phosphoserine-33 RPA32 (Bethyl Laboratories) which is identical in mouse. These antibodies have been characterized for specificity by western blotting (unpublished observations). Negative control sections were incubated in comparable dilutions of immunoglobulin G or with primary or secondary antibody omitted.

Sections were studied, analyzed, and imaged using a Olympus brightfield microscope with a ProgRes C14 Plus digital camera and Progres CapturePro imaging software. Phospho-p53 and phospho-Rad17 immunoreactivities were quantified by counting the numbers of neurons with immunopositive nuclei in lesioned (ipsilateral) and non-lesioned (contralateral) sides in serial sections through the LGN and lumbar spinal cord. Phospho-RPA32 immunoreactivity was quantified by counting the numbers of immunopositive neurons or axons in lesioned (ipsilateral) and non-lesioned (contralateral) sides in serial sections through the LGN and lumbar spinal cord.

Statistical analysis

Neuronal, nuclear, and axonal counts and immunoblot optical densities were used to determine group means and variances and comparisons among groups were analyzed using a one-way analysis of variance and a Newman-Keuls post-hoc test.

RESULTS

Verification of human DNA repair gene transduction in mouse CNS

To confirm the adenoviral-mediated human gene transduction in the CNS of mice, western blotting was done (Fig. 1A-C). Lumbar spinal cord extracts of mice with skeletal muscle infection of Ad-hOGG1 revealed increased expression of OGG1 in the transduced ipsilateral side of spinal cord compared to non-transduced contralateral lumbar spinal cord and cervical spinal cord (Fig. 1A). OGG1 levels in the ipsilateral lumbar spinal cord were approximately 50% higher compared to the contralateral lumbar spinal cord. Western blotting for V5-tagged hOGG1 was used as an alternative approach to demonstrate the efficacy of human DNA repair gene transduction (Fig. 1B). As expected, no band was seen in contralateral control spinal cord with only endogenous mouse OGG1 (Fig. 1B). LGN extracts of mice with occipital cortex infection of Ad-hAPE revealed increased expression of APE in the transduced ipsilateral side of dorsal thalamus compared to non-transduced thalamus (Fig. 1C). The level of hAPE in the transduced LGN corresponded to about a 40% increase in total APE compared to contralateral control LGN.

Target delivery of recombinant adenovirus and its retrograde transport by infected neuronal populations is an excellent strategy for robust expression of exogenous genes in neurons. For example, injection of adenovirus into the gastrocnemius muscle results in robust gene expression in motor neurons of lumbar spinal cord (Fig. 1D). Direct imaging of GFP fluorescence shows strong expression in motor neuron cell bodies, dendrites and axons resulting in detailed visualization of motor neuron morphology (Fig. 1D).

Immunohistochemistry was used to demonstrate human gene transduction in mouse neurons in vivo. Immunohistochemical localization of V5-hOGG1 and V5-hAPE demonstrated enforced expression of these genes in thalamic neurons and spinal motor neurons (Fig. 1E-G). Spinal cord motor neurons unequivocally identified by Hb9-driven eGFP were found to be enriched in OGG1 immunoreactivity (Fig. 2). OGG1 immunoreactivity in motor neurons was detected in the nucleus and as numerous discrete particles throughout the cytoplasm (Fig. 2), most likely representing mitochondria.

DDR protein localizations in brain and spinal cord and in injured thalamic neurons and spinal motor neurons after target deprivation and axotomy

Previous studies have shown that retrogradely degenerating dLGN and spinal motor neurons after axotomy and target deprivation in vivo undergo oxidative stress and accumulate perinuclear mitochondria and DNA damage, in the form of OHdG, DBS-SSBs, and DNA-DSBs (Al-Abdulla and Martin, 1998; Martin et al., 1999; Martin and Liu, 2002; Martin et al., 2005; Martin et al., 2011). The activation of p53 is also found in injured pre-apoptotic dLGN and motor neurons, consistent with the presence of DNA damage; p53 gene null mutation nearly completely or partially rescues the neurons in vivo (Martin et al., 2001;

Martin and Liu, 2002). Other DDR markers have not been studied in these two mouse models of lesion-induced retrograde neurodegeneration: 1) occipital cortex ablation that induces widespread apoptosis of dLGN neurons (Al-Abdulla and Martin, 1998; Martin et al., 2001; Martin et al., 2011); and 2) sciatic nerve avulsion that induces apoptosis of lumbar spinal cord motor neurons (Martin et al., 1999; Martin and Liu, 2002; Martin et al., 2005). Both lesions were made unilaterally, so the contralateral neurons served as a within-section control.

p53 activation, assessed by accumulated phospho-p53, was seen in lesioned (ipsilateral) dLGN neurons and spinal motor neurons (Fig. 3A-D), consistent with previous descriptions (Martin et al., 2001; Martin et al., 2003; Martin et al., 2005). The enhanced phospho-p53 immunoreactivity appeared as nuclear and cytoplasmic (Fig. 3A-D), particularly in lesioned neurons. The ability of hAPE and hOGG1 gene expression to alter the p53 DDR was evaluated. Enforced hOGG1 and hAPE expression significantly attenuated the enhancement of phospho-p53 in injured dLGN and motor neurons (Fig. 3E, F). The beneficial effects were more robust with hAPE compare to hOGG1.

There are no reports on the localization of phospho-RPA32 in the mammalian CNS, so a brief description is provided. Phospho-RPA32 immunoreactivity was ubiquitous in the mouse brain and spinal cord (Fig. 4). In non-lesioned side of the mouse CNS, phospho-RPA32 was localized very differently in brain compared to spinal cord. In brain, phospho-RPA32 was localized to the neuropil (Fig. 4A,D) and subsets of neurons (Fig. 5A). Phospho-RPA32 had a prominent axonal localization and was seen in the neuropil in a synaptic terminal-like pattern, notably evidenced in the hippocampal intradentate or hilar region (Fig. 4D). The axonal localization was seen in forebrain white matter (striatal bundles) and diencephalic white matter tracts (fasciculus retroflexus) (data not shown) and was also very evident in spinal cord white matter (Fig. 4E). Interestingly, the ipsilateral loss of phospho-RPA32 immunoreactivity in the spinal cord dorsal column white matter bundles was an effective readout for the efficacy of the sciatic nerve avulsion (Fig. 4E). RPA32 seemed mostly confined to neurons because no obvious glial cell body labeling was seen in the CNS parenchyma and white matter. In the brain on the side of the occipital cortex lesion, phospho-RPA32 accumulated in cortical neurons (Fig. 4B) and in axonal swellings (Fig. 4C) and did not seem to be upregulated in glia (Fig. 4B,C). Phospho-RPA32 was highly enriched in spinal cord neurons at baseline and was found in the neuronal cell bodies and proximal dendrites of spinal motor neurons (Fig. 5C-F). Within neuronal cell bodies of motor neurons, phospho-RPA32 immunoreactivity was present in the cytoplasm and nucleus. Within the nucleus of motor neurons, phospho-RPA32 was localized as discrete speckles or foci (Fig. 5E,F).

After injury the number of neuronal cell bodies in the ipsilateral dLGN positive for phospho-RPA32 immunoreactivity increased after cortical ablation (Fig. 5G). More prominently, motor neurons became strongly immunoreactive for phospho-RPA32 after sciatic nerve avulsion (Fig. Fig. 5E,F). Enforced hOGG1 and hAPE expression significantly attenuated the RPA32 activation in injured neurons (Fig. 5G,H).

There are no descriptions of the localization of phospho-Rad17 in the mammalian CNS. In naïve adult mouse CNS phospho-Rad17 appeared to be constitutively expressed because immunoreactivity was localized in the nucleus of cells throughout the CNS (Fig. 6). There was evidence for neuron-specific localization (Fig. 6), but no indication of an axonal or putative synaptic localization of phospho-Rad17. In mice without lesions, some neurons, such as spinal motor neurons, showed cytoplasmic localization of phospho-Rad17 immunoreactivity (Fig. 6E-F), but other neurons did not have detectable cytoplasmic phospho-Rad17. After cortical ablation, ipsilateral dLGN neurons showed enhanced nuclear phospho-Rad17 (Fig. 6C,D), and after peripheral nerve avulsion, spinal motor neurons also showed enhanced nuclear phospho-Rad17 immunoreactivity (Fig. 6G-J). Injured spinal motor neurons beautifully revealed the cytoplasm-to-nucleus redistribution of phospho-Rad17 (Fig. 6G,H). Enforced hOGG1 and hAPE expression significantly attenuated the Rad17 activation in injured neurons (Fig. 6I,J).

Enforced hAPE and hOGG1 gene expression protects mature neurons from remote retrograde apoptosis in vivo

The ability of hAPE and hOGG1 gene expression to protect neurons was evaluated in the cortical ablation and nerve avulsion models of retrograde neurodegeneration (Fig. 7). Seven days after occipital cortex ablation, mice without human DNA repair gene transduction or with Ad-GFP gene transduction showed about a ~85% loss of dLGN neurons on the lesion side (Fig. 7A-D,I), consistent with other studies (Martin et al., 2001, 2011). Mice expressing hOGG1 in thalamic neurons registered a significant increase in neuronal number compared to control lesioned mice as evidenced by a 65% loss of dLGN neurons (Fig. 7E,I). In contrast, mice expressing hAPE in thalamic neurons showed a far more prominent rescue of dLGN neurons (Fig. 7F,I). After sciatic nerve avulsion, mice without human DNA repair gene transduction or with Ad-GFP gene transduction showed about a ~60% loss of lumbar spinal motor neurons on the lesion side (Fig. 7G,J) consistent with other studies (Martin et al., 2005). The loss of motor neurons with enforced expression hOGG1 was modestly attenuated (Fig. 7J), but enforced expression of hAPE in motor neurons mediated a prominent rescue of spinal motor neurons (Fig. 7H,J).

Discussion

This study advances the understanding of the molecular regulation of neuronal cell death in the mammalian CNS. We tested the hypothesis that enforcing human DNA repair gene expression would protect differentiated mature neurons from injury-induced apoptosis in mouse CNS. We used conditional adenovirus-mediated transduction of hOGG1 and hAPE in combination with adult mouse models of axotomy and target deprivation that specifically induce neuronal apoptosis in brain and spinal cord (Martin et al., 2001; Martin and Liu, 2002; Martin et al., 2003; Martin et al., 2005; Martin et al., 2011). This neuronal apoptosis is associated with mitochondrial accumulation, oxidative stress, and DNA damage (Martin et al., 2001; Martin and Liu, 2002; Martin et al., 2003; Martin et al., 2005; Martin et al., 2011). We found that enforced expression of hAPE and hOGG1 in neurons mitigated DDR, identified by immunoreactivity for phospho-p53, phospho-RPA32, and phospho-Rad17, and increased the survival of selectively vulnerable thalamic neurons and spinal cord motor

neurons. The attenuation of DDR and suppression of apoptosis in different populations of adult CNS neurons by DNA repair enzymes supports the hypothesis that DNA damage is a primary trigger for the in vivo degeneration of neurons, other than cerebellar neurons that can degenerate putatively in response to faulty DNA repair ensuing from mutations in ATM (Aguilar et al., 1968).

We studied the CNS localizations of phospho-activated p53, RPA32, and Rad17 as readouts for DRR. To this end, we provide a synopsis of the localization of RPA32 and Rad17 in the adult mouse brain and spinal cord because the localizations of RPA32 and Rad17 have not been shown before in normal and injured CNS. The antibody we used for phospho-RPA32 is well characterized and used widely (Feng et al., 2009; Vassin et al., 2009; see Bethyl Laboratories for other references). The antibody we used for phospho-Rad17 is also well characterized (Wang et al., 2006; Verdun and Karlseder, 2006).

RPA32 is part of a heterotrimeric single-stranded DNA-binding protein composed of two other subunits (RPA70 and RPA14) (Treuner et al., 1999; Binz et al., 2004). The RPA complex, the most abundant single strand-specific DNA binding protein, functions in many aspects of eukaryotic DNA biology, including the repair of DNA-DSBs, possibly through stabilizing locally unwound DNA and the targeting of repair endonucleases to DNA damage sites (Binz et al., 2004). It is thought that the RPA-DNA complex, formed by the accumulation of RPA, signals DNA damage for the activation of DDR (Binz et al., 2004). RPA is phosphorylated dramatically in response to DNA damage and during apoptosis in non-neural cells (Liu et al., 2006). We found a surprisingly high basal level of phospho^{Ser33}-RPA32 immunoreactivity ubiquitously throughout the adult mouse CNS. We observed phospho-RPA32 in the cell body and nucleus of neurons, axons, and within putative synaptic terminals. This distribution is interesting given its purported nuclear function as a DDR protein. An explanation for this pattern of immunoreactivity may reside in possible non-nuclear functions of single strand-DNA binding proteins involving mitochondrial DNA (Broderick et al., 2010); or, RPA32 may have yet to be discovered functions.

Rad17 is a DDR sensor protein that functions in the early recruitment and retention of the MRE11-Rad50-Nbs1 complex at DNA-DSBs (Wang et al., 2014). Rad17 is phosphorylated by ATM at serine⁶⁴⁵ and facilitates DNA repair through homologous recombination (Wang et al., 2014). Activated Rad17 immunoreactivity was also detected surprisingly at a constitutive presence in adult mouse CNS. It was localized primarily in the nucleus of apparently different types of neuronal and non-neuronal cells, but was found accumulated selectively in the cytoplasm of some spinal motor neurons. After injury, phospho-Rad17 accumulated in the nucleus of injured neurons, and there was a generalized upregulation in small microglia-like cells throughout the brain and spinal cord parenchyma.

These immunohistochemical results demonstrate that the DDR is rapidly and robustly inducible in adult CNS in response to axotomy and target deprivation of neurons and also appears activated secondarily in non-neuronal cells, possibly as a protective mechanism; thus, rapid and robust DNA damage is likely to be elevated in these cells. The activation of DDR in injured neurons independently validates previous data showing by immunohistochemical detection of OHdG and single-stranded DNA, comet assay, TUNEL,

and Southern blotting that DNA damage accumulates early after injury in these neurons (Al-Abdulla and Martin, 1998; Al-Abdulla et al., 1998; Liu and Martin, 2001; Martin and Liu, 2002; Natale et al., 2002; Martin et al., 2003). Our new observations also suggest that the steady-state threat of DNA-DSBs may be greater than anticipated previously in postmitotic terminally differentiated neurons in the adult CNS and that neuronal injury, and possibly aging, can exacerbate DNA-DSB formation. A high baseline level of DDR protein in the adult CNS is consistent with a recent astonishing finding that physiological brain activity causes DNA-DSBs, as inferred by phospho-H2AX immunoreactivity (Suberbielle et al., 2013). We have shown previously that p53 is activated during retrograde apoptosis of thalamic and spinal motor neurons (Martin and Liu, 2002; Martin et al., 2003; Martin et al., 2005). p53 is also activated in degenerating upper and lower motor neurons in human ALS (Martin 2000, 2001). Our new results extend previous findings of DNA damage accumulation in adult neurons after axotomy and target deprivation by showing that the DNA-DSB signaling pathways of the DDR are also activated as demonstrated by the upregulation of phospho-RPA32 and phospho-Rad17. In mice with enforced expression of hOGG1 the DDR responses, as detected by immunohistochemistry for phospho-p53, phospho-RPA32, and phospho-Rad17, were modestly attenuated, and this outcome was associated with modest neuroprotection. In contrast, enforced expression of hAPE eventuated in major suppression of the DDR response and greater protection of injured neurons in thalamus and spinal cord. These findings suggest that, in retrogradely dying mature neurons, DNA single-strand nicking and break formation may evolve into DNA-DSBs and that enforcement of DNA-SSB repair can interrupt the formation of DNA-DSBs, resulting in prominent neuroprotection.

Choice of model

We have shown that target ablation and axotomy in rodents can serve as models of regionally discrete, precisely-timed homogeneous neurodegeneration that resembles apoptosis (Martin et al 1999; Martin et al., 2001; Martin and Liu, 2002; Martin et al., 2003; Martin et al., 2005; Martin et al., 2011). The progression of this degeneration is entirely synchronous in different neurons, and the precise locations and amounts of neurodegeneration and neuronal loss are predictable. These models have extraordinary value for understanding the molecular mechanisms of neuronal cell death. As experimental tools these models offer major advantages over transgenic mouse models generated by expression of human mutant genes linked to Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (Martin, 2012). Many of these transgenic mouse models do not have the phenotype of neuronal loss or, when present, have chronic and asynchronous neurodegeneration and neuronal loss that often involves equivocal cell death forms that may not recapitulate the neurodegeneration seen in human disease (Martin et al., 2007; Martin and Liu, 2004; Martin, 2012). Most transgenic mouse models of AD expressing human mutant APP and/or presenilin can generate considerable deposits of parenchymal amyloid without neuronal loss during aging, and a variety of transgenic mouse models with null or point mutations in PD- and ALS-linked genes fail to exhibit age-related neurodegeneration (Martin, 2012).

In contrast, occipital cortex ablation and sciatic nerve avulsion in rodents induce major neuronal loss in the thalamus and lumbar spinal cord, respectively. The loss of neurons in the thalamic dLGN is ~90 in adult rat and mouse at 7 days after brain injury (Martin et al., 2001; Martin and Liu, 2002; Martin et al., 2003; Martin et al., 2005; Martin et al., 2011). The loss of neurons has been demonstrated directly by stereological cell counting and has been confirmed by DNA fragmentation assays and electron microscopy. The cell death is specific for neurons, and the neurons that die early are the thalamocortically projecting dLGN neurons, as determined by prelabeling neurons undergoing DNA fragmentation with retrograde tracer (Al-Abdulla et al., 1998; Al-Abdulla and Martin, 1998; Al-Abdulla and Martin, 2002), consistent with a target deprivation induced cell death. There is a delayed degeneration of dLGN interneurons (Al-Abdulla and Martin, 2002). dLGN neuron degeneration after cortical ablation is apoptosis as determined light microscopy and electron microscopy (Al-Abdulla et al., 1998; Al-Abdulla and Martin, 1998; Al-Abdulla and Martin, 2002). Similarly, in adult rat and mouse, sciatic nerve avulsion reliably causes loss (~60%) of motor neurons in lumbar spinal cord by an unequivocal apoptotic process (Martin et al., 1999). The apoptosis in thalamus and spinal cord induced by these lesions is bax-dependent (Martin and Liu, 2002), thus implicating intrinsic direct mitochondrial death pathways, and is also driven by p53 (Martin and Liu, 2002). The neurons pass through a chromatolytic stage before they undergo apoptosis. During the pre-apoptotic stages of neurodegeneration injured motor neurons accumulate DNA-SSBs by 5 days as by comet assay (Liu and Martin, 2001), and p53 accumulates in nuclei of motor neurons destined to undergo apoptosis (Martin and Liu, 2002). p53 is activated functionally by 4-5 days postlesion, as revealed by serine³⁹²-phosphorylated p53 (Martin, 2001; Martin and Liu, 2002) that regulates its tetramerization enabling DNA binding (Kim et al., 2004). During the early chromatolytic stage of the injury response, the target deprived neurons accumulate significant intracellular Ca²⁺ and show elevated ROS production and oxidative damage to DNA and protein in a process eventuating in somatodendritic attrition (Martin et al., 2005; Martin et al., 2011).

The role of DNA damage has been vague with regard to it being a definitive direct upstream cause of neuronal cell death or a consequence of the degeneration. This study clarifies this uncertainty. We show here that enforced neuronal expression of hOGG1 and hAPE attenuates the DDR and protects against the apoptosis of neurons in these models. Conditional neuronal enforcement of hAPE produced better outcomes than enforcement of hOGG. This could mean that the accumulation of oxidatively damaged DNA and DNA-SSBs is an upstream trigger for neuronal apoptosis. However, we used wildtype hAPE which is a multifunctional protein possessing DNA BER activity and redox regulatory activity on transcription factors (Tell et al., 2009). Thus it is possible that the redox related functions of hAPE promote neuroprotection as well. Other studies have shown that modulation of APE levels can influence neuronal survival. Adenoviral mediated enforced expression of APE in mouse hippocampus protected neurons from excitotoxic cell death (Cho et al., 2010). DNA repair competent APE, but not DNA repair-disabled, protected hippocampal neurons from ischemic brain injury (Leak et al., 2015). In cell culture, DNA repair active/redox inactive APE protected hippocampal and dorsal root ganglion neurons from irradiation damage (Vasko et al., 2011), but the redox function of APE also appeared to convey some protection of neurons against oxidative stress (Vasko et al., 2005). Recombinant adenoviruses

expressing mutant variants of hAPE are necessary to decipher the contributions of DNA repair and redox activities to the neuroprotection of hAPE in our in vivo models of retrograde neuronal apoptosis.

In neurological disorders without defined genetic mutations in DDR or DNA repair-related genes, the evidence for DNA damage as etiological to the neurodegeneration is circumstantial. A clear discrepancy is evident from the neuropathological patterns seen in AT, XP, and AOA1 that are very different from those seen in most age-related, adult-onset neurodegenerative diseases. Yet, biochemical evidence for DNA-SSBs in neocortex of people with AD reveals two-fold increases compared to aged control subjects (Mullaart et al., 1990), and many cortical neurons in cases of AD show DNA-SSBs and DNA-DSBs in brain sections (Cotman and Su, 1996; Adamec et al., 1999). The A β protein, derived from aberrant processing of the integral membrane amyloid precursor protein (Martin et al., 1991) and that accumulates in the aging and AD brain (Glenner and Wong, 1984), has a neurotoxicity in cultured human primary cortical neurons appears to involve DNA damage triggered by ROS (Zhang et al., 2002). Cultured rodent cortical neurons are exquisitely sensitive to DNA damaging insults and robustly activate p53-dependent apoptosis (Park et al., 1998; Martin et al., 2009). A β might also exert effects in the AD brain by binding to and activating the triggering p53 promoter activation (Ohyagi et al., 2005). In the brains of PD patients, increased levels of DNA lesions have been detected compared to age-matched controls (Zhang et al., 1999). Transgenic mice expressing human familial PD-linked mutant α -synuclein show early accumulation of DNA damage within neurons and their mitochondria (Martin et al., 2006). DNA damage could be involved in the pathogenesis of ALS as well (Bradley and Krasin, 1982; Martin, 2001, 2008). DNA damage might be caused by oxidative stress from wildtype and mutant superoxide dismutase-1 (SOD1) gain-in-function as shown in cell culture and transgenic mouse models of ALS (Estevez et al., 1999; Martin et al., 2007, 2009; Wong and Martin, 2010). OHdG adducts are elevated in postmortem CNS tissue extracts from individuals with ALS (Fitzmaurice et al., 1996). OHdG-DNA lesions have been shown to accumulate specifically in vulnerable neurons in individuals with ALS (Martin, 2001, 2002). This finding is important because DNA damage is a strong signal for apoptosis in cells, including postmitotic differentiated neurons (Jayaraman and Prives, 1995; Park et al., 1998; Morris and Geller, 1997; Lesuisse and Martin, 2002a,b; Martin et al., 2009). The finding that p53 is overactive in ALS (Martin, 2000), further implicates DNA damage or aberrant cellular senescence as upstream pathogenic events in ALS. Indirect evidence for DNA damage in ALS is also available. The protein levels and enzyme activity of APE are elevated selectively in vulnerable regions in ALS (Shaikh and Martin, 2002). APE polymorphisms could contribute to increased risk for some forms of ALS (Olkowski, 1998; Tomkins et al., 2000). While these numerous examples suggest provocatively that DNA damage may have some role in the mechanisms of human adult onset neurodegeneration causality is still elusive. As shown here animal models appropriately applied can help resolve the uncertainty.

Conclusion

Our experiments show that conditional enforcement of hAPE and hOGG1 attenuate the DDR and exert neuroprotection in neurons known to be undergoing mitochondrial

abnormalities, oxidative stress, and DNA damage in the adult CNS. Thus DNA damage contributes mechanistically for the degeneration of different types of adult CNS neurons instead of being merely a secondary event. Modulation of DNA repair in neurons could be a therapeutic target for nervous system diseases in which DNA damage-induced neuroapoptosis contributes to the neuropathology.

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Figure 1.

Enforcement of OGG1 and APE in mouse CNS using recombinant adenovirus encoding hOGG1 and hAPE. **A-C.** Western blots showing the levels of immunoreactivity for OGG1 (A), V5-tagged hOGG1 (B), and APE in spinal cord (A,B) or brain (C) extracts of mice transduced with recombinant adenovirus encoding hOGG1 or hAPE. Ponceau S-stained membranes are used to show protein loading. **D.** Direct fluorescence image of mouse lumbar spinal cord demonstrating robust gene transduction in ipsilateral spinal motor neurons (open arrow) by unilateral injection of Ad-eGFP into gastrocnemius muscle. Scale bar = 50 μ m. **E.** Immunohistochemistry for V5-tagged hOGG1 reveals the ipsilateral expression of hOGG1 in mouse lumbar spinal cord. Scale bar = 75 μ m. **F.** Immunohistochemistry for V5-tagged hOGG1 shows the expression of hOGG1 in mouse

ventral horn spinal motor neurons. Scale bar = 40 μm . **G.** Immunohistochemistry for V5-tagged hAPE shows the expression of hAPE in mouse dLGN neurons of thalamus. Scale bar = 40 μm .

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Figure 2.

OGG1 is enriched in mouse spinal cord motor neurons. Transgenic mice expressing eGFP selectively in motor neurons (B) were used to show the localizations of DNA repair gene OGG1 (C) directly in mouse motor neurons. DAPI (A) was used as a DNA stain for the nucleus (D, asterisk). Scale bar = 10 μ m.

Figure 3.

Phospho-p53 immunoreactivity in mouse thalamus and spinal cord after occipital cortex ablation and sciatic nerve avulsion and effects of enforced expression of OGG1 and APE.

A,B. Immunohistochemistry showing the localization of phospho-p53 in non-lesioned (Contra) and in target deprived lesioned (Ipsi) dLGN. Arrows (B) identify immunopositive cells. Scale bar = 100 μ m. **C,D.** Immunohistochemistry showing the localization of phospho-p53 in non-lesioned (Contra) and in axotomized (Ipsi) spinal motor neurons. Arrows (D) identify immunopositive motor neurons. Scale bar = 40 μ m. **E,F.** Graphs of the numbers of

immunopositive dLGN neurons (E) and spinal motor neurons (F) showing nuclear phospho-p53 immunoreactivity with and without (control) enforced expression of OGG1 and APE. Asterisks denote significant difference from control (* $p < 0.05$, ** $p < 0.01$).

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Figure 4.

Phospho-RPA32 immunoreactivity in mouse forebrain and spinal cord after occipital cortex ablation and sciatic nerve avulsion. **A,B.** Parietal cortex in the non-lesioned contralateral cerebral cortex (A, arrow identifies a sharply in-focus capillary) shows less neuronal cell body phospho-RPA32 immunoreactivity compared to the lesioned ipsilateral cerebral cortex (B) where subsets of cortical pyramidal neurons show strong immunostaining (B, arrow). Scale bar (same for B,C) = 50 μ m. **C.** At locations near the vicinity of the cortical trauma, axonal swellings (arrows) are positive for phospho-RPA32 immunoreactivity, demonstrating

an axonal localization. **D.** In normal mouse hippocampus phospho-RPA32 immunoreactivity is concentrated in axonal synaptic terminal fields in CA3 (arrows) and mossy fibers (open arrow) within the dentate gyrus (DG). Scale bar = 80 μ m. **E.** Phospho-RPA32 immunoreactivity is present in axons of the normal spinal cord dorsal column white matter (Contra, arrows) and is lost in the ipsilateral dorsal column after sciatic nerve avulsion (Ipsi, arrows). Scale bar = 80 μ m.

Figure 5. Phospho-RPA32 immunoreactivity in mouse thalamus and spinal cord after occipital cortex ablation and sciatic nerve avulsion and effects of enforced expression of OGG1 and APE. **A,B.** Immunohistochemistry showing the localization of phospho-RPA32 in non-lesioned (Contra) and in target deprived lesioned (Ipsi) dLGN. Black line delineates the dLGN. Scale bar = 100 μ m. **C,D.** Immunohistochemistry showing the localization of phospho-RPA32 in non-lesioned (Contra) and in axotomized (Ipsi) spinal motor neurons. Scale bar = 80 μ m. **E,F.** High magnification images showing the cytoplasmic and nuclear (arrows) localization

of phospho-RPA32 in non-lesioned (Contra) and in axotomized (Ipsi) spinal motor neurons. Scale bar = 20 μm . **G,H.** Graphs of the numbers of immunopositive dLGN neurons (G) and spinal motor neurons (H) with phospho-RPA32 immunoreactivity with and without (control) enforced expression of OGG1 and APE. Asterisks denote significant difference from control (* $p < 0.05$, ** $p < 0.01$).

Figure 6.

Phospho-Rad17 immunoreactivity in mouse thalamus and spinal cord after occipital cortex ablation and sciatic nerve avulsion and effects of enforced expression of OGG1 and APE. **A,B.** Immunohistochemistry showing the localization of phospho-Rad17 in non-lesioned (**A**, Contra) and in target deprived lesioned (**B**, Ipsi) dLGN. Arrows (**B**) identify representative neurons with strong immunoreactivity in the ipsilateral dLGN. Scale bar = 80 μ m. **C,D.** High magnification images showing the upregulated nuclear (arrows) localization of phospho-Rad17 in ipsilateral target deprived dLGN neurons (**D**, arrows) compared to the

non-lesioned contralateral dLGN neurons (C). Scale bar = 20 μm . **E,F.** Immunohistochemistry showing the localization of phospho-RPA32 in non-lesioned (Contra) and in axotomized (Ipsi) spinal cord motor neurons. Black line delineates the ventral horn containing the motor neurons. Scale bar = 100 μm . **G,H.** High magnification images showing the cytoplasm-to-nucleus redistribution of phospho-Rad17 (arrows) in ipsilateral lesioned motor neurons (H, arrow) compared to the non-lesioned contralateral motor neurons (G, arrow). Asterisk (in G) identifies the nucleus. Scale bar = 20 μm . **I,J.** Graphs of the numbers of immunopositive dLGN neurons (I) and spinal motor neurons (J) showing nuclear phospho-Rad17 immunoreactivity with and without (control) enforced expression of OGG1 and APE. Asterisks denote significant difference from control (* $p < 0.05$, ** $p < 0.01$).

Figure 7.

Enforcement of OGG1 and APE in mouse CNS using recombinant adenovirus encoding hOGG1 and hAPE protects neurons from apoptosis. **A,B.** Nissl staining shows the loss of neurons in the ipsilateral dLGN without enforced DNA repair (A) compared to the contralateral dLGN (B). The ipsilateral dLGN appear hypercellular due to the accumulation of small inflammatory cells. Scale bar = 100 μ m. **C,D.** High magnification images showing the depletion of neurons in the ipsilateral dLGN without enforced DNA repair (C) compared to the contralateral dLGN (D). Scale bar = 40 μ m. **E,F.** Nissl staining shows that enforced

DNA repair with Ad-hOGG1 and Ad-hAPE protects the ipsilateral dLGN from neuronal loss. Scale bar = 100 μm . **G.** Nissl staining of spinal cord sections after sciatic nerve avulsion reveals the loss of motor neurons in the ipsilateral side compared to the contralateral side. Black lines delineate the ventral horn motor neuron pools. Scale bar = 100 μm . **H.** Nissl staining shows that enforced DNA repair with Ad-hAPE protects the ipsilateral motor neurons. Black lines delineate the ventral horn motor neuron pools. Scale bar = 100 μm . **I,J.** Graphs of the number of neurons in the dLGN (I) and lumbar spinal cord (J) with and without (control) enforced expression of OGG1 and APE. Asterisks denote significant difference from control (* $p < 0.05$, ** $p < 0.01$)