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Malfunctioning DNA Damage Response (DDR) Leads to the Degeneration of Nigro-Striatal Pathway in Mouse Brain

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Abstract Pronounced neuropathology is a feature of ataxia-telangiectasia (A-T) and Nijmegen breakage syndrome (NBS), which are both genomic instability syndromes. The Nbs1 protein, which is defective in NBS, is a component of the Mre11/RAD50/NBS1 (MRN) complex. This complex plays a major role in the early phase of the cellular response to double strand breaks (DSBs) in the DNA. Among others, MRN is required for timely activation of the protein kinase ATM (A-T mutated), which is disrupted in patients with A-T. Earlier reports show that Atm-deficient mice exhibit severe degeneration of tyrosine hydroxylase (TH)-positive dopaminergic nigro-striatal neurons and their terminals in the striatum. This cell loss is accompanied by a large reduction in immunoreactivity for the dopamine transporter protein (DAT) in the striatum. To test whether Nbs1 inactivation also affects the integrity of the nigro-striatal pathway, we examined this pathway in a murine model with conditional inactivation of the *Nbs1* gene in central nervous system (Nbs1-CNS-Δ). We report that this model has a reduction in TH-positive cells in the substantia nigra. This phenomenon was seen at very early age, while Atm−/− mice showed a progressive age-dependent reduction. Furthermore, we observed an age-dependent increase in the level of TH in the striatum of Atm−/− and Nbs1-CNS-Δ mice. In addition to the altered expression of TH, we also found a reduction of DAT in the striatum of both Atm−/− and Nbs1-CNS-Δ mice at 60 days of age. Finally, microglial recruitment and alterations in the levels of various neurotrophic factors were also observed. These results indicate that malfunctioning DNA damage response severely affects the integrity of the nigro-striatal pathway and suggest a new neurodegenerative pathway in Parkinsonian syndromes.

Keywords Ataxia-telangiectasia · Nijmegen breakage syndrome · Atm · Nbs1 · DNA damage response · Nigro-striatal pathway · Dopaminergic neurons · Glial cells

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Introduction

All prokaryote and eukaryote cells are continuously exposed to exogenous and endogenous agents that damage DNA. The many types of DNA lesions that result from these insults are rapidly detected, leading to activation of an intricate web of signaling pathways known as the DNA damage response (DDR) (Stucki and Jackson 2004; Harrison and Haber 2006; Su 2006). This response culminates in activation of cell-cycle checkpoints and appropriate DNA repair pathways, and, in certain contexts, initiation of programmed cell death especially in the

CNS (Barzilai et al. 2008). The DNA damage response is a hierarchical process that is executed through a series of steps; The damage is detected by *sensor* proteins (Stucki and Jackson 2004; Hu et al. 2005; Zgheib et al. 2005; Zhang and Powell 2005; Kim et al. 2006; Stucki and Jackson 2006) that recognize the lesions themselves or chromatin alterations that may follow their induction. Transducers (Shiloh 2003; Shechter et al. 2004; Shiloh and Lehmann 2004; Shiloh 2006; Hurley and Bunz 2007) are brought into action to convey the damage signal to downstream *effectors* (Harrison and Haber 2006; Shiloh 2006; Su 2006; Hurley and Bunz 2007). This relay system from transducers to effectors enables a few DNA lesions to modulate numerous pathways. The transducers might also be involved in the assembly of DNA repair complexes at the sites of DNA damage (Shiloh 2003; Bassing and Alt 2004; Lieber et al. 2004; Wyman and Kanaar 2006). Defects in the repair of (or response to) DNA damage have been associated with severe genetic disorders that usually involve cancer predisposition (Bassing et al. 2003; Celeste et al. 2003; Varley and Haber 2003; Varley 2003; Arai et al. 2004; Narod and Foulkes 2004; Staalesen et al. 2004; Tort et al. 2005), immunodeficiency (O'Driscoll et al. 2004), and neurodegeneration (Thompson and West 2000; Caldecott 2004; Barzilai 2007; Lee and McKinnon 2007). Defective DDR in proliferating cells can lead to cancer, while DDR defects in neurons may result in neurodegeneration. Mature neurons are highly differentiated, post-mitotic cells that cannot be replenished after disease or trauma. Their high metabolic activity generates large amounts of reactive oxygen species with DNA damaging capacity. Moreover, their intense transcriptional activity increases the potential for genomic DNA damage. Respectively, neurons have elaborate mechanisms to defend the integrity of their genome, thus ensuring their longevity and functionality in the face of these threats. Until recently, most DDR research was focused on neuronal cells. However, over the past two decades, there has been a substantial increase in our understanding of the role of glial cells in supporting the neuronal cell DDR and longevity (Barzilai 2010).

Loss or inactivation of ATM leads to a severe genomic instability syndrome, ataxia-telangiectasia (A-T). A-T is characterized by progressive cerebellar degeneration, immunodeficiency, genome instability, gonadal dysgenesis, extreme radiosensitivity, and high incidence of lymphoreticular malignancies (Chun and Gatti 2004; Lavin 2008). One of the most severe symptoms of A-T—cerebellar ataxia—develops progressively into general motor dysfunction; it is accompanied by a marked loss of Purkinje and granule neurons in the cerebellum (Chun and Gatti 2004; Biton et al. 2008). Interestingly, Atm deficiency in mice is also associated with degeneration of the nigro-striatal pathway. Eilam et al. (1998) found that Atm-deficient mice exhibit severe degeneration of tyrosine hydroxylase-positive (TH), dopaminergic nigro-striatal neurons, and their terminals in the striatum. This cell loss is accompanied by a large reduction in immunoreactivity for dopamine transporter in the striatum (Eilam et al. 1998). Eilam et al. (1998) also reported that dopaminergic neurons were formed normally in the Atm−/− mice and lost during the first few months (Eilam et al. 2003).

Hypomorphic *NBS1* mutations underlie the Neijmegen breakage syndrome (NBS), characterized by immunodeficiency, genomic instability, radiation sensitivity, and predisposition to lymphoid malignancies. Interestingly, the neurological manifestations of NBS include microcephaly and mental deficiency rather than cerebellar degeneration and ataxia (Digweed and Sperling 2004). A recent study showed that DNA signaling in the nervous system differs between A-TLD and NBS and may explain their different neuropathologies (Shull et al. 2009).

A major tool in the investigation of human genetic disorders is the corresponding knockout murine model. *Atm*-deficient mice exhibit many of the characteristics of human A-T; retarded growth, immunodeficiency, cancer predisposition, radiosensitivity, infertility, and a cellular phenotype similar to that of A-T cells. With that said, they barely show the most cardinal feature: namely, neuronal degeneration and associated neuromotor dysfunction (Barlow et al. 1996; Elson et al. 1996; Xu et al. 1996; Borghesani et al. 2000). On the other hand, careful analysis of these mice show subtle neurological abnormalities in several instances (Kuljis et al. 1997; Eilam et al. 1998; Barlow et al. 1999; Watters et al. 1999; Borghesani et al. 2000; Chiesa et al. 2000; Allen et al. 2001; Eilam et al. 2003; Watters 2003; Mount et al. 2004). Furthermore, there is a reduction in in vitro survival of Atm−/− Purkinje cells and their dendritic branching compared to wild-type (WT) cells (Chen et al. 2003; Gueven et al. 2006).

To analyze the role of the Nbs1 protein in the CNS, the NBS1 gene was specifically inactivated in the murine central nervous system (Nbs1-CNS-Δ) (Frappart et al. 2005). This mouse exhibits a dramatic neurological phenotype that combines the microcephaly typical of human NBS patients with proliferation arrest of granule cell progenitors and apoptosis of post-mitotic neurons in the cerebellum that leads to severe ataxia. This phenotype can be explained by the requirement of the MRN complex for processes that are not under ATM control, such as ATR-dependent branches of the DDR (Uziel et al. 2003; Falck et al. 2005; Stiff et al. 2005; Adams et al. 2006; Myers and Cortez 2006). This valuable mouse model facilitates investigation of the role of the DDR in the nervous system, which is critical for understanding the

molecular pathogenesis of A-T. Furthermore, we recently reported that conditional inactivation of the murine Nbs1 gene has a profound effect on the integrity and the functionality of the glial cells, which suggests their crucial role in the pathogenesis of NBS. Interestingly, in Nbs1-CNS-Δ mice, the dramatic reduction in the numbers of Purkinje and granule cells was also linked to a reduction of microglial cells but not to astrocytes (GFAP+), suggesting an impairment in astrocytic functionality. Indeed, Nbs1 levels were dramatically reduced in adult astrocyte isolated from Nbs1-CNS-Δ mice, suggesting a major role in cerebellar pathology (Galron et al. 2011).

The nigrostriatal pathway is a neural pathway that connects the substantia nigra with the striatum. It is one of the four major dopamine pathways in the brain, and is particularly involved in the production of movement, as part of a system called the basal ganglia motor loop (Fallon and Moore 1978). Loss of dopamine neurons in the substantia nigra is one of the main pathological features of Parkinson disease, leading to a marked reduction in dopamine function in nigrostriatal pathway. The symptoms of the disease typically do not show themselves until 80–90% of dopamine function has been lost (Gaig and Tolosa 2009).

Here we report that malfunctioning DDR leads to the degeneration of the nigro-striatal pathway, which was associated with reduced TH-positive cells and alterations in TH and DAT levels in the striatum. In addition, we found reduced functionality of astroglial cells which was accompanied by the generation of oxidative stress, a significant reduction in microglial recruitment and alterations in the levels of various neurotrophic factors.

Experimental Procedures

Generation of Various Nbs1-CNS-Δ and Atm−/− Genotypes

Atm+/− mice (SV129 background) (Barlow et al. 1996) were a generous gift from Dr. Anthony Wynshaw-Boris (University of California, San Diego, CA). Offspring of these mice were genotyped using PCR-based assays based on mouse-tail DNA prepared with the GenElute Mammalian Genomic DNA Miniprep kit (Sigma, St. Louis, MO). Mice (129/Sv and C57BL/6 background) with conditional inactivation of the *Nbs1* gene in CNS (Nbs1-CNS-Δ) were generated by taking *NBS1* exon 6, floxed by two loxP sites, resulting in *Nbs1*-floxed mice. Since the nestin protein is specifically expressed in the CNS (neurons and glia), the *Nbs1* gene can be specifically deleted in the nervous system when crossing the *Nbs1*-floxed mice with Nestin-Cre transgenic mice (Frappart et al. 2005).

Atm−/− mice (Barlow et al. 1996) were a generous gift from Dr. Anthony Wynshaw-Boris (University of California, San Diego, CA). Offspring of these mice were genotyped using PCR-based assays based on mouse tail.

Genotyping Using PCR

At the age of 3 weeks (after weaning), the mice were marked by a numbered earring, and a piece of 0.5 cm of the tip of the tail was taken to genotype each mouse. Mice were genotyped by PCR-based assays using mouse-tail DNA, prepared with the Genelute mammalian genomic DNA kit (Sigma) according to the manufacture's protocol. PCR-based reaction was done following DNA preparation. Products of the reaction were separated on 2% agarose gel, in TAE running buffer at 120 V for 30–50 min, and visualized with ethidium bromide under UV light.

Striatum and Cerebellum Desiccations

Striatum desiccations were performed on Nbs1-CNS-Δ, Atm−/−, and WT mice at the age of 10, 30, and 60 days old mice. The cerebella of the WT and mutant mice were dissected at the age of 60 days.

Total Protein Extracts

Striatal tissue was washed with ice-cold phosphate-buffered saline and homogenized in ice-cold homogenation buffer [for total striatum, 120 mM NaCl, 50 mM Tris buffer pH 7.4, 1 mM EDTA, 0.5% NP40, 1:50 phosphatase inhibitor cocktail (I and II Sigma), and 1:100 protease inhibitor cocktail. Protein concentration was determined according to the method of Bradford using BSA as standard.

Odyssey Analysis of Mid-saggital Brain Sections

The sections were fixed in 4% fixative (4% formaldehyde in PBS) for 10 min and placed in PBS. The sections were then washed in PBS for an additional 10 min and later incubated with blocking solution containing 1% BSA (Sigma, St. Louis, MO) and 10% normal donkey serum (NDS) (Jackson ImmunoResearch, Baltimore, MD) in PBS for 1 h at room temperature. The sections were incubated overnight with a primary antibody (see relevant section) in 0.25% Triton X100 at 4°C. The slides were then washed three times with PBS and incubated with the appropriate secondary antibody (Li-Cor antibody) for 1 h at room temperature. After one wash with PBS, sections were mounted with aqueous mounting medium containing anti-fading agents (Biomeda, Burlingame, CA). Observations and photography were carried out using a Li-Cor imaging system.

Quantitative RT-PCR Analysis

Two retinas from each animal were pooled and subjected to RNA extraction using MasterPure RNA Purification kit (Tamar MCR85102). Total RNA concentration was determined with nanodrop analysis assay (260/280 nm), and the purity of the extraction was further determined (240/260 nmN1.9). The total RNA concentration was diluted with molecular water (DNase, RNase free) (Sigma W4502) to achieve 500 ng, which was later converted into cDNA using Verso cDNA kit. The quantification of NBS1 mRNA expression was conducted via reverse transcription followed by quantitative real-time polymerase chain reaction (QRT-PCR) using TaqMan (Absolute Blue QPCR ROX Mix, Tamar AB-4138). Reverse transcriptase PCR assays were designed by Applied Biosystems (Carsland, CA) and compared with β-actin (ACTB) RNA levels. The comparative Ct method was used for quantification of transcripts according to the manufacturer's protocol. The primers used were Nbs1 MM01210905M1 (Applied Biosystem, Carsland, CA). Measurement of ΔCt was performed in duplicates or triplicates. All reactions were performed with primer concentration of 0.25 μM in a total volume of 10 μl reaction.

Tissue Preparation

Mouse brains were dissected and fixed in 4% fixative (4% formaldehyde in PBS) for 24 h. The brains were then infiltrated for cryo-protection with 30% sucrose (Merck) for 24 h at 4°C. Fixed brains were embedded in tissue freezing medium (Leica Instruments GmbH, Nussloch, Germany) and quickly frozen in liquid nitrogen. Cross-sections (10 μM) were placed on subbed slides (0.5% gelatin, containing 0.05% chromium potassium sulfate) and stored at −20°C.

Immunostaining of Paraffin Sections

Immunostaining of paraffin-embedded tissue was performed as previously described (Chen et al. 2003; Gueven et al. 2006). Briefly, paraffin sections of cerebella or striata from WT and Nbs1-CNS-Δ mice were dewaxed, digested with trypsin, and stained for nitrotyrosine (Cell Signalling; No. 9691; 1:50).

Immunohistochemical Analysis of Mesencephalic Sections

Sections were washed in PBS for 30 min and then incubated with blocking solution containing 1% BSA (Sigma, St. Louis, MO) and 10% normal donkey serum (NDS) (Jackson ImmunoResearch, Baltimore, MD) in PBS for 1 h at room temperature. The sections were incubated overnight with a primary antibody (see relevant section) in 0.25% Triton X-100 at 4°C. The slides were washed three times with PBS and incubated with the appropriate secondary antibody for 1 h at room temperature. After one wash with PBS and two washes in a buffer containing 10 mM Tris/1 mM EDTA, the sections were incubated with the nucleic acid dye Sytox blue (Molecular Probes, Invitrogen, Carlsbad, Germany) for 30 min. Slides were washed three times with the same buffer and mounted with aqueous mounting medium containing anti-fading agents (Biomeda, Burlingame, CA). Observations and photography were carried out with a Zeiss (Oberkochen, Germany) LSM 510 META confocal microscope.

Statistical Analysis

Statistical analysis was performed using two-tailed Student's *t* test.

Results

It has been suggested that neuronal attrition in A-T patients stems primarily from cytoplasmatic functions of ATM (Watters et al. 1999; Watters 2003; Li et al. 2009). Based on our previous results (Dar et al. 2006, 2011; Levine-Small et al. 2011), we hypothesized that malfunctioning DDR is the main cause for neuronal degeneration in genomic instability disorders including A-T. However, Atm-deficient mice display very mild cerebellar defects but exhibit severe age-dependent degeneration of tyrosine hydroxylase-positive, dopaminergic nigro-striatal neurons (Eilam et al. 1998, 2003). In contrast, specific inactivation of the *Nbs1* gene in the CNS caused marked cerebral, cerebellar, and retinal attrition in the Nbs1-CNS-Δ mice (Frappart et al. 2005; Assaf et al. 2008; Baranes et al. 2009; Galron et al. 2011). The fact that Nbs1, which is an exclusive component of the DDR machinery and one of the Atm activators (Uziel et al. 2003; Falck et al. 2005; Dar et al. 2011) plays such an essential role in CNS homeostasis can serve as a good model system to test our hypothesis that malfunctioning DDR is indeed the major contributor to CNS degeneration observed in genomic instability disorders.

Malfunctioning DDR Causes Substantia Nigra Attrition

The nigro-striatal pathway is composed of dopamiergic neurons, which reside in the substantia nigra and form synapses in the striatum. To further study the role of malfunctioning DDR on the nigro-striatal pathway, we exposed brain slices derived from 2-month-old WT, Atm−/−,

and Nbs1-CNS-Δ mice to anti-TH antibody and analyze the TH levels in the substantia nigra pars compacta (SNc), ventral tegmental area (VTA), and the striatum. Figure 1 shows that malfunctioning DDR markedly reduces the size of the SNc in both Atm−/− and Nbs1-CNS-Δ brains. However, high levels of TH immunoreactivity were detected in the striata of Atm−/− and Nbs1-CNS-Δ mice. These findings imply that malfunctioning DDR is capable of severely reduced the TH immunoreactivity in mutant mice substantia nigra, thereby hampering the nigro-striatal pathway.

Reduction in TH-Positive Neuron Staining in SNc of Atm−/− and Nbs1-CNS-Δ Mice

To quantify the amount of dopaminergic neurons in the various genotypes as a function of age, we stained mesencephalic slices with anti-TH antibodies and counted the TH-positive cells in a series of slices. Whereas specific inactivation of Nbs1 resulted in significant reduction of TH-positive cells starting at the age of 10 days, Atm deletion led to significant decrease in TH-positive cells only from the age of 1 month (Fig. 2). Significant reduction in the number of TH-positive cells was detected at the age of 2 months in both models. These results suggest that Nbs1 conditional deletion causes earlier attrition of TH-positive cells as compared to Atm−/− mice.

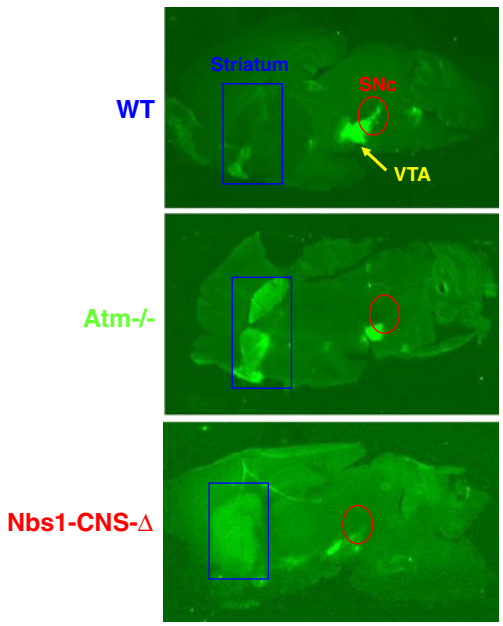

Fig. 1 Atm and Nbs1 inactivation alters the TH expression levels. Mid-saggital sections derived from 2-month-old WT, Atm, and Nbs1-CNS-Δ, immunoreacted with dopaminergic cells marker (anti-TH antibody). The TH expression levels in the various genotypes were analyzed using Odyssey system

Alterations in the TH Protein Levels in the Striata of Atm−/− and Nbs1-CNS-Δ Mice

The immunohistochemical analysis of the 2-month-old mesencephalic slices revealed a high level of TH immunoreactivity in the striata of Atm−/− and Nbs1-CNS-Δ mice. To temporally quantify the alterations in the level of the TH protein, we extracted total proteins from the striata of the WT and the mutant mice and analyzed them using western blot analysis. We found reduced levels of the TH protein in the striata of Atm-deficient and Nbs1-CNS-Δ mice at the age of 10 days and 1 month (Fig. 3). In agreement with the immunohistochemical analysis (Fig. 1), we found a significant increase in the levels of TH protein in the striata of 2-month-old Atm−/−, Nbs1-CNS-Δ mice (Fig. 3).

Reduction in Dopamine Transporter Protein (DAT) Levels in the Striatum of 2-Month-Old Mice Atm−/− and Nbs1-CNS-Δ

Alterations in the levels of the striatal TH immunostaining can be explained either by a specific reduction in the number of dopaminergic fibers in the striatum following the attrition of the dopaminergic cell bodies in the SNc or down-regulation of TH in existing dopaminergic fibers. To distinguish between these two possibilities, we analyzed the levels of DAT protein in the striatum and used this protein as a selective marker of dopaminergic terminals. Western blot analysis revealed a significant reduction in the level of the striatal DATprotein in Atm-deficient and Nbs1-CNS-Δ at the age of 2-month-old mice (Fig. 4). No alterations in the striatal DAT protein were found in 10-day-old Atm−/− and Nbs1-CNS-Δ mice (Fig. 4).

Generation of Oxidative Stress in the Cerebellum and Substantia Nigra of Nbs1-CNS-Δ Mice

Atm deficiency in the CNS leads to the generation of oxidative stress (Barlow et al. 1999; Kamsler et al. 2001; Stern et al. 2002; Gueven et al. 2006). It has been proposed that Atm can function as redox sensor (Kruger and Ralser 2011), can activate catalase in the peroxisomes (Watters et al. 1999) or directly sense or modulate redox homeostasis (Watters 2003). To assess whether alterations in the redox state stem primarily from the accumulation of unrepaired DNA damage, we analyze the redox state in the cerebellum and substantia nigra derived from Nbs1-CNS-Δ mice. We previously reported that Nbs1 inactivation leads to cerebellar atrophy (Frappart et al. 2005; Assaf et al. 2008; Dar et al. 2011). Unlike Atm, Nbs1 is a component of the MRN complex and functions exclusively in DDR machinery. Using nitrotyrosine as a marker of oxidative stress, we found an increased level of oxidative damage from undetected

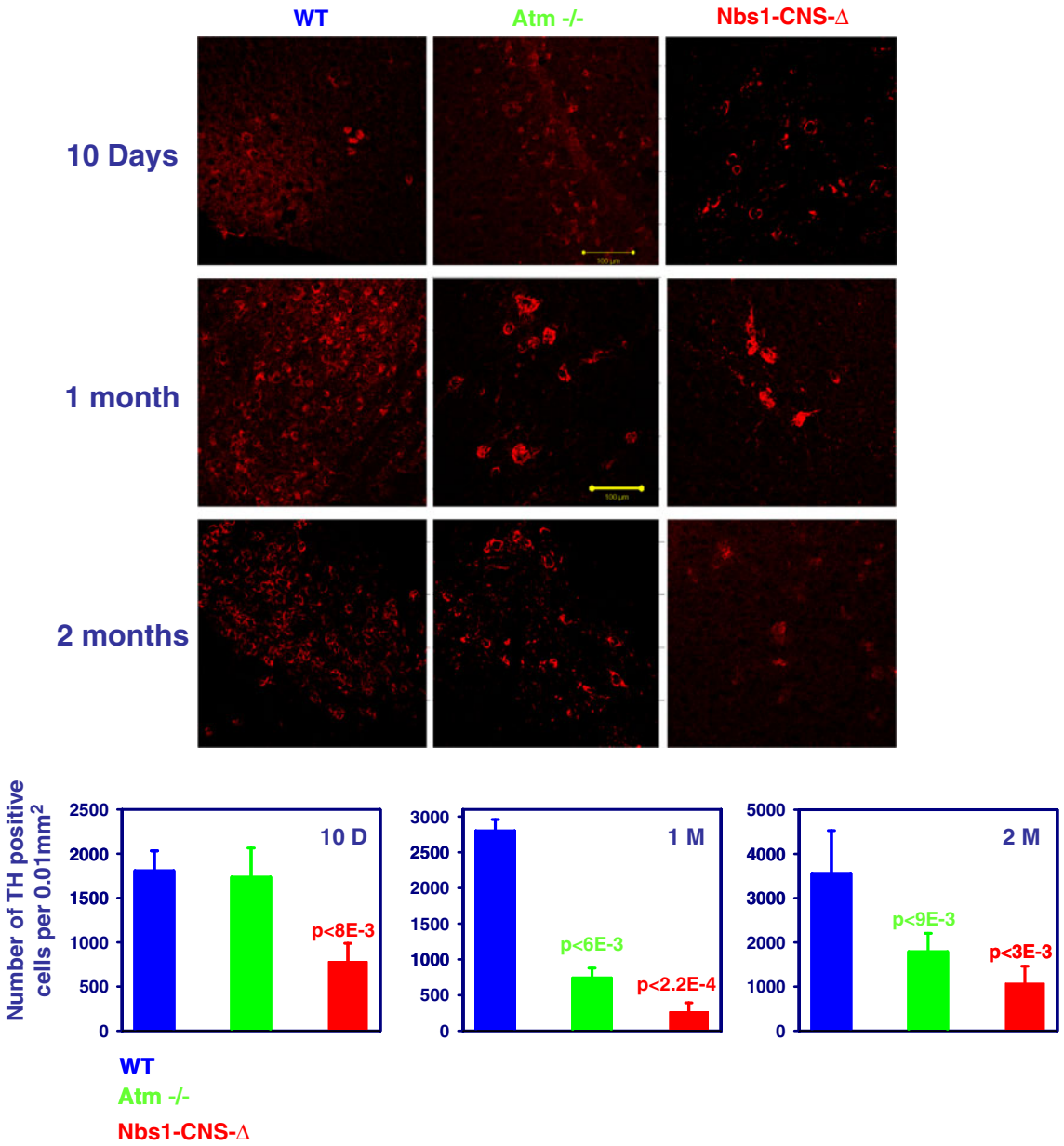

Fig. 2 Atm and Nbs1 inactivation associates with reduction of TH-positive cells in SNc derived from the mutant mice. Mesencephalic sections were dissected from the various genotypes at the indicated ages, were fixed and the frozen section stained with anti-TH antibody. The TH-positive cells (*red*) were visualized using confocal microscopy and counted. *Bar*=100 μm

levels in WT to marked staining in the cerebella and substantia nigra derived from the Nbs1-CNS-Δ mice (Figs. 5 and 6). Collectively, these results strengthen the notion that the accumulation of unrepaired DNA damage is the main cause for the generation of oxidative stress.

Reduced Levels of Nbs1 in Astrocytes may Affect Their Activity in Nbs1-CNS-Δ Mice

Eilam et al. (2003) reported massive gliosis in the SNc, striatum, and other parts of the brain of 5–6-month-old Atm−/− mice. By tracking glial fibrillary acidic protein (GFAP), an intermediate filament protein found in the cytoskeleton of astrocyte, we could determine whether malfunctioning DDR can lead to gliosis (Streit 2006). We report high levels of GFAP, especially at the age of 10 days and some moderate elevation at 1 and 2-month-old Nbs1-CNS-Δ mice (Fig. 7). No signs of gliosis were detected in Atm−/− mice up to the age of 2 months (Fig. 7). These data suggest that specific deletion of Nbs1 in the CNS may generate early gliosis in the striatum compared to Atm−/− mice.

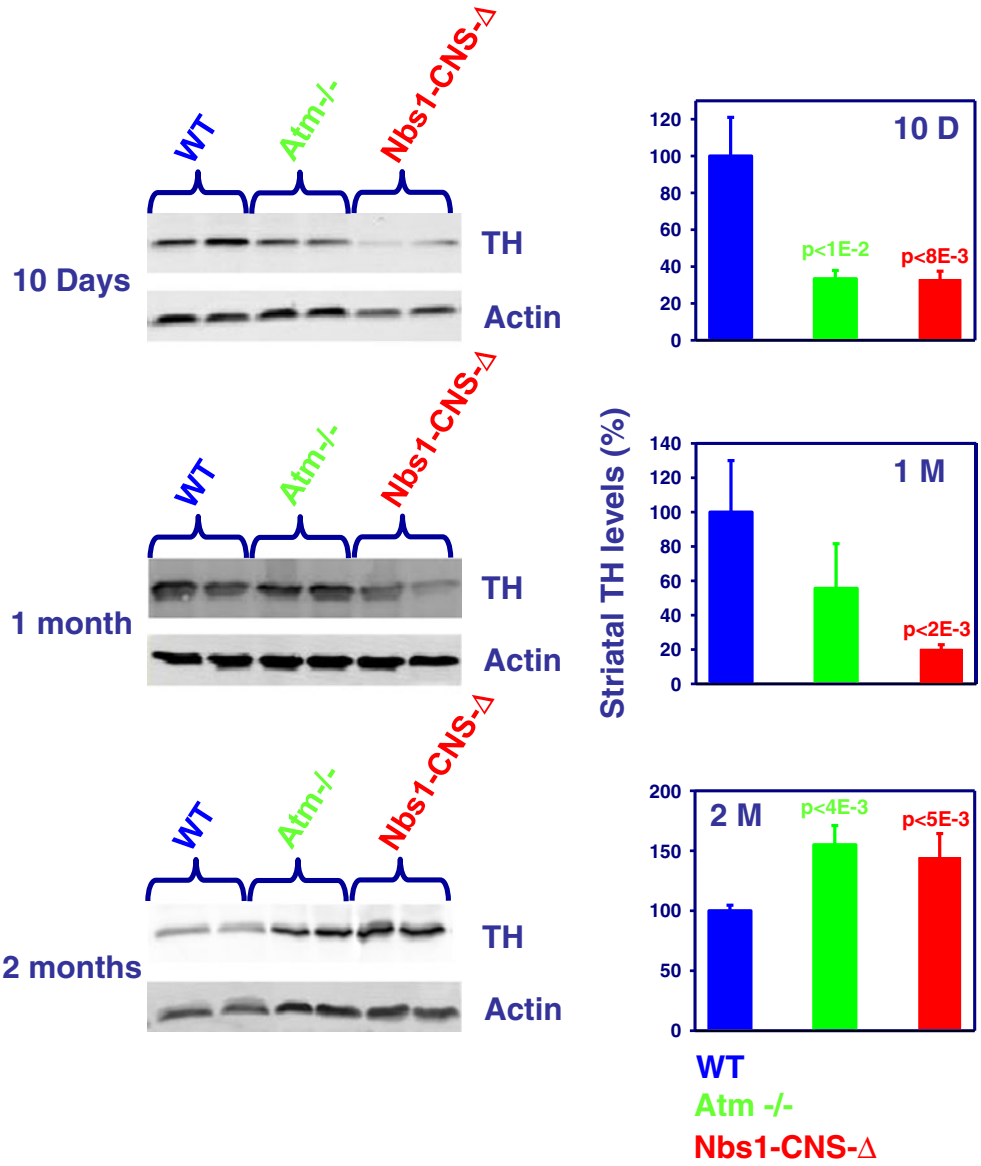

Fig. 3 Age-dependent alterations in TH derived from the striata of Atm−/− and Nbs1-CNS-Δ mice. Western blot analysis displaying age-dependent alterations in the expression of striatal TH. WB results were quantified using the Odyssey system (n=5)

Impaired Microglia Recruitment into the Striata of Atm-Deficient and Nbs1-CNS-Δ Mice

Based on our previous results that Nbs1-CNS-Δ cerebella is associated with cerebellar attrition and reduced secretion of chemokine CCL2 (Galron et al. 2011), we wanted to see whether nigro-striatal pathway degeneration was associated with impaired micorglial recruitment in the striatum. We witnessed significant reduction in the CD11b mRNA levels in the striata of 2-month-old Atm-deficient and Nbs1-CNS-Δ mice (Fig. 8). Because in our previous study we found that reduction of mRNA levels was accompanied with equivalent reduction in CD11b protein levels (Galron et al. 2011), we can assume that the reduction of mRNA levels was associated with similar reduction in the CD11b protein level. Collectively, these results suggest that malfunctioning DDR in the CNS impairs the functionality of astrocytes.

Reduced BDNF Gene Expression Levels in the Striatum of Nbs1-CNS-Δ Mice

Neurotrophic factors play an important role in the maintenance of the CNS homeostasis. Astrocytic cells are known to produce and secrete their own repertoire of neurotrophic factors, including brain-derived neurotrophic factor (BDNF) and neurotrophic factor-3 (NT3) (Ma et al. 2005). Recent data suggests that a dysfunction in glial cell activity may contribute to the pathogenic process that leads to neurodegenerative diseases (Ma et al. 2005). Quantitative RT-PCR analysis revealed a significant reduction in the levels of BDNF in the striata of 2-month-old mutant mice

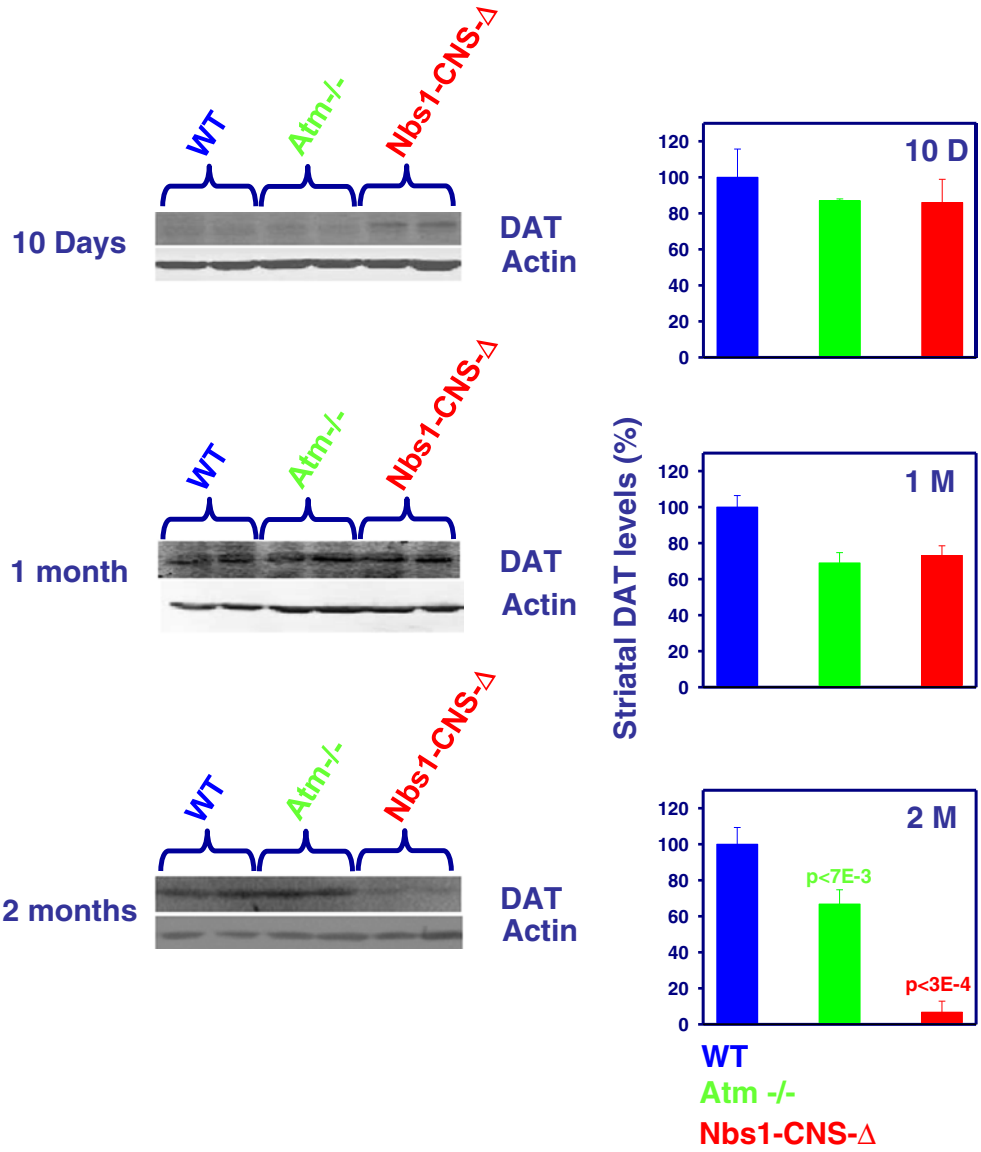

Fig. 4 Age-dependent alterations in DAT derived from 2-month-old striata of Atm−/− and Nbs1-CNS-Δ mice. Western blot analysis displaying age-dependent alterations in the expression of striatal DAT. WB results were quantified using the Odyssey system (n=5)

(Fig. 9). As previously mentioned in Nbs1-CNS-Δ mice, the reduction of mRNA levels was associated with similar reduction in the protein level (Galron et al. 2011). No alterations in the levels NT3 and VEGF levels were observed in the striatum of Nbs1-CNS-Δ mice (Fig. 9).

Discussion

It has been previously reported by Eilam et al. (1998) that Atm deficiency leads to severe degeneration of TH-positive cells in the nigro-striatal pathway. Furthermore, Eilam et al. (2003) found that Atm−/− mice exhibited a progressive, age-dependent, reduction in dopaminergic cells of the substantia nigra, followed by a reduction in projection neurons of the striatum. To test the notion that malfunctioning DDR is indeed the main cause for the demise of the nigro-striatal pathway in Atm-deficient mice, we expanded this study and found that conditional inactivation of the *NBS1* gene, a key component of the DDR machinery and an important regulator of Atm in the CNS resulted in higher and earlier attrition of the nigro-striatal pathway. In general, Atm −/− animals display mild neurological deficits, whereas Nbs1-CNS-Δ mice exhibit marked neurological abnormalities (Frappart et al. 2005; Assaf et al. 2008; Dar et al. 2011). These observations can be explained by the fact that the MRN complex, which is a major, early sensor/mediator of DSB is operated upstream of Atm and is necessary for proper Atm activation (Bakkenist and Kastan 2003; Uziel et al. 2003; Falck et al. 2005; Lavin 2007; Lee and Paull 2007). Due to the physical association and functional cooperation between the three core components of the MRN complex, even partial loss of one of them leads to abrogation of the complex functions (Van Den Bosch et

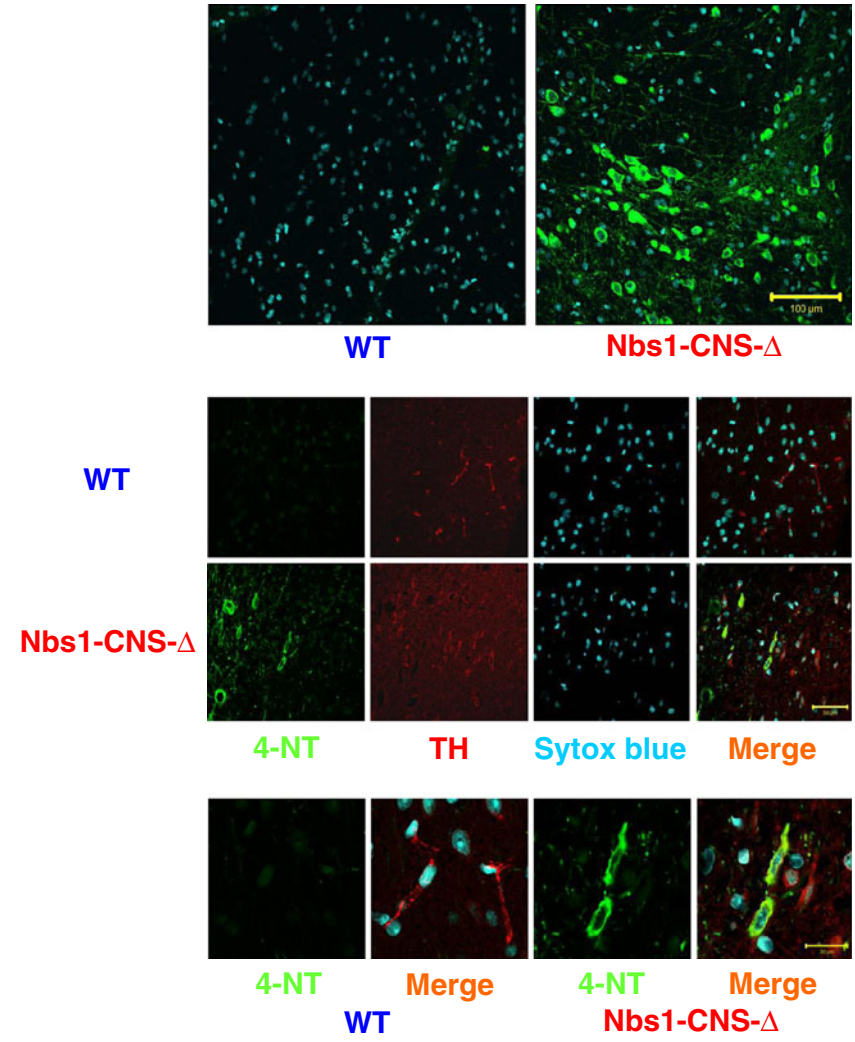

Fig. 5 Increased levels of 4-nitrotyrosine (4-NT) in the substantia nigra of Nbs1-CNS-Δ mice. Mesencephalic brain sections derived from 2-month-old WT and stained with anti-4-nitrotyrosine antibody (*green*) and tyrosine hydroxylase antibody (red, marker of dopaminergic neurons). In addition, DNA nuclei were stained with Sytox blue (*light blue*). The figure shows three different magnifications. Note that the levels of proteins with oxidative damage are markedly higher in the substantia nigra derived from Nbs1-CNS-Δ mice. *Bars* (from top down)=100, 50, and 20 μm

al. 2003). Thus, it is safe to assume that Nbs1 loss is most likely analogous to MRN inactivation. Our results further suggest that Nbs1 activates other components of the DDR rather than Atm and strengthen the notion that accumulation of DNA damage has a key role in the degeneration of the nigro-striatal pathway in these mutant mice. The notion that malfunctioning DDR and not cytoplasmic functions of certain DDR components is responsible for the attrition of certain CNS cellular populations is further supported by the findings showing that cerebellar demise in AOA1 and SCAN1 patients is indistinguishable from that of A-T patients (Hirano et al. 2007; Sugawara et al. 2008). The genes that are responsible for these diseases (AOA1 and SCAN1) operate exclusively in the DNA single strand breaks repair mechanism. Collectively, all these data strongly support the notion that cerebellar and nigro-striatal demise in A-T and NBS stems from defective DDR machinery.

No significant differences in the number of dopamiergic neurons between Atm−/− and WT were found in 10-day-old mice. In contrast, Nbs1-CNS-Δ mice showed a significant reduction already at the age of 10-day-old that persisted for at least 2 months (Fig. 2). This suggests that Atm deficiency causes progressive postnatal demise of dopaminergic neurons, whereas Nbs1 deficiency either accelerates the postnatal deterioration of dopaminergic neurons or causes prenatal developmental defects in the dopaminergic neurons. Interestingly, at the age of 10 days, Atm−/− and Nbs1-CNS-Δ mice exhibited a reduction in striatal TH protein levels. Surprisingly, a significant increase in the TH protein was monitored at the age of 2 months (Figs. 1 and 3). The mechanism by which TH levels exceed those of WT is unknown; however, some mode of compensation occurs in Atm- and Nbs1-deficient striata. It is possible that sprouting of dopaminergic neurons in the SNc is responsible for this intriguing phenomenon. In

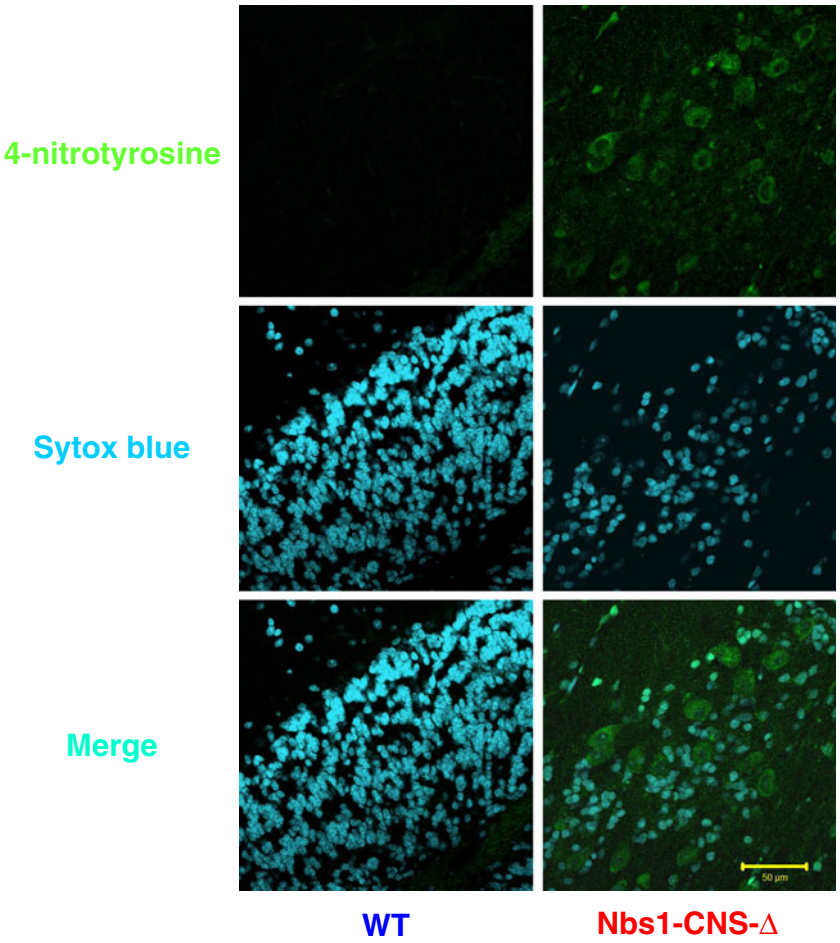

Fig. 6 Oxidative stress in the cerebellum of Nbs1-CNS-Δ mice. Cerebellar sections derived from 2-month-old WT and Nbs1-CNS-Δ mice and stained with anti-4-nitrotyrosine antibody (*green*). DNA nuclei were stained with Sytox blue (*light blue color*). Note that the levels of proteins with oxidative damage are markedly higher in the cerebellum derived from Nbs1-CNS-Δ mice. *Scale bar*=50 μm

the CNS, sprouting can occur at the margins of traumatic wounds or in the terminal fields of pathways with lesions. Furthermore, studies have demonstrated that intrastriatal implantation of IL-1b can enhance compensatory sprouting from residual dopaminergic neurons in the ventral tegmental area of the midbrain and can induce behavioral improvement in hemiparkinsonian rats (Wang et al. 1994). Since IL-1b does not directly stimulate sprouting, it is possible that it does so by stimulating the release glial derived growth factors (Cintra et al. 1991; Stromberg et al. 1993; Chadi et al. 1994; Ho et al. 1995). The fact that mutations in the *ATM* and the *NBS1* genes did not severely reduce the levels of glial derived growth factors suggests that the growth factor levels are sufficient to support sprouting of dopaminergic neurons. To our knowledge, there is no supporting data showing that astorcyte are capable of compensating for TH in the nigro-striatal pathway. Thus, we may assume that the only cells capable of expressing TH are the dopaminergic cells. In contrast to TH elevation in mutant striata, significant reduction in the striatal levels of DAT was observed in Atm−/− and Nbs1-CNS-Δ mice. DAT is essential for the maintenance of normal dopamine homeostasis in the brain. DAT-mediated re-uptake system governs dopamine action at the synaptic level (Torres 2006). DAT can be considered a specific marker for dopaminergic neurons because it is expressed exclusively in neurons that synthesize dopamine as a neurotransmitter (Torres 2006). However, DAT is also expressed in midbrain glial cells (Karakaya et al. 2007). An interesting question stems from this: are reduced striatal glial cells responsible for DAT reduced levels? Our results do not favor this possibility because no reduction in striatal GFAP expressing cells was noticed in the Atm and Nbs1 mutant mice (Fig. 7). If functional sprouting occurs, we would not expect to detect a significant reduction in DAT levels. The mechanism that down-regulates the expression levels of DAT in Atm and Nbs1 mutant mice and the purpose of this down-regulation are not known. However, it is tempting to speculate that increased striatal TH expression in conjunction with reduced DAT levels is the way by which Atm-deficient and Nbs1-CNS-Δ mice are capable of maintaining sufficient levels of dopamine in the synaptic cleft in the striatum.

Oxidative stress is one of the hallmarks of A-T (Rotman and Shiloh 1997; Barlow et al. 1999; Kamsler et al. 2001; Gueven et al. 2006). Interestingly, the Atm-deletion-

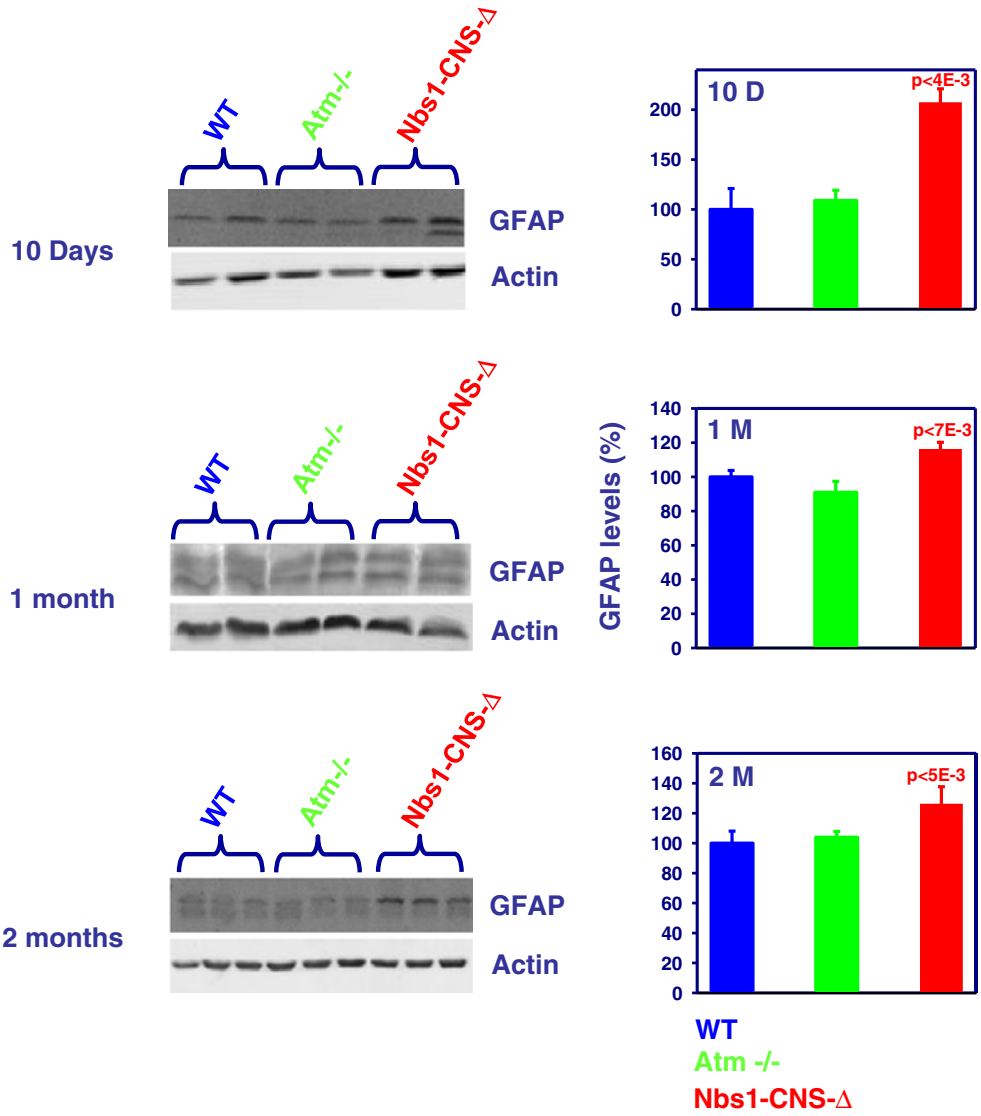

Fig. 7 Glial cell alterations in striata derived from Atm−/− and Nbs1-CNS-Δ mice. The expression of GFAP (marker of astrocytes) was quantified using the Odyssey system and normalized to the level of actin. Note that specific Nbs1 deletion increases the expression of GFAP

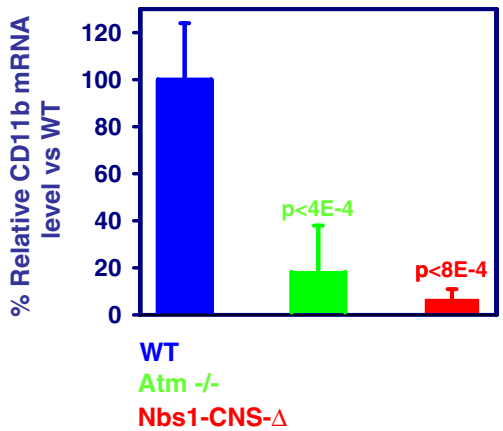

Fig. 8 Reduction in CD11b mRNA levels in the striata derived from 2-month-old Atm−/− and Nbs1-CNS-Δ mice. RT-PCR analysis measuring the mRNA levels of CD11b vs. house-keeping gene ACTB. Note that the reduced mRNA levels of CD11b reflects a significant reduction in the recruitment of microglia cells both Atm−/− and Nbs1-CNS-Δ striata

induced oxidative stress was detected primarily in the cerebellum but not in the rest of the brain (Kamsler et al. 2001; Stern et al. 2002), suggesting that Atm inactivation selectively affects certain cell populations. However, it is unknown whether Atm itself is capable of controlling the redox state of the cell as previously suggested (Watters et al. 1999; Watters 2003) or whether the accumulation of DNA damage in Atm-deficient cells is the main cause for the generation of oxidative stress. No systematic study has been done regarding the effects of Nbs1 deletion on the redox state. Some indirect evidence for the generation of oxidative stress in Nbs1-deficient cells has been reported by Melchers et al. (2009). Melchers et al. (2009) showed higher expression of oxidative stress associated proteins in irradiated Nbs1-deficient hepatocytes as compared to irradiated WT hepatocytes. Furthermore, evidence for oxidative stress has been obtained in untreated Nbs1-deficient differentiated neurons. Remarkably, Atr-deleted

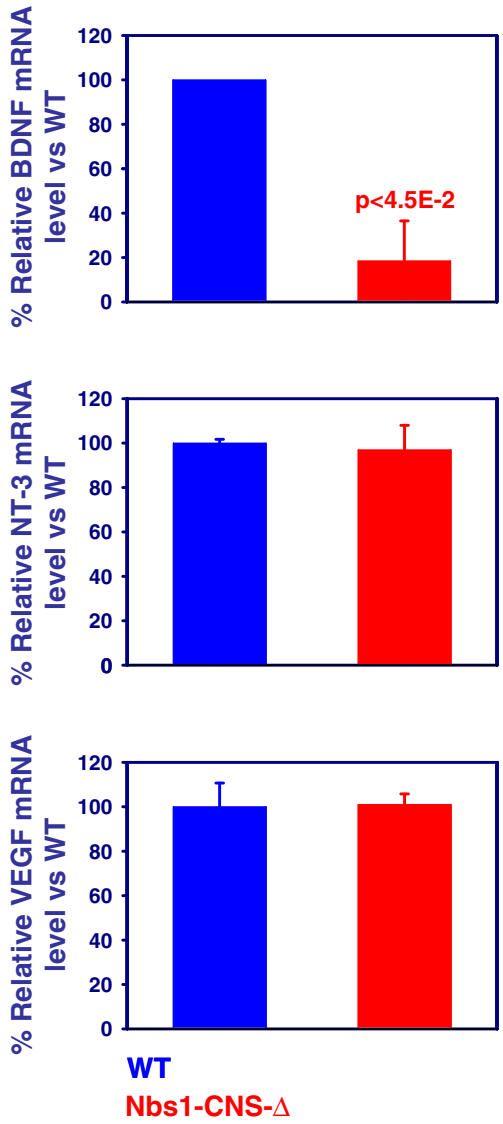

Fig. 9 Alterations in neurotrophic factors' gene expression levels in striata derived from Atm−/− and Nbs1-CNS-Δ mice. The mRNA levels of BDNF, NT3, and VEGF were quantified using RT-PCR. *Error bars* represent SD

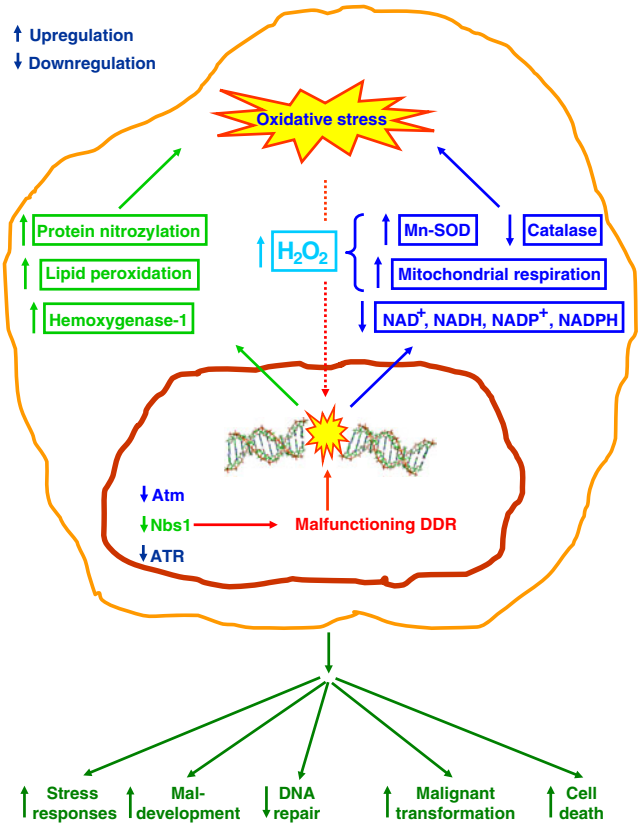

Fig. 10 Effect of defective DDR pathways on the cellular stress responses. Accumulation of DNA damage initiates the activation of the DDR machinery. In WT cell, the functional DDR leads DNA repair and return to homeostasis. On the other hand, defective DDR machinery caused by either Atm or Nbs1 deletion leads to cumulative DNA damage, resulting in the generation of oxidative stress and other stress responses. The generated oxidative stress further accelerates the DNA damage, thereby elevating the cellular stress and expediting its demise

differentiated neurons also display increased level of oxidative stress (Zhongwei Zhou, unpublished results). As shown in Fig. 10, the common denominator among Atm, Nbs1, and Atr is their involvement in the DDR machinery. The fact that inactivation of each protein leads to the generation of oxidative stress strengthen the notion that malfunctioning DDR but not cytoplasmic effect of Atm is responsible for the generation of oxidative stress and other cellular stress responses. Further support to this notion comes from the fact that Atm resides mainly in the nuclei of neuronal cells (Dar et al. 2006). Does oxidative stress play a role in neurodegeneration? Continuous and elevated oxidative stress does not seem to have any effect on the organization or morphology of either Purkinje or cerebellar granule neurons. The brain slices derived from Sod1−/−//Sod2+/−//Atm−/− were indistinguishable from those generated from Sod1+/+//Sod2+/+//Atm+/+ mice (Dar et al., unpublished results). Collectively, these results strengthen our notion that malfunctioning DDR is the main source for the generation of oxidative stress. One of the most intriguing questions in the field of genomic instability disorders is the fact that certain cellular populations are affected, whereas the rest of the brain seems to function normally. Based on our results, it is tempting to speculate that oxidative stress is one of the contributors to this selectivity.

Glial cells are not only essential for maintaining a healthy well-functioning brain but are known to protect the brain and enhance functional recovery from injury (Escartin and Bonvento 2008; Sofroniew and Vinters 2010). Moreover, glial cells play an essential role in synaptic transmission, thereby modulating neuronal activity (Giaume et al. 2007). It is also known that activation of glial cells in the CNS is a first defense mechanism against pathological

abnormalities that occur in neurodegenerative diseases (Farfara et al. 2008). Here we show that specific inactivation of the *NBS1* gene in the CNS significantly reduces the microglial cell level in the striatum (Fig. 6). In our previous study (Galron et al. 2011), we found that reduced microglial recruitment in the cerebellum was associated with reduced levels of CCL2 and CCR. It is highly conceivable that reduced secretion of CCL2 also occurs in the striatum. Different signals can mediate and influence the fate of glial cell activation. It has been shown that in CCR2−/− mice, microglia failed to migrate in the CNS (El Khoury et al. 2007). CCR2, which alter microglia's ability to sense their environment, can lead to neurological diseases such as Alzheimer's disease (Hanisch and Kettenmann 2007). Astrocytes have been implicated as the major source of CCL2/monocyte chemoattractant protein-1 in the CNS (Kalehua et al. 2004). We speculate that the dramatic reduction in the number of microglial cells in Nbs1-CNS-Δ mice, which stems from reduced astrocytic cell functionality, generates a non-permissive environment affecting the ability of the TH-positive cells to survive. Astrocytes are the most abundant cells in the CNS and most likely play an important role in the homeostasis and maintenance of the brain (Nimmerjahn 2009). They emerged as highly sophisticated cells participating in a large and diverse variety of functions vital for normal brain development and adult physiology and pathology (Nimmerjahn 2009) and influence neuronal survival following stress (Swanson et al. 2004).

Altogether, our study strongly supports the notion that the neurological deficits in A-T and NBS stem primarily from malfunctioning DDR and not from cytoplasmic roles of Atm. This impression is further strengthened by the fact that specific deletion of a central and exclusive component of the DDR machinery such as Nbs1 results in cerebella as well as nigro-striatal attrition. Moreover, our results demonstrate an important regulatory mechanism of glial cells in the mouse brain and a likely important factor in the etiology of genomic instability disorders in humans, such as A-T and NBS, which are known to severely affect the CNS. These results further demonstrate the value of animal models in the investigation of the molecular mechanisms underlying human disease.

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