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Accelerated Age-Related Cognitive Decline and Neurodegeneration, Caused by Deficient DNA Repair

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Age-related cognitive decline and neurodegenerative diseases are a growing challenge for our societies with their aging populations. Accumulation of DNA damage has been proposed to contribute to these impairments, but direct proof that DNA damage results in impaired neuronal plasticity and memory is lacking. Here we take advantage of Ercc1Δ/Δ mutant mice, which are impaired in DNA nucleotide excision repair, interstrand crosslink repair, and double-strand break repair. We show that these mice exhibit an age-dependent decrease in neuronal plasticity and progressive neuronal pathologival, suggestive of neurodegenerative processes. A similar phenotype is observed in mice where the mutation is restricted to excitatory forebrain neurons. Moreover, these neuron-specific mutants develop a learning impairment. Together, these results suggest a causal relationship between unrepaired, accumulating DNA damage, and age-dependent cognitive decline and neurodegeneration. Hence, accumulated DNA damage could therefore be an important factor in the onset and progression of age-related cognitive decline and neurodegenerative diseases.

Introduction

Accumulated DNA damage is thought to be an important factor underlying aging (Hoeijmakers, 2009). Several studies show that aging is accompanied by accumulation of neuronal DNA damage in rodents and humans (Sohal et al., 1994; Hamilton et al., 2001; Dorszewska and Adamczewska-Goncerzewicz, 2004; Gedik et al., 2005). Furthermore, the brain is particularly vulnerable to oxidative stress since it exhibits very high oxygen metabolism, has abundant lipid content and relatively low levels of antioxidants compared to other organs (Cai et al., 1996; Leutner et al., 2001; Serrano and Klann, 2004; Möller et al., 2010). Therefore, neurons may be especially prone to DNA lesions resulting from oxidative stress.

Evidence linking DNA damage to cognitive impairment follows from animals and patients receiving genotoxic chemotherapeutic drugs (Ahles and Saykin, 2007; Konat et al., 2008). Additionally, mutations in DNA repair genes may cause neurological impairments, progressive neurodegeneration, and segmental accelerated aging as in a variety of progeroid conditions like xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy, which are caused by defective nucleotide excision repair (NER) (Nance and Berry, 1992; Lehmann, 2003; Kraemer et al., 2007). However, in above examples, it cannot be ruled out that the cognitive dysfunction is secondary to the large impact of chemotherapy or progeroid syndrome on overall health.

Interestingly, increased oxidative DNA damage has been observed in subjects with mild cognitive impairments as well as late-Alzheimer’s Disease (Keller et al., 2005; Wang et al., 2005; Lovell and Markesbery, 2007), suggesting a correlation between age-related accumulation of DNA damage and cognitive decline. However, a direct method to reliably quantify physiological levels of heterogeneous types of DNA damage in mammalian organs and tissues is extremely difficult (and controversial) and only possible for a very limited subset of lesions (Dizdaroglu et al., 2002). Moreover, there is a chicken and egg problem in the interpretation of studies trying to correlate neuronal degeneration with signs of DNA damage.

To circumvent these problems, we took advantage of a well-established mouse mutant with a mutation in the excision repair cross-complementing group 1 (Erc1l) gene. ERCC1 is involved in multiple DNA repair pathways: nucleotide excision repair (Houtsomuller et al., 1999), interstrand crosslink repair (Bergstrahl and Sekelsky, 2008; Bhagwat et al., 2009), and double-strand break repair (Zhu et al., 2003; Ahmad et al., 2008). Consistently, ERCC1-deficient cells show increased sensitivity to agents or
treatments that damage DNA, and hence it is expected that these animals accumulate DNA damage at a higher rate than control animals (Sijbers et al., 1996; Muñoz et al., 2005; Niedernhofer et al., 2006; Ahmad et al., 2008; Hoeijmakers, 2009). Recently it was shown that these animals have age-related neuronal changes in the spinal cord as well as in neuromuscular junctions of the skeletal muscle (de Waard et al., 2010). Here we show that global Ercc1 mutants as well as neuron-specific Ercc1 mutants exhibit an age-dependent decrease in neuronal plasticity, and progressive neuronal pathology, suggestive of neurodegenerative processes. These results suggest a causal relationship between un repaired, accumulating DNA damage and age-dependent cognitive decline and neurodegeneration.

**Materials and Methods**

**Generation and breeding of mutant mice.** The generation and characterization of nucleotide excision repair-deficient Ercc1<sup>−/−</sup> and Ercc1<sup>+/−</sup> mice has been previously described (Weeda et al., 1997). Ercc1<sup>−/−</sup> mice were obtained by crossing Ercc1<sup>+/−</sup> (in the C57BL6/J background) with Ercc1<sup>+/−</sup> mice (in the FVB background) to yield Ercc1<sup>−/−</sup> with an F1 C57BL6/J/FVB hybrid background. Wild-type littermates were used as controls. All animals used in the studies described in this paper were of the same F1 C57BL6/J/FVB hybrid background. Typical unfavorable characteristics, like blindness in an FVB background or deafness in a C57BL6/J background, do not occur in this hybrid background.

For the conditional mutant studies, we made use of a mouse line that has loxp sites inserted into its Ercc1 gene (floxed Ercc1, Ercc1<sup>fl</sup>/H11001) (Doig et al., 2006). To achieve Ercc1 gene inactivation, we used a transgenic line with Cre recombinase under the control of the CaMKII promoter (Tisien et al., 1996a,b). Expression from this promoter is specific for postmitotic excitatory neurons (Madsen et al., 2010). Ercc1<sup>−/−</sup> CaMKII-Cre<sup>+</sup> mice were obtained by crossing Ercc1<sup>−/−</sup> (in the FVB background) with Ercc1<sup>+/−</sup> CaMKII-Cre<sup>+</sup> mice (in the C57BL6/J background), to yield hybrid Ercc1<sup>−/−</sup>CaMKII-Cre<sup>+</sup> mice. Ercc1<sup>−/−</sup>CaMKII-Cre<sup>+</sup> mice are heterozygous for Ercc1 in their entire body, except for the postmitotic postmitotic neurons in the forebrain, which are homozygous for Ercc1 after Cre excision of the floxed allele. These mice will be referred to as Ercc1<sup>−/−</sup> in the remainder of the study. As controls, we used Ercc1<sup>−/−</sup> CaMKII-Cre<sup>+</sup> littermates (referred to as Ercc1<sup>−/−</sup>), which are wild type in their entire body, except for the excitatory postmitotic neurons in the forebrain, which are heterozygous. For water maze learning and fear conditioning, we also included Ercc1<sup>−/−</sup>CaMKII-Cre<sup>+</sup> control mice, which are heterozygous for Ercc1 and not different from Ercc1<sup>−/−</sup>CaMKII-Cre<sup>+</sup> mice with respect to water maze learning and fear conditioning (data not shown).

Animals were screened for discomfort and weighed once a week. Animals were maintained in a controlled environment (19–24°C, 12 h light:12 h dark cycle), received standard rodent maintenance chow and not different from Ercc1<sup>−/−</sup>CaMKII-Cre<sup>+</sup> mice with respect to water maze learning and fear conditioning (data not shown).

**Antibodies.** Primary antibodies [supplier; applications: immunohistochemistry (IHC); immunofluorescence (IF); and dilutions] reported in this study are as follows: rabbit anti-ATF3 (Santa Cruz Biotechnology; IHC; 1:1000); rabbit anti-GFAP (DAKO; IHC; 1:10,000; IF; 1:5000); rabbit anti-cleaved caspase 3 (Asp175, Cell Signaling Technology; IHC; 1:1000); mouse anti-MAP2 (clone AP20, Millipore; Sigma, IF; 1:200), rabbit anti-p53 (Leica; IHC; 1:1000), and mouse anti-NeuN (clone A60, Millipore; IF; 1:1000).

For avidin–biotin–peroxidase immunocytochemistry biotinylated secondary antibodies from Vector Laboratories, diluted 1:200 were used. FITC-, Cy3-, and Cy5-conjugated secondary antibodies raised in donkey (Jackson ImmunoResearch) diluted at 1:200 were used for confocal immunofluorescence.

**Electrophysiology.** After the animals had been killed, sagittal slices (400 μm) were made and submerged in ice-cold artificial CSF (ACSF) using a vibratome, and hippocampi were dissected out. These sagittal hippocampal slices were maintained at room temperature for at least 1.5 h to recover before experiments were initiated. Then they were placed in a submerged recording chamber and perfused continuously at a rate of 2 ml/min with ACSF equilibrated with 95% O<sub>2</sub>, 5% CO<sub>2</sub> at 31°C. ACSF contained the following (in mM): 120 NaCl, 3.5 KCl, 2.5 CaCl<sub>2</sub>, 1.3 MgSO<sub>4</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 10 glucose. Extracellular recording of field EPSP (fEPSPs) were made in CA1 stratum radiatum with platinum/iridium electrodes (Frederick Haer). A bipolar Pt/Ir was used to stimulate Schaffer–collateral/commissural afferents with a stimulus duration of 100 μs. Stimulus response curves were obtained at the beginning of each experiment, 20 min after placing the electrodes, with 10, 20, 40, 60, and 100 μs stimuli. For analyses, the data from the strongest stimulation were used. LTP was evoked using two different stimulus trains: (a) 10 Hz (1 train of 10 for 10 Hz) and (b) 100 Hz (1 train of 1 s 100 Hz). The 10 Hz protocol was performed at two-thirds and the 100 Hz protocol at one-third of the maximum fEPSP. fEPSP measurements were obtained by placing the electrodes, with 10, 20, 40, 60, and 100 μs stimuli. For analyses, the data from the strongest stimulation were used. LTP was evoked using two different stimulus trains: (a) 10 Hz (1 train of 10 for 10 Hz) and (b) 100 Hz (1 train of 1 s 100 Hz). The 10 Hz protocol was performed at two-thirds and the 100 Hz protocol at one-third of the maximum fEPSP. We used a Leica DM-DB microscope and a Leica DC300 digital camera. Sections stained for immunofluorescence were analyzed with a Zeiss LSM 510 confocal laser scanning microscope. ATF3-, p53-, and cleaved caspase 3-positive cells were quantified in the neocortex of serial parasagittal sections [1–1.5 mm lateral from the midline; corresponding to plates 109–116 in the stereotaxic atlas of Paxinos and Franklin (2001)] using an Olympus microscope fitted with a Lucivid miniature monitor (MicroBrightField). Cells were plotted in an area of 3–6 mm<sup>2</sup> (depending on the age and genotype of the mice) extending from the visual cortex to the frontal cortex and including all cortical laminae, and the number of labeled cells per square millimeter cortical area was determined.

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**Immunohistochemical and histopathological procedures.** For immunocytochemistry and immunofluorescence, mice were anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde. The brain was carefully dissected out and postfixed overnight in 4% paraformaldehyde. Routinely, brain tissue was embedded in gelatin blocks (Jaarsma et al., 2000) and sectioned at 40 μm with a freezing microtome, and sections were processed, free-floating, using a standard avidin–biotin–peroxidase complex method (ABC, Vector Laboratories) with diaminobenzidine (0.05%) as the chromogen, or single-, double-, and triple-labeling immunofluorescence (Jaarsma et al., 2000; Vlug et al., 2005). In addition, a selected number of frozen sections were processed for a silver or thionine staining procedure that selectively labels dying neurons and their processes (Jaarsma et al., 2000).

Immunoperoxidase-stained sections were analyzed and photographed using a Leica DM-DB microscope and a Leica DC300 digital camera. Sections stained for immunofluorescence were analyzed with a Zeiss LSM 510 confocal laser scanning microscope. ATF3-, p53-, and cleaved caspase 3-positive cells were quantified in the neocortex of serial parasagittal sections [1–1.5 mm lateral from the midline; corresponding to plates 109–116 in the stereotaxic atlas of Paxinos and Franklin (2001)] using an Olympus microscope fitted with a Lucivid miniature monitor (MicroBrightField). Cells were plotted in an area of 3–6 mm<sup>2</sup> (depending on the age and genotype of the mice) extending from the visual cortex to the frontal cortex and including all cortical laminae, and the number of labeled cells per square millimeter cortical area was determined.

**Water maze.** To test spatial memory, we used the water maze. Before the test, the mice were housed temporarily (24 h/in; 5 d). Occlusion of the pool was 1.2 m in diameter and has an 11 cm diameter platform submerged 1 cm below the surface. The water is painted milk-white with nontoxic paint and water temperature is kept constant at 26°C. We used dimmed lighting, and mouse tracking is performed using SMART version 2.0 (Panlab). Mice were given 2 trials/d, with 30 s intertrial interval for 5 consecutive days. At a training session, the mouse was placed on the platform for 30 s. Then it was placed in the water at a pseudorandom start position, and it was given a maximum of 60 s to find the platform. If the mouse did not find the platform in 60 s, it was placed back on the platform. After 30 s on the platform, this training procedure was performed once more. The platform remained at the same position during all trials.

One hour after the training on day 5, a probe trial was given to test spatial learning. Mice were placed on the platform for 30 s, after which the platform was removed and the mice were placed in the pool at the opposite side of the former platform location. The mice were then allowed to search for the platform for 60 s.

**Fear conditioning.** Fear conditioning was performed in a conditioning chamber (Med Associates) equipped with a grid floor via which the foot shock was administered. The floor of the conditioning chamber and the grid were divided into 4 areas of 3–6 mm<sup>2</sup> (depending on the age and genotype of the mice) extending from the visual cortex to the frontal cortex and including all cortical laminae, and the number of labeled cells per square millimeter cortical area was determined.

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done once per minute. Potentiation was measured as the normalized increase of the mean fEPSP slope for the duration of the baseline. Only stable recordings were included, and this judgment was made blind to genotype. Average LTP was defined as the mean last 10 min of each protocol. We used maximally two slices per mouse per protocol.

Results

**Ercc1** mice develop mild age-related neurodegenerative changes

To examine whether a DNA repair defect is indeed sufficient to induce age-related neurodegeneration and cognitive decline, we made use of the **Ercc1** mouse mutant. This mutant carries a null mutation in one allele, whereas the protein derived from the second allele shows reduced activity, due to a 7 aa C-terminal truncation (Weeda et al., 1997). This hypomorphic mutation results in increased sensitivity to DNA damaging treatments such as gamma and UV radiation and Mitomycin C treatment, due to severely impaired nucleotide excision repair, interstrand cross-link repair, and double-strand break repair (Weeda et al., 1997; Niederhofer et al., 2006; Ahmad et al., 2008). **Ercc1** mutants and their wild-type littermates were analyzed at 1 and 4 months of age. The age of 4 months was chosen since at this age animals display clear signs of premature aging, without significant mortality. The maximum lifespan of these mice in the genetically homogeneous F1 C57BL/6J-FVB/N hybrid genetic background used here is ~6 months (de Waard et al., 2010).

Macroscopically, the brains of **Ercc1** mice are smaller, but proportional to their reduced body size (de Waard et al., 2010). The gross histological organization analyzed in thionine stained sections appeared normal in all brain areas, including hippocampus (Fig. 1A), indicating that there is no evident developmental perturbation or massive neuronal degeneration. Immunostaining for glial fibrillary acidic protein (GFAP), which is known to be upregulated in response to neuronal injury and degeneration, showed increased GFAP staining, indicative of reactive astrogliosis, throughout the brain in 4-month-old **Ercc1** mice, while at 1 month GFAP staining was the same as in **Ercc1**/+ mice (Fig. 1A). These data indicate the occurrence of neurodegenerative changes in the **Ercc1** brain at 4 months of age but not at 1 month. Further analysis of thionine-stained sections revealed sporadic changes in pyknotic nuclei, indicative of cells undergoing apoptotic cell death, in hippocampus (Fig. 1B) as well as other brain areas of 4-month-old **Ercc1** mice. To further examine cell death, we immunostained for activated caspase 3, a final executioner caspase in multiple cell death pathways (Logue and Logue, 1999), and p53, which is known to be activated by multiple types of DNA damage and to mediate neuronal degeneration (Levine et al., 2006), and ATF3, which also is induced following genotoxic stress via p53-dependent and -independent pathways (Hai et al., 1999; Fan et al., 2002; Turchi et al., 2009). Already at 1 month of age, **Ercc1** mice showed cells with intensely p53-labeled nuclei throughout the brain, and this number was significantly increased at 4 months of age (Fig. 1C,F). Instead, no p53-positive cells were observed in the neocortical systems of **Ercc1**/+ mice. Double labeling with the neuronal marker NeuN showed that in cortex and hippocampus >95% of the p53-positive cells were neurons (Fig. 1F). ATF3 labeling, like p53, was absent in most brain areas of **Ercc1** mice except for weakly labeled neuronal nuclei in the dentate gyrus, olfactory bulb, and pyriform cortex. However, significant amounts of cells with intensely labeled ATF3 nuclei occurred in the brain of **Ercc1** mice (Fig. 1G). Similar to p53, double labeling with NeuN indicated that the vast majority of ATF3-positive cells were neurons (data not shown). The frequencies of ATF3-positive and p53-positive cells were ~10-fold the frequency of caspase 3-positive cells (Fig. 1C). Some p53- and ATF3-positive neurons displayed morphological abnormalities such as eccentric flattened nuclei, suggestive of compromised health (Fig. 1G). Together, these neuropathological data indicate that 4-month-old **Ercc1** mice show a low number of neurons in the process of dying while a greater number of neurons show signs of genotoxic stress and poor condition.

**Ercc1** mice show reduced hippocampal synaptic plasticity

We investigated basal synaptic transmission properties of **Ercc1** mice, using fEPSP measurements of the Schaffer collateral pathway. The ratio between the fEPSP slope and the fiber volley, which is a measure of the efficacy of the synapses, did not differ significantly between genotypes at either 1 month or at 4 months of age, suggesting no changes in basal synaptic transmission [ratio 2.9 ± 0.10 (n = 41 slices from 9 animals) and 2.7 ± 0.11 (n = 35 slices from 8 animals) for 1-month-old **Ercc1**/+ and **Ercc1** mice, respectively; ratio 3.5 ± 0.33 (n = 66 slices from 20 animals) and 2.9 ± 0.28 (74 slices from 20 animals) for 4-month-old **Ercc1**/+ and **Ercc1** mice, respectively; F(1,74) = 2.5, p = 0.11;
Figure 1. Young adult Ercc1<sup>−/−</sup> mice display reactive astrogliosis, mild neuronal degeneration, and signs of genotoxic stress. A, Coronal brain slices stained with thionine (upper panels) or processed for GFAP immunoperoxidase histochemistry (lower panels) illustrating normal gross histoarchitecture of the dorsal hippocampus and surrounding brain structures in Ercc1<sup>−/−</sup> mice, and increased GFAP staining throughout the brain of 4-month-old Ercc1<sup>−/−</sup> mice (scale bar, 500 μm). B, Light photomicrographs illustrating dying cells in hippocampus (CA1), cortex (NCx), and corpus callosum of 4-month-old Ercc1<sup>−/−</sup> mice. Dying cells are identified by their pyknotic nucleus in thionine-stained sections (white arrow and inset in second image), or by active caspase3 staining. In many occasions, caspase 3-positive cells can be easily differentiated in neurons (arrows) or glial cells (arrowheads; scale bar, 25 μm). C, Quantification of cortical cell density positive for ATF3, p53 or caspase 3 (y-axis on left indicates values for ATF3 and p53; y-axis on the right indicates values for caspase 3). All data are reported as mean ± SEM. Two-way ANOVA revealed a significant effect for genotype, age and their interaction for ATF3 (all \( p < 0.0001 \)), p53 (all \( p < 0.0001 \)), and caspase 3 (all \( p < 0.01 \)). D, Silver staining showing argyrophilic somatodendritic neuronal profiles indicative of dying neurons in 4-month-old Ercc1<sup>−/−</sup> hippocampus (arrows in upper row; scale bar, 50 μm), and argyrophilic degenerating axons in fimbria-fornix (FF) (arrow in middle row; scale bar, 250 μm) and striatal capsula interna (CI) bundles (arrow in lower row; scale bar, 50 μm) of 4-month-old Ercc1<sup>−/−</sup> brain. Note the absent and highly sporadic argyrophilic fiber degeneration in Ercc1<sup>+/−</sup> mice and 1-month-old Ercc1<sup>−/−</sup> brain, respectively. E, Confocal immunofluorescent images of CA1 area in the hippocampus showing unaltered level of MAP2 and increased GFAP immunoreactivity in 4-month-old Ercc1<sup>−/−</sup> mice (scale bar, 100 μm). F, Confocal image showing p53-NeuN double-labeled cells in 4-month-old Ercc1<sup>−/−</sup> cortex and hippocampus (scale bar, 50 μm). G, ATF3-immunoperoxidase histochemistry illustrating multiple ATF3-positive cells in 4-month-old Ercc1<sup>−/−</sup> cortex. Inset shows enlargement of neuron with flattened eccentric nucleus (scale bar, 100 μm). Th, Thalamus; Str, striatum; Am, amygdala; cc, corpus callosum; DG, dentate gyrus; py, pyramidal layer; rad, stratum radiatum; ml, molecular layer; gr, granule layer.
Ercc1 mice, which are homozygous Ercc1 knock-outs in aCaMKII-expressing cells (mostly excitatory postsynaptic neurons of the hippocampus and cortex) and heterozygous for Ercc1 in the remainder of their body. As controls we used Ercc1+/− CaMKII-Cre− mice, which are heterozygous for the Ercc1 gene, and Ercc1+/+ CaMKII-Cre− littermate controls, which are heterozygous for the Ercc1 gene in the aCaMKII-expressing cells, and wild type in the rest of their body (hereafter referred to as Ercc1+/−; see Materials and Methods). This breeding strategy was chosen instead of the preferred Ercc1+/− with CaMKII-Cre− breeding, because the latter breeding strategy frequently results in germ-line deletion of the floxed allele. Importantly, mice that are either heterozygous for Ercc1 (Ercc1+/− mice or Ercc1−/− mice without Cre) do not show a phenotype with respect to lifespan, body weight, general appearance, brain pathology, and synaptic plasticity (Weeda et al., 1997 and data not shown).

Like Ercc1+/− mice, the obtained Ercc1+/− mice showed an age-related increase in GFAP staining. However, increased GFAP staining was restricted to forebrain areas, in particular hippocampus and cortex (Fig. 3A), consistent with the Cre expression-dependent restricted ablation of Ercc1 in these mice. Changes in GFAP staining in hippocampus and cortex were detectable at the age of 4 and 6 months, but not yet at 2 months (Fig. 3A). Accordingly, immunohistochemistry for active caspase 3, p53, and ATF3 revealed no labeled cells in cortex and hippocampus of Ercc1+/− mice at 2 months of age, while sporadic active caspase 3-positive cells and higher levels of ATF3- and p53-positive cells occurred at 4 and 6 months of age (Fig. 3B, C). Positive cells only occurred in forebrain areas, again consistent with the restricted ablation of Ercc1. Regarding active caspase 3-labeled cells, we only observed cells with neuronal or undefined morphologies (Fig. 3B), but no labeled cells with clear glial morphologies such as observed in the brain of Ercc1+/− mice (Fig. 1B). Also ATF3- and p53-positive cells were neurons, as >95% was NeuN positive in double labeling experiments. The occurrence of neuronal degeneration in 4 and 6-month-old Ercc1−/− forebrain was further confirmed by silver degeneration staining producing sporadic argyrophilic neurons in the cortex and hippocampus, as well as argyrophilic fiber staining in the fimbria-fornix, capsule interna, and corpus callosum (data not shown).

In conclusion, these immunohistochemistry data show that the Ercc1−/− mice have pathologies in hippocampus and cortex similar to those of the Ercc1+/− mice, albeit that they develop at a somewhat slower time course, establishing a cell intrinsic cause of the neuronal phenotype and demonstrating that a DNA repair defect in forebrain neurons is sufficient to cause increased expression of markers that are indicative for genotoxic stress and neuronal degeneration.

A DNA repair defect in excitatory postnatal forebrain neurons causes gradual neuronal degeneration and reactive astrocytosis

Although the aforementioned results showed that the global Ercc1−/− mutation significantly affects neuronal health and plasticity, it cannot be ruled out that these outcomes are secondary to the reduced fitness of these animals due to liver, kidney, and other pathology (McWhir et al., 1993; Weeda et al., 1997; Selfridge et al., 2001; Lawrence et al., 2008; de Waard et al., 2010). To examine the direct effect of a DNA repair defect in neurons, and to rule out possibly confounding effects of systemic aging pathology, we made use of the Cre-loxP system to generate mutant mice with a neuron-specific ablation of Ercc1.

Ercc1−/− mice containing a floxed Ercc1 gene were crossed with Ercc1+/− CaMKII-Cre mice resulting in the desired Ercc1−/−CaMKII-Cre mice (hereafter referred to as Ercc1−/− mice), which are homozygous Ercc1 knock-outs in aCaMKII-expressing cells (mostly excitatory postsynaptic neurons of the hippocampus and cortex) and heterozygous for Ercc1 in the remainder of their body. As controls we used Ercc1+/− CaMKII-Cre− mice, which are heterozygous for the Ercc1 gene, and Ercc1+/+ CaMKII-Cre− littermate controls, which are heterozygous for the Ercc1 gene in the aCaMKII-expressing cells, and wild type in the rest of their body (hereafter referred to as Ercc1+/−; see Materials and Methods). This breeding strategy was chosen instead of the preferred Ercc1+/− with CaMKII-Cre− breeding, because the latter breeding strategy frequently results in germ-line deletion of the floxed allele. Importantly, mice that are either heterozygous for Ercc1 (Ercc1+/− mice or Ercc1−/− mice without Cre) do not show a phenotype with respect to lifespan, body weight, general appearance, brain pathology, and synaptic plasticity (Weeda et al., 1997 and data not shown).

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In conclusion, these immunohistochemistry data show that the Ercc1−/− mice have pathologies in hippocampus and cortex similar to those of the Ercc1+/− mice, albeit that they develop at a somewhat slower time course, establishing a cell intrinsic cause of the neuronal phenotype and demonstrating that a DNA repair defect in forebrain neurons is sufficient to cause increased expression of markers that are indicative for genotoxic stress and neuronal degeneration.

A DNA repair defect in excitatory postnatal forebrain neurons causes a reduction of LTP

We studied the electrophysiological properties of the Ercc1−/− mouse hippocampus to determine whether neuronal DNA damage is sufficient to cause the aging-like phenotype at the level of synaptic plasticity. Similar to Ercc1−/− mice, Ercc1−/− mice show no detectable impairment in synaptic transmission. The ratio between fEPSP slope and fiber volley does not differ at both 3 and 6 months of age [ratio 2.9 ± 0.16 (n = 36 slices from 5 animals) and 2.8 ± 0.16 (n = 55 slices from 12 animals) for 3-month-old Ercc1−/− and Ercc1+/−, respectively; ratio 2.1 ± 0.13 (n = 34 slices from 5 animals) and 1.9 ± 0.06 (63 slices from 9 animals) for 6-month-old Ercc1+/− and Ercc1−/−, respectively; F(1,89) = 2.9, p =
0.09; $F_{(1,95)} = 2.2, p = 0.14$ one-way ANOVA for 3 and 6 months old, respectively]. Next we investigated the ability to induce LTP at the Schaffer-collateral synapse using a 10 Hz stimulus. Although LTP was observed in all four groups, 6-month-old Ercc1f/- mice showed significantly less LTP than their littermate Ercc1f/+ controls, whereas no significant difference was observed for the 3-month-old mice (Fig. 4A, B; $F_{(1,33)} < 0.001, p = 1.0; F_{(1,23)} = 5.8, p < 0.05$, one-way ANOVA for 3 and 6 month old, respectively). Similar results were obtained when a strong 100 Hz stimulus was applied: 6-month-old Ercc1f/- mice but not 3-month-old mice showed significantly reduced LTP as compared to their age-matched controls (Fig. 4C, D; $F_{(1,29)} = 1.6, p = 0.20; F_{(1,30)} = 5.6, p < 0.05$, one-way ANOVA for 3 and 6 month old, respectively). Hence, by restricting the defect in DNA repair to the excitatory neurons of the hippocampus, the mice are overall healthy, but hippocampal plasticity is still affected in an age-dependent fashion.

### A DNA repair defect exclusively in neurons causes impaired cognitive function

Having shown that the Ercc1f/- mice are physically in good condition, but with regard to the hippocampus still display an accelerated aging phenotype similar to the Ercc1A/- mice, both in cellular pathology and in vitro synaptic plasticity, we tested the ability of the mutants to learn by using the Morris water maze test. In this test, which relies on a functional hippocampus, animals are trained over several days to locate a submerged platform in a circular pool filled with opaque water using distal visual cues. All four groups showed a significant reduction of their latency times to find the platform across training days (Fig. 5A, B; effect of training: $F_{(4,148)} = 28.5, p < 0.001; F_{(4,124)} = 21.4, p < 0.001$, repeated-measures ANOVA for 3 and 6 month old, respectively). There was no significant effect of genotype in both age groups (Fig. 5A, B; effect of genotype: $F_{(1,37)} = 0.2, p = 0.66; F_{(1,31)} = 3.7, p = 0.06$, repeated-measures ANOVA for 3 and 6 month old, respectively), and swim speed was not different between genotypes (18.1 ± 1.0 and 16.5 ± 0.5 cm/s for 3-month-old Ercc1f/+ and Ercc1f/-, respectively; 15.9 ± 0.3 and 16.3 ± 0.7 cm/s for 6-month-old Ercc1f/+ and Ercc1f/-, respectively; $F_{(1,37)} = 3.3, p = 0.08; F_{(1,34)} = 0.4, p = 0.51$ one-way ANOVA for 3 and 6 month old, respectively). These data indicate that mice in all four groups were able to execute this task and motivated to
expression of fear in mice. At 3 months of age, the freezing response of the Ercc1<sup>V<sup>−/−</sup></sup> mice was indistinguishable from that of their Ercc1<sup>V<sup>+/+</sup></sup> littermate controls (Fig. 5G; F<sub>1,42</sub> = 0.9, p = 0.36, one-way ANOVA). However, at 6 months the Ercc1<sup>V<sup>−/−</sup></sup> mice froze significantly less than their littermate Ercc1<sup>V<sup>+/+</sup></sup> controls (Fig. 5H; F<sub>1,40</sub> = 5.7, p < 0.05, one-way ANOVA). Neither the 3-month-old groups nor the 6-month-old groups showed a difference in baseline freezing behavior (Fig. 5G,H; F<sub>1,42</sub> = 0.5, p = 0.47; F<sub>1,32</sub> = 0.09, p = 0.77, one-way ANOVA for 3 and 6 months old, respectively), suggesting that reduced freezing is a consequence of the failure to form a contextual representation of the fear conditioning box, and not of reduced fear in general. The combination of reduced learning in the water maze, reduced levels of freezing after context conditioning, and the impaired hippocampal LTP strongly indicate impaired hippocampal function. However, we want to emphasize that the cognitive deficit is most likely not restricted to the hippocampus, but probably affects all the αCaMKII-expressing cells of the forebrain, as is also suggested by the pathological findings.

Discussion

Although accumulation of DNA damage has been put forward as a potential cause for cognitive decline, there is no direct causal proof that a defect in DNA repair can induce cognitive deficits. This study shows that defective DNA repair, either in the entire body or in neurons alone, causes an accelerated aging-like phenotype of the brain with respect to both cellular pathology and synaptic plasticity deficits. In addition, we show that in otherwise healthy animals, homozygous deletion of Ercc1 restricted to αCaMKII-expressing neurons (mostly excitatory neurons of the forebrain) is sufficient to affect learning.

Our pathological data indicate that both the global and neuron-specific Ercc1 mutant mice, while showing respectively minimal and no degenerative changes at juvenile age, develop signs of genotoxic stress and mild neuronal degeneration as well as astrocytosis during young adult life. The increased expression of GFAP, indicative of reactive astrocytosis, in Ercc1 mutant mice is also seen in the brains normally aging rats, mice, and humans (O’Callaghan and Miller, 1991; Nichols et al., 1993; Takahashi et al., 2006). In addition, the increased p53 expression we found is observed in normal aging rat brain (Chung et al., 2000; Doroszewska and Adamczewska-Goncerzwicz, 2004). Together, these results show that (neuronal) DNA damage results in brain pathology that shares characteristics with normal aging. However, it is important to point out that although the Ercc1 mutants show global functional and structural deterioration; some aspects of neuroaging are not modeled, such as accumulation of lipofuscin and the aggregation of proteins.

The pattern and time course of neuronal degeneration is compatible with a model where neurons are afflicted by stochastic DNA lesions that can either cause neuronal degeneration by blocking or deregulating expression of essential genes, or trigger genotoxic stress response pathways, for instance, by stalling of RNA polymerase II (de Waard et al., 2010; Garinis et al., 2009; Mitchell et al., 2003; Hoeijmakers, 2009; Nouspikel, 2007; Brooks, 2008). Ercc1 mutants show impaired nucleotide excision repair, interstrand crosslink repair, and double-strand break repair, and hence may accumulate different types of DNA lesions (Houtsmuller et al., 1999; Zhu et al., 2003; Ahmad et al., 2008; Bergstrahl and Sekelsky, 2008; Bhagwat et al., 2009). Oxidative base

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Figure 4. *Ercc1<sup>V<sup>−/−</sup></sup>* mice show reduced synaptic plasticity at 6 months of age. A, LTP (100 Hz) in 3-month-old mice shows no difference between *Ercc1<sup>V<sup>−/−</sup></sup>* and *Ercc1<sup>V<sup>+/+</sup></sup>* mice (n = 16 slices from 5 animals, n = 19 slices from 8 animals for *Ercc1<sup>V<sup>−/−</sup></sup>* and *Ercc1<sup>V<sup>+/+</sup></sup>* , respectively). B, LTP (100 Hz) in 6-month-old mice shows reduced LTP in *Ercc1<sup>V<sup>−/−</sup></sup>* mice (n = 14 slices from 4 animals and n = 11 slices from 5 animals for *Ercc1<sup>V<sup>−/−</sup></sup>* and *Ercc1<sup>V<sup>+/+</sup></sup>* , respectively). C, LTP (100 Hz) in 3-month-old mice shows no difference between *Ercc1<sup>V<sup>−/−</sup></sup>* and *Ercc1<sup>V<sup>+/+</sup></sup>* mice (n = 13 slices from 4 animals, n = 18 slices from 10 animals for *Ercc1<sup>V<sup>−/−</sup></sup>* and *Ercc1<sup>V<sup>+/+</sup></sup>* , respectively). D, LTP (100 Hz) in 6-month-old mice shows reduced LTP in *Ercc1<sup>V<sup>−/−</sup></sup>* mice (n = 15 slices from 5 animals and n = 17 slices from 7 animals for *Ercc1<sup>V<sup>−/−</sup></sup>* and *Ercc1<sup>V<sup>+/+</sup></sup>* , respectively). All data are reported as mean ± SEM. Filled circles represent *Ercc1<sup>V<sup>−/−</sup></sup>*. Open circles represent *Ercc1<sup>V<sup>+/+</sup></sup>*. Significantly different (p < 0.05) from age-matched *Ercc1<sup>V<sup>−/−</sup></sup>* mice.
DNA damage such as 7,8-dihydro-8-oxoguanine (8-oxoG) is repaired by the base excision repair pathway and may not accumulate in Ercc1 mutant mice. However, other oxidative lesions such as malondialdehyde adducts and 8,5'--cyclopurine-2'-deoxyribose nucleotides are both NER substrates and potential threats to transcription (Fishel et al., 2007; Nousiainen & Pääbo, 2008). However, directly determining physiological levels of heterogeneous types of DNA damage in mammalian organs and tissues in a quantitatively reliable manner is extremely challenging and reliable for a very limited subset of lesions (Dizdaroglu et al., 2002; Himmelstein et al., 2009). Hence, the precise lesions that accumulate in Ercc1-deficient neurons remain to be determined.

In addition to the neuropathological changes, we observed an age-dependent impairment in synaptic plasticity in the global Ercc1 mutant and in synaptic plasticity as well as learning in the neuron-specific Ercc1 mutant. The strong resemblance of phenotypes in the global and the neuron-specific Ercc1 mutants indicate that synaptic plasticity and learning defects in the global Ercc1−/− mutant result from the Ercc1 deficiency in principal forebrain neurons, rather than from indirect effects in other tissues.

How does the lack of a fully functional DNA repair system cause a reduction of LTP and impaired learning? There are several possibilities. For instance, it could be a direct result of the observed neuronal degeneration. However, this seems unlikely, because as judged by histology, the overall loss of excitatory neurons appears to be very limited. This is in agreement with the electrophysiology, which showed no significant change in the presynaptic nerve terminal volley (a measure for the number of stimulated fibers) and no change in the size of the excitatory postsynaptic potential (EPSP) as a function of stimulation strength (a measure for both the number of activated neurons and the efficacy of synaptic transmission). In addition, since LTP is a relative measure of change of synaptic strength, neurons will only contribute to this measure as long as they are able to show synaptic transmission. Hence, LTP will not be affected by a decreased number of neurons. It is therefore more likely that unpaired DNA damage interferes with transcription of genes necessary for normal neuronal functioning, which results in decreased neuronal plasticity. In this respect, it is interesting to note that the induction phase of LTP appears to be normal in the Ercc1 mutants but that the later phase is affected. This impairment coincides with the start of the mRNA and protein synthesis-dependent phase of LTP (called late phase LTP; L-LTP) (Kelleher et al., 2004). Notably, L-LTP has been shown to be affected in aged animals (Bach et al., 1999).

Alternatively, the deficits in plasticity and learning could arise from impaired insulin-like growth factor 1 (IGF1) signaling. Both DNA damage and aging result in a reduction of the somatotroph axis by downregulating growth hormone (GH)/IGF1 signaling (Niedernhofer et al., 2006; van de Ven et al., 2006; van der Pluijm et al., 2007; Garinis et al., 2009), which appears to operate in a cell autonomous way, presumably via DNA damage-induced stalling of RNA polymerase II (Garinis et al., 2009), which appears to operate in a cell autonomous way. It has been shown that IGF1 regulates synaptic plasticity in the adult CNS (Torres-Aleman, 1999; Sonntag et al., 2000) and that age-related behavioral impairments can be alleviated by IGF1 (Markowska et al., 1998; Shi et al., 2005), thereby suggesting that IGF1 reduction could be a factor in age-related reduction of synaptic plasticity. In addition, it has recently been shown that the related family member IGF2 facilitates the sta-
bility of LTP and is critical for memory consolidation (Chen et al., 2011).

Finally, we cannot exclude that the plasticity deficits are caused by reactive glia, because even though the homozygous Ercc1 gene deletion was restricted to αCaMKII-expressing neurons, we observed a robust age-dependent increase of GFAP staining, indicative of reactive astrocytosis. GFAP is known to be upregulated in response to neuronal injury and degeneration, and indeed, increased GFAP staining was not observed in wild-type and heterozygous Ercc1 mutants. To what extent reactive astrocytes can contribute to LTP and learning impairment remains, however, to be investigated (Wenker, 2010).

The observed plasticity deficits and behavioral phenotype of the Ercc1 mouse, parallel findings in aged animals, as impairments in LTP, contextual conditioning, and spatial learning are commonly observed in aged rodents (Bach et al., 1999; Foster et al., 2001; Liu et al., 2002; Watson et al., 2002; Barnes, 2003; Blalock et al., 2003; Blank et al., 2003; Erickson and Barnes, 2003; Foster et al., 2003; Rosenzweig and Barnes, 2003; Verbistsky et al., 2004; Moyer and Brown, 2006; Kaczorowski and Disterhoft, 2009). In addition, some AD mouse models also show reduced LTP and learning (Lambert et al., 1998; Chapman et al., 1999; Walsh et al., 2002; Wang et al., 2002; Jacobsen et al., 2006; Laurén et al., 2009). Also, humans show an age-related deficit in performance on a virtual Morris water maze (Driscoll et al., 2003).

Together, our results show that unrepaired DNA damage is sufficient to cause progressive neuronal pathology, neuronal plasticity deficits, and cognitive decline. The phenotype of the Ercc1 mutants is reminiscent of the global structural and functional deterioration associated with aging. Therefore, we propose that the mouse model(s) mimic(s) aspects of neuroaging. Further experiments are required to more precisely determine which types of toxic DNA lesions accumulate in the Ercc1 nervous system, to which extent these lesions occur in the aging brain, and by which mechanisms these lesions affect neuronal function. Nevertheless, at this point the mice represent a unique model system to identify conditions that accelerate or prevent the accumulation of toxic DNA lesions. The results of these studies may prove to be important for the development of better therapeutic strategies to battle age-related cognitive decline or to prevent the devastating effects of neurodegenerative diseases.

References


