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A single strand that links multiple neuropathologies in human disease

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The development of the human central nervous system is a complex process involving highly coordinated periods of neuronal proliferation, migration and differentiation. Disruptions in these neurodevelopmental processes can result in microcephaly, a neuropathological disorder characterized by a reduction in skull circumference and total brain volume, whereas a failure to maintain neuronal health in the adult brain can lead to progressive neurodegeneration. Defects in the cellular pathways that detect and repair DNA damage are a common cause of both these neuropathologies and are associated with a growing number of hereditary human disorders. In particular, defects in the repair of DNA single strand breaks, one of the most commonly occurring types of DNA lesion, have been associated with three neuropathological diseases: ataxia oculomotor apraxia 1, spinocerebellar ataxia with neuronal neuropathy 1 and microcephaly, early-onset, intractable seizures and developmental delay. A striking similarity between these three human diseases is that they are all caused by mutations in DNA end processing factors, suggesting that a particularly crucial stage of DNA single strand break repair is the repair of breaks with 'damaged' termini. Additionally all three disorders lack any extraneurological symptoms, such as immunodeficiency and cancer predisposition, which are typically found in other human diseases associated with defective DNA repair. However despite these similarities, two of these disorders present with progressive cerebellar degeneration, whereas the third presents with severe microcephaly. This review discusses the molecular defects behind these disorders and presents several hypotheses based on current literature on a number of important questions, in particular, how do mutations in different end processing factors within the same DNA repair pathway lead to such different neuropathologies?

Keywords: DNA single strand break repair; neurodegeneration; microcephaly; ataxia oculomotor apraxia; spinocerebellar ataxia

Abbreviations: AOA = ataxia oculomotor apraxia; APTX = aprataxin; MCSZ = microcephaly, early-onset, intractable seizures and developmental delay; SCAN1 = spinocerebellar ataxia with axonal neuropathy 1; SETX = senataxin

Introduction

The development of the human CNS is a remarkably complex process involving highly coordinated periods of neuronal proliferation, migration and differentiation (de Graaf-Peters and Hadders-Algra, 2006; Lagercrantz *et al*., 2010). Once neurodevelopment is completed, the post-mitotic neurons will then be required to be functional for decades, and thus the maintenance of these long-lasting post-mitotic neurons is critical for the health of the adult CNS. Owing to the inherent complexity involved in developing and maintaining the human CNS, disruptions in any of the processes, through exposure to infectious or toxic agents, traumatic injury or the inheritance of

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genetic mutations, can result in a number of different neuropathologies (Sarnat and Flores-Sarnat, 2004).

Primary microcephaly is a neurodevelopmental disorder that presents at birth and is characterized by a reduced skull circumference, clinically defined as three or more standard deviations below the age- and sex-adjusted mean (Mochida and Walsh, 2001; Carmichael and Woods, 2006; Cox *et al*., 2006). The reduced head circumference closely correlates with reduced brain volume, as it is the outward pressure of the growing developing brain that drives skull bone growth. Microcephaly typically results from perturbations in cell proliferation and neuronal generation, which ultimately leads to a significant reduction in brain growth *in utero*. Indeed, a significant portion of hereditary microcephalic disorders arises from defects in neurogenic mitosis (Cox *et al*., 2006; Nicholas *et al*., 2009; Thornton and Woods, 2009). Progressive neurodegeneration is a second pathology that can occur due to a failure to maintain neuronal health, which leads to a progressive loss of neuronal structure and function and a decline in the total number of neurons due to apoptotic cell death. Although the most common risk factor for developing a neurodegenerative disorder is age, there is a heterogeneous group of disabling neurodegenerative diseases that manifest mostly in children and young adults. These are characterized by the progressive degeneration of the cerebellum and are collectively called autosomal recessive cerebellar ataxias (Hindle, 2010; Hung *et al*., 2010; Anheim *et al*., 2012). Ataxia is the most debilitating symptom of these disorders and is typified by a lack of coordination of muscle movement that affects movement, speech and balance.

A common cause of both microcephalic disorders and neurodegenerative cerebellar ataxias is the inheritance of mutations in genes that are involved in the DNA damage response (McKinnon and Caldecott, 2007; O'Driscoll and Jeggo, 2008; Jeppesen *et al*., 2011). Cellular DNA is under constant attack from both endogenous and exogenous agents, and without the network of cellular pathways that function to efficiently detect, signal and repair DNA damage, collectively termed the DNA damage response, the cell would rapidly cease to function (Hoeijmakers, 2001; Jackson and Bartek, 2009). Microcephaly is a frequent symptom of many syndromes associated with defective DNA repair, demonstrating that an efficient response to DNA damage is critical for normal neurodevelopment (O'Driscoll and Jeggo, 2008). This is likely due to the rapidly dividing nature of the neural progenitor stem cells in the developing brain, which would make them very sensitive to persisting DNA damage (Hoshino and Kameyama, 1988). Additionally, there are also a number of cerebellar ataxias that arise from mutations in proteins involved in DNA repair (Jeppesen *et al*., 2011). Neurons are highly metabolically active and are associated with high rates of transcription and translation, as well as high levels of mitochondrial activity (Flangas and Bowman, 1970; Sarkander and Uthoff, 1976). As a result, the brain has a very high oxygen demand, producing an environment with high levels of metabolic byproducts, such as reactive oxygen species, meaning that over their long lifespan, neurons are likely to be exposed to high levels of oxidative DNA damage rendering them heavily dependent on DNA repair mechanisms (Barzilai, 2007; Chen *et al*., 2007; Weissman *et al*., 2007). The link between DNA damage and neurodegeneration is further demonstrated by the observation that elevated levels of DNA damage, in particular oxidative DNA damage, as well as decreased rates of DNA repair, are seen in post-mortem regions of the brain derived from patients with the age-related neurodegenerative disorder, Alzheimer's disease (Lovell *et al*., 2000; Iida *et al*., 2002; Shao *et al*., 2008).

Single strand break repair

A DNA lesion that is of particular physiological relevance to neuronal cells is the DNA single strand break. DNA single strand breaks are discontinuities in a single strand of the DNA double helix and are one of the most commonly occurring types of DNA lesion. It has been estimated that as many as 10 000 single strand breaks can arise in a single cell per day (Lindahl, 1993; Ward, 1998; Caldecott, 2001). Although they can arise from a variety of sources, those arising from the disintegration of the sugar phosphate backbone of DNA following oxidative attack by reactive oxygen species are one of the most frequent types of single strand breaks (Ward *et al*., 1987; Evans, 1997; Ward, 1998; Cadet *et al*., 2003). Therefore, due to the high oxygen environment of the adult brain, post-mitotic neurons would be exposed to high levels of DNA single strand breaks during their long life. In addition to single strand breaks directly arising from oxidized DNA, they can also arise indirectly through the removal of damaged bases during base excision repair, and as the result of aborted DNA topoisomerase 1 activity (TOP1) (Roca, 1995; Pourquier *et al*., 1997; Pommier *et al*., 2003; Robertson *et al*., 2009). Although single strand breaks are not considered to be as genotoxic as other types of DNA damage, such as the DNA double strand break, they can be deleterious to the cell if left unrepaired in any number owing to their potential to collapse replication forks and transcription bubbles on collision (Kuzminov, 2001; Kouzminova and Kuzminov, 2006; Deckbar *et al*., 2007).

Despite the variety of ways single strand breaks can occur, they are all repaired by overlapping repair pathways collectively termed single-strand break repair (reviewed in Caldecott, 2008) (Fig. 1). Single strand break repair typically involves four stages of repair: detection of the break, end processing of damaged termini, gap-filling and DNA ligation. Owing to the large numbers of single strand breaks that constantly arise within the cell, a rapid and efficient response is vital to ensure continued cellular viability. This is in part facilitated by the enzyme poly(ADP-ribose) polymerase 1, which immediately binds to DNA breaks and auto-modifies itself with long branching chains of poly(ADP-ribose) (D'Amours *et al*., 1999; Kim *et al*., 2005). This facilitates the rapid accumulation of single strand break repair factors with the aid of the core single strand break repair protein X-ray cross-complementing protein 1 (XRCC1) (Caldecott *et al*., 1996; Masson *et al*., 1998; El-Khamisy *et al*., 2003). XRCC1 interacts with a large number of DNA repair proteins and facilitates the rapid and efficient repair of single strand breaks by functioning as a molecular scaffold and helping to coordinate the downstream steps of single strand break repair (Caldecott, 2003). XRCC1 also supports rapid repair by stimulating the activities of a number of its interacting partners (Whitehouse *et al*., 2001; Marsin *et al*., 2003; Campalans *et al*., 2005; Mani *et al*., 2007; Lu *et al*., 2010). Indeed, XRCC1

defective cell lines exhibit significantly reduced rates of single strand break repair following exposure to numerous DNA damaging agents, including oxidative and alkylating agents (Caldecott, 2003). The crucial role XRCC1 plays in ensuring cell survival is also demonstrated by the observation that *XRCC1*$^{-/-}$ mice are not viable past embryonic Day 6.5 (Tebbs *et al*., 1999, 2003).

As the majority of single strand breaks possess 'damaged' termini and need to be restored to the conventional 3′-hydroxyl and 5′-phosphate chemistries before DNA ligation can occur, a DNA end processing step is required for most single strand break repair reactions. This is the most enzymatically diverse stage of single strand break repair owing to the variety of different chemistries that can arise at DNA termini. Once end processing is complete, any single strand gaps are filled in by one of two sub-pathways: short-patch single strand break repair or long-patch single strand break repair. In short-patch single strand break repair, a single nucleotide is incorporated, and the resulting nick is ligated, whereas long-patch single strand break repair involves the synthesis of 2–12 nucleotides, displacing a 5′ single-strand DNA flap, which is cleaved by flap endonuclease 1 before ligation occurs (Dianov *et al*., 1992; Frosina *et al*., 1996; Kim *et al*., 1998; Caldecott, 2008).

Owing to the high levels of single strand breaks that can arise in neurons, it is likely that the CNS is heavily dependent on single strand break repair pathways both for the period of rapid proliferation during neurodevelopment and for the maintenance of the adult CNS over its long lifetime. This is supported by the discovery of three neuropathological diseases associated with defective single strand break repair: ataxia oculomotor apraxia 1 (AOA1), spinocerebellar ataxia with axonal neuropathy 1 (SCAN1) and microcephaly, early-onset, intractable seizures and developmental delay (MCSZ) (Date *et al*., 2001; Moreira *et al*., 2001; Takashima *et al*., 2002; Shen *et al*., 2010). The striking similarity between these three human diseases is that they are all caused by mutations in DNA end processing factors that are found in complexes with XRCC1, suggesting that a particularly crucial stage of single strand break repair is the repair of single strand breaks with 'damaged' termini (Whitehouse *et al*., 2001; Clements *et al*., 2004; Chiang *et al*., 2010). In keeping with this premise, it has also been reported that a brain-specific deletion of *XRCC1* in mice leads to a severe neuropathology, including significant loss of neurons within both the cerebellum and hippocampus (Lee *et al*., 2009). Interestingly, all three of these human disorders lack any extraneurological symptoms, such as immunodeficiency and cancer predisposition, which are typically found in other human diseases associated with defects in the repair of DNA double strand breaks, rather than single strand breaks, such as ataxia-telangiectasia (Taylor and Byrd, 2005). This suggests that despite patients with ataxia-telangiectasia exhibiting a neuropathology that is strikingly similar to AOA1 and SCAN1, only the nervous system is exquisitely sensitive to loss of single strand break repair processes. However, there are also significant differences in the clinical presentations of these aforementioned neuropathological diseases. In particular, both AOA1 and SCAN1 are characterized by a progressive neurodegeneration of the cerebellum, whereas MCSZ presents with severe microcephaly at birth without exhibiting any signs of neurodegeneration. This suggests that, although single strand break repair is evidently critical for both neurogenesis in the developing brain and maintaining neuronal homeostasis in the adult brain, the defective repair of specific DNA lesions may have different impacts on replicating neuronal progenitor cells and post-mitotic neurons. This review presents several hypotheses based on current literature in an attempt to shed light on a number of important questions, in particular, how do mutations in different end processing factors within the same DNA repair pathway lead to such different neuropathologies?

Neurological dysfunction and defective single strand break repair

Ataxia oculomotor apraxia 1

Amongst the heterogeneous group of autosomal recessive cerebellar ataxias is a distinct sub-group that is also associated with oculomotor apraxia, defined as the inability to control eye movements, in particular with saccade movements. There are least two defined disorders within this group that present solely with neurological symptoms, namely AOA1 and AOA2 (Date *et al*., 2001; Moreira *et al*., 2001, 2004). AOA1 is one of the most common autosomal recessive ataxias and presents with early onset ataxia, oculomotor apraxia, cerebellar atrophy, axonal motor neuropathy and hypoalbuminaemia. Similar to ataxia-telangiectasia, a severe loss of Purkinje cells has been observed post-mortem in patients with AOA1 (Sekijima *et al*., 1998; Sugawara *et al*., 2008). The prominent symptom at the onset of the disease is typically gait ataxia, which occurs between early childhood and adolescence, and patients can be severely disabled and wheel chair-bound by later life. There is phenotypic variability between patients with AOA1 and oculomotor apraxia, despite being the hallmark of the disease, can be absent in a subset of patients or associated with a later age of onset (Le Ber *et al*., 2003; Yokoseki *et al*., 2011).

AOA1 is caused by mutations in the gene aprataxin (*APTX*), an end processing factor that catalyses the release of adenylate groups covalently linked to the 5′-phosphate terminus of a DNA single strand break (Fig. 1F) (Date *et al*., 2001; Moreira *et al*., 2001; Ahel *et al*., 2006; Rass *et al*., 2008). Such lesions are usually generated as normal intermediates during a DNA ligation reaction by an ATP-dependant DNA ligase and are very transient. However, if a DNA ligase attempts to prematurely ligate a single strand break that still retains a 'damaged' 3′ terminus, then the ligation reaction will stall, and a stable abortive ligation intermediate will be generated that requires processing to repair (Fig. 1F) (Shuman, 1995; Sriskanda and Shuman, 1998; Ellenberger and Tomkinson, 2008). In addition, it has also been proposed that APTX can process other types of 'damaged' single strand breaks, specifically those possessing 3′-phosphate and 3′-phosphoglycolate termini, although this activity is much less efficient than its 5′ adenylate hydrolysis activity (Takahashi *et al*., 2007).

APTX possesses three distinct domains, a catalytic HIT (histidine triad) domain, a C-terminal zinc finger motif (DNA-binding domain) and an N-terminal fork-head-associated (phosphoprotein binding) domain (Moreira *et al*., 2001). Currently, 22 pathogenic *APTX* mutations and whole gene deletions have been identified in patients with AOA1, the vast majority of which reside within the catalytic HIT domain, implicating the adenylate hydrolysis activity

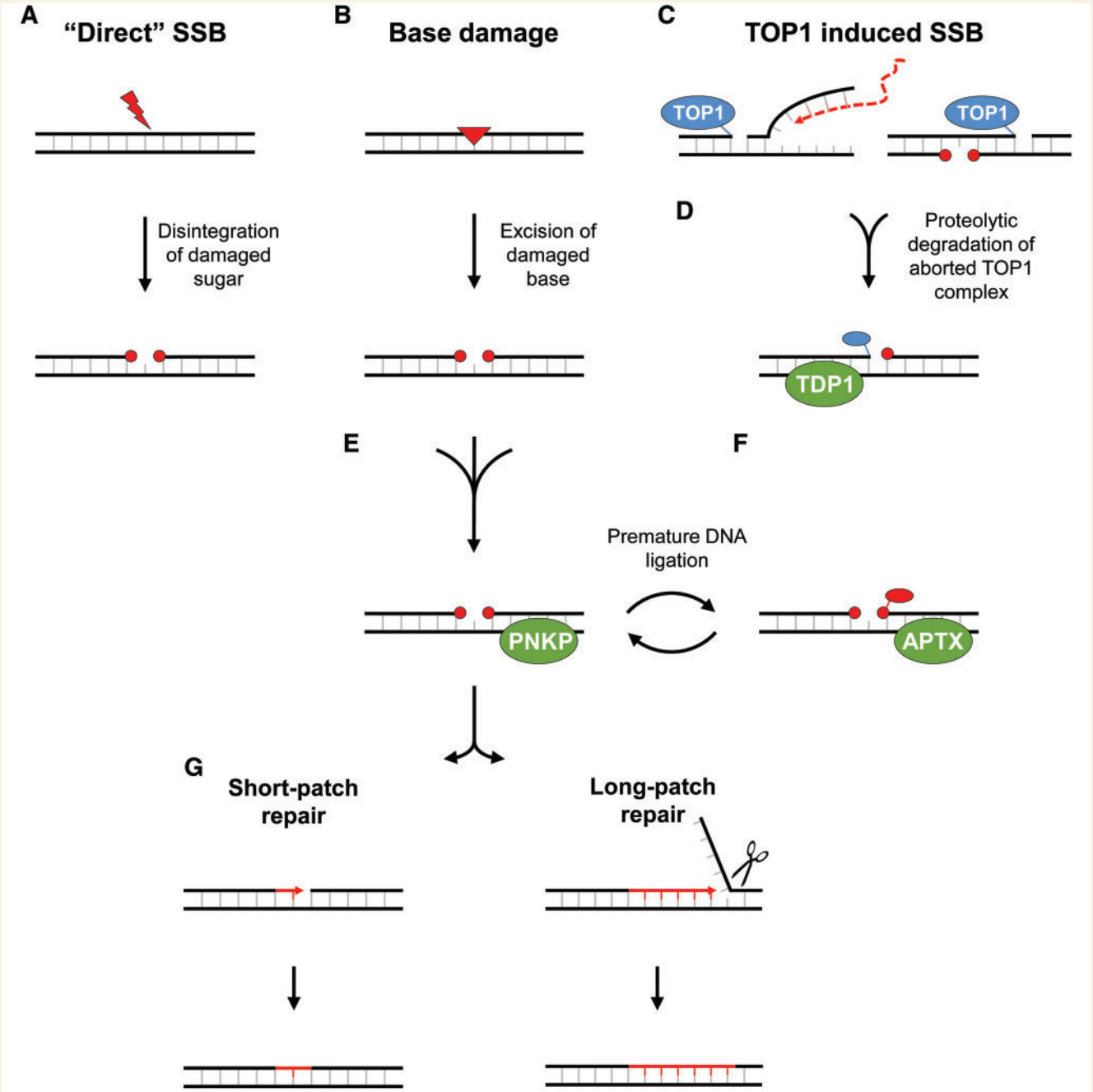

Figure 1 A simplified model of DNA single strand break repair. DNA single strand breaks can arise from a variety of sources and typically possess 'damaged' termini (red circles). (**A**) DNA single strand breaks arising directly from the disintegration of damaged sugars following DNA damage are rapidly detected by the sensor molecule poly(ADP-ribose) polymerase 1. (**B**) Single strand breaks also result from the action of repair enzymes that recognize and remove damaged DNA bases (red triangle). (**C**) A third type of single strand break can also arise if topoisomerase 1 (TOP1) comes into close proximity to elongating replication forks, transcription bubbles or other DNA lesions. Despite arising in different manners, all single strand breaks are repaired in overlapping repair pathways collectively called single-strand break repair. End processing: due to the presence of 'damaged' DNA termini, the majority of single strand breaks require processing. (**D**) The stalled TOP1 protein attached to the single strand break is degraded to a peptide, which is then processed by TDP1 (mutated in SCAN1). (**E**) A crucial stage of single strand break repair is the processing of a particularly common type of 'damaged' termini (a 3′ phosphate) that can arise from all three types of single strand break, by PNKP (mutated in MCSZ). (**F**) At this stage, if a DNA ligase attempts to prematurely ligate a DNA single strand break before the 'damaged' termini have been processed, then an aborted ligation intermediate can arise, which requires processing by APTX (mutated in AOA1). Gap filling and DNA ligation: once the 'damaged' DNA termini have been repaired, then any gaps are filled by DNA polymerases in one of two sub-pathways. (**G**) In short-patch repair, a single nucleotide is incorporated, and the DNA is ligated. (**F**) In long-patch repair, the DNA polymerase synthesizes a strand of 2–12 nucleotides, displacing a single-stranded flap, which is removed, and then the DNA is ligated. SSB = single strand break.

as being critical for maintenance of neuronal homeostasis (Date *et al*., 2001; Moreira *et al*., 2001; Le Ber *et al*., 2003; Caldecott, 2008; Castellotti *et al*., 2011; Crimella *et al*., 2011). Indeed, cell-free extracts prepared from cells derived from patients with AOA1 completely lack the ability to repair an adenylated single strand break *in vitro* (Ahel *et al*., 2006; Reynolds *et al*., 2009). In cells, APTX co-associates with the single strand break repair machinery mediated through an interaction with phosphorylated XRCC1 and therefore as a consequence co-localizes with XRCC1 at sites of DNA damage (Clements *et al*., 2004; Gueven *et al*., 2004, 2007; Jakob *et al*., 2005; Harris *et al*., 2009). Thus, APTX has been proposed to play a role in single strand break repair as a way of resetting an aborted ligation event and allowing repair to continue (Ahel *et al*., 2006; Rass *et al*., 2007).

A number of different studies have reported that AOA1 cell lines exhibit sensitivity to various DNA damaging agents. Transformed AOA1 lymphoblastoid cell lines and primary fibroblasts have been reported to be sensitive to treatment with the alkylating agent methyl methane sulphonate and the oxidizing agent hydrogen peroxide (Clements *et al*., 2004; Gueven *et al*., 2004). In addition, it has been suggested that AOA1 primary fibroblasts are mildly sensitive to ionizing radiation and buthionine-(S,R)-sulphoximine, an inhibitor of the synthesis of the reactive oxygen species scavenger glutathione (Clements *et al*., 2004; Gueven *et al*., 2004; Hirano *et al*., 2007). Taken together, these data provide support for an important role for APTX in the promotion of cellular survival following alkylating or oxidative damage, which is consistent with a role for APTX in the repair of abortive ligation intermediates. As breaks arising directly from the disintegration of oxidized sugars or from the cleavage of oxidative and alkylation base damage can result in single strand breaks with a blocked 3′ terminus, it is plausible that an increased level of aborted ligations will occur following alkylating or oxidative DNA damage.

The evidence for a global single strand break repair defect in AOA1 cell lines, however, has proved more inconsistent. Although several studies have reported that APTX defective cell lines show normal rates of single strand break repair on treatment with hydrogen peroxide or methyl methane sulphonate when measured by alkaline comet assay or NAD(P)H depletion assay, another study reported the opposite (Gueven *et al*., 2004; El-Khamisy *et al*., 2009; Reynolds *et al*., 2009; Crimella *et al*., 2011). Additionally, a single study has reported reduced rates of repair of TOP1-induced lesions in an AOA1 cell line, perhaps reflecting the proposed role of APTX in 3′ end processing (Mosesso *et al*., 2005; Takahashi *et al*., 2007). It has been proposed that defects in APTX-dependent single strand break repair can be masked by redundant repair pathways that can remove an adenylated single strand break in the absence of APTX. In agreement with this, an APTX-dependent single strand break repair defect was observed in quiescent *APTX*$^{-/-}$ mouse astrocytes on incubation with aphidicolin, an inhibitor of long-patch repair polymerases, suggesting that long-patch single strand break repair can compensate for the loss of APTX (Reynolds *et al*., 2009). Intriguingly, it has also been shown that deleting Hnt3, the *Saccharomyces cerevisiae* homolog of APTX, in an apn1Δ apn2Δ (3′ end-processing factors) or a *rad27*Δ (FEN1) background conferred sensitivity to hydrogen peroxide and methyl methane sulphonate, supporting this notion (Daley *et al*., 2010).

These findings offer an explanation for the lack of extraneurological symptoms in patients with AOA1. Long-patch single strand break repair, which is believed to predominantly use DNA replicative factors such as DNA polymerase δ, DNA polymerase ε, proliferating cell nuclear antigen and flap endonuclease 1, would be significantly attenuated in non-cycling cells (DiGiuseppe and Dresler, 1989; Frosina *et al*., 1996; Klungland and Lindahl, 1997; Kim *et al*., 1998; Caldecott, 2008). Accordingly, although the loss of APTX would not be expected to have a significant impact on cycling cells due to the existence of redundant repair mechanisms, post-mitotic neurons (which would not possess S-phase and G2 associated repair pathways) would be more dependent on APTX-dependent single strand break repair. Additionally, it is possible that post-mitotic neurons have a higher incidence of aborted ligations than other cell types, as the elevated level of oxidative DNA damage in the adult brain would result in higher levels of single strand breaks with blocked 3′ termini, further increasing the reliance of neurons on APTX-dependent repair pathways (Barzilai, 2007; Weissman *et al*., 2007). Therefore, unrepaired adenylated single strand breaks could accumulate in post-mitotic neurons in the absence of APTX. As DNA damage has the potential to block RNA polymerase II-dependent transcription and disrupts pre-messenger RNA processing, an accumulation of this type of DNA damage would result in an increase in the levels of collapsed transcription forks, ultimately leading to a higher occurrence of neuronal cell death in AOA1 individuals over time (Kroeger and Rowe, 1989; Bendixen *et al*., 1990; Zhou and Doetsch, 1994; Wu and Liu, 1997).

Spinocerebellar ataxia with axonal neuropathy 1

SCAN1 is an adult onset autosomal recessive ataxia that is characterized by progressive cerebellar ataxia, dysarthia (difficulties with speech) and progressive atrophy of the muscles of the fingers and feet (Takashima *et al*., 2002). All individuals with SCAN1 are also clinically associated with mild hypercholesterolaemia and borderline hypoalbuminemia. SCAN1 typically has a later age of onset than AOA1 and seems to have a milder clinical presentation, and to date, no individual with SCAN1 has presented with oculomotor apraxia or demonstrated any cognitive decline.

SCAN1 is caused by mutations in tyrosyl-DNA phosphodiesterase 1 (*TDP1*), a phosphodiesterase that can selectively hydrolyse a phosphotyrosyl linkage at the 3′ termini of DNA single strand breaks or double strand breaks (Fig. 1D) (Yang *et al*., 1996; Interthal *et al*., 2001). In eukaryotic cells, these linkages arise as the result of abortive TOP1 activity, which lead to DNA breaks with a TOP1 peptide covalently linked to the 3′ terminus of a single strand break (Pourquier *et al*., 1997; Pommier *et al*., 2003). TOP1 plays an important role in the relaxation of DNA supercoiling that is generated ahead of elongating DNA replication forks and transcription bubbles. It functions by nicking one strand of the DNA double helix and allowing the DNA to unwind before religating it (Roca, 1995; Wang, 2002). Under normal conditions, the reaction mechanism of TOP1 proceeds very rapidly, and a TOP1–single strand break is a transient intermediate. However, collision of a replication fork or RNA polymerase with the TOP1-DNA complex will result in a stable abortive intermediate, probably owing to the misalignment of the 5′ terminus within the active site (Fig. 1C) (Avemann *et al*., 1988; Bendixen *et al*., 1990; Ryan *et al*., 1991;

Wu and Liu, 1997). To date, only nine patients with SCAN1 from a single Saudi Arabian family have been discovered, all homozygous for a single amino acid substitution of histidine 493 (H493R), which resides within a highly conserved catalytic motif present within the phosphodiesterase domain (Takashima *et al*., 2002). This mutation results in ~25-fold reduction in TDP1 activity *in vitro* and also causes the accumulation of covalent TDP1-DNA intermediates (Interthal *et al*., 2005; Walton *et al*., 2010).

In agreement with this, SCAN1 cell lines have been shown to accumulate more DNA breaks in the presence of the TOP1 inhibitor camptothecin than control cells using the alkaline comet assay (El-Khamisy *et al*., 2005). Camptothecin increases the half-life of TOP1 cleavage complexes and is used to experimentally increase the cellular level of abortive TOP1-single strand breaks (Pourquier *et al*., 1997). Levels of these TOP1-single strand breaks did not decrease when subsequently incubated in camptothecin-free media (El-Khamisy and Caldecott, 2006). Interestingly, only about half of these breaks were dependent on replication, suggesting that collision with the transcription machinery is responsible for as many abortive TOP1-single strand breaks as with collision with the replication machinery (El-Khamisy and Caldecott, 2006). This is an important observation, as the clinical presentation of SCAN1 is restricted to post-mitotic neurons, which are transcriptionally active and could therefore potentially possess a higher level of TOP1-single strand breaks than other cell types, resulting in a higher dependence on TDP1 (Flangas and Bowman, 1970; Sarkander and Uthoff, 1976). In addition, SCAN1 cell lines also displayed defective single strand break repair of oxidative damage induced by hydrogen peroxide and ionizing radiation (El-Khamisy *et al*., 2005, 2007; Katyal *et al*., 2007). It has been proposed that this reflects an increase in TOP1-single strand breaks in the presence of oxidative DNA damage, as the close proximity of DNA lesions to the topoisomerase cleavage complex increases the likelihood of an abortive reaction (Pourquier *et al*., 1997; El-Khamisy *et al*., 2007). Alternatively, this could reflect a defect in the processing of oxidative DNA breaks or base damage, as TDP1 has been shown to be able to process several types of 3′ adducts and hydrolyze abasic sites within DNA (Inamdar *et al*., 2002; Zhou *et al*., 2005; Lebedeva *et al*., 2012).

Importantly, *TDP1*$^{-/-}$ mice display a progressive decrease in cerebellar size that is consistent with the clinical presentation of the disease, although the mice do not seem to exhibit any obvious ataxic phenotype (Katyal *et al*., 2007). SCAN1 is a relatively late onset disorder, and it is possible that the mice do not live long enough to display these symptoms. Alternatively, the ataxia in individuals with SCAN1 could be due to the extra TDP1-single strand breaks that are generated as a result of the H493R mutation (Interthal *et al*., 2005). In a scenario similar to AOA1, it has been proposed that the neurodegeneration seen in individuals with SCAN1 can be explained by a gradual accumulation of unrepaired TDP1-single strand breaks and TOP1-single strand breaks that would eventually impact on global transcription levels, leading to neuronal cell death (El-Khamisy and Caldecott, 2006). Additionally, it is also likely that post-mitotic neurons lack redundant DNA repair pathways that can repair TOP1-single strand breaks lesions in the absence of TDP1.

Biochemical analysis of the *Pyrococcus furiosus* homologues of human Mre11 and Rad50, which function together with a third protein, Nbn, as the core DNA double strand break sensor complex in human cells, has shown that this nuclease complex is able to cleave a DNA 3′ phosphotyrosyl linkage *in vitro* and then subsequently resect the DNA to generate 3′ single-stranded DNA that can support DNA synthesis (Sacho and Maizels, 2011). If an analogous repair mechanism exists in mammalian cells, it is likely to occur within the homologous recombination double strand break repair pathway, which is predominantly active within late-S and G2 phases of the cell cycle, and would not be functional within post-mitotic neurons. Thus, neuronal cells are likely to be more heavily dependent on TDP1-dependent repair pathways than cycling cells.

Microcephaly, early-onset, intractable seizures and developmental delay

Recently, a third human neurological disorder associated with defective single strand break repair has been discovered. MCSZ is a developmental disorder characterized by microcephaly, infantile-onset seizures, developmental delay and variable behavioural problems, in particular hyperactivity (Shen *et al*., 2010). Despite possessing microcephaly from birth, individuals with MCSZ retain normal brain structures and, in contrast to AOA1 and SCAN1, do not exhibit signs of neurodegeneration or ataxia. Intriguingly, MCSZ is also the only DNA repair syndrome where seizures are universally present.

MCSZ is caused by mutations in polynucleotide kinase phosphatase (PNKP), a bi-functional end processing factor with 5′ DNA kinase and 3′ DNA phosphatase activity. PNKP contains an N-terminal forkhead-associated domain, which contains significant homology to the forkhead-associated domain within APTX, and like APTX, it interacts with phosphorylated XRCC1 and co-associates with single strand break repair machinery (Whitehouse *et al*., 2001; Loizou *et al*., 2004; Luo *et al*., 2004; Bernstein *et al*., 2005). Four different mutations have been found in PNKP in seven families with individuals suffering from MCSZ (Shen *et al*., 2010). Two point mutations reside in the DNA phosphatase domain of PNKP (L176F and E326K) and two frame-shift-mutations in the DNA kinase domain that both result in the production of a truncated PNKP protein (T424Gfs48X and exon15Δfs4X). PNKP is considered to be a particularly important end processing factor within single strand break repair owing to the large volume of single strand breaks possessing 3′-phosphate termini that can arise in a cell. 3′-Phosphate termini occur at ~70% of single strand breaks induced by reactive oxygen species, at single strand breaks generated by the removal of damaged bases by repair enzymes, and at single strand breaks following cleavage of a 3′-phosphotyrosyl bond by TDP1 (Fig. 1) (O'Connor and Laval, 1989; Ward, 1998; Caldecott, 2001; Interthal *et al*., 2001). Therefore, as a result, it is very likely that the 3′-phosphatase activity of PNKP is essential for cell survival.

So far, all of the MCSZ causing mutations have proved to be hypomorphic and retain varying levels of PNKP activity (Reynolds *et al*., 2012). Indeed at 30°C, only a single PNKP mutation (L176F) exhibited significantly reduced levels 3′ phosphatase activity compared with wild-type protein, whereas the other three

mutations possessed normal levels of activity. In contrast, only a single PNKP mutation (E326K) retained normal levels of 5′ kinase activity, and three of four mutations (L176F, T424Gfs48X and exon15Δfs4X) displayed greatly reduced or ablated kinase activity. However, at more physiological temperatures, all mutant proteins exhibited significantly reduced stability *in vitro*, and in agreement with this, all MCSZ cell lines studied to date possess more than a 10-fold reduced cellular level of PNKP protein. Crucially, all MCSZ cell lines tested exhibit a significantly reduced rate of single strand break repair following exposure to oxidative DNA damage (Reynolds *et al*., 2012). This observation could provide an explanation for the clinical presentation of MCSZ. As MCSZ cell lines exhibit significantly reduced rates of repair of oxidative DNA lesions, this could have an impact on the rapidly replicating neuronal progenitor cells in the ventricular zone and subventricular zone during neurodevelopment. A significant decrease in the rates of PNKP-dependent single strand break repair would lead to high levels of unrepaired single strand breaks that would be converted to double strand breaks on collision with elongating replication forks (Kuzminov, 2001; Kouzminova and Kuzminov, 2006; Deckbar *et al*., 2007). As neurons in the subventricular zone are reported to be very sensitive to the presence of unrepaired double strand breaks and readily undergo apoptosis, defective PNKP activity would lead to increased levels of apoptosis in the developing brain, thus leading to a global reduction in the numbers of neurons generated, ultimately resulting in a reduced total brain volume (Hoshino and Kameyama, 1988; Hoshino *et al*., 1991; Gatz *et al*., 2011). The lack of neurodegeneration could be explained by the hypomorphic nature of the MCSZ mutations. As none of the disease-causing mutations ablate DNA 3′ phosphatase activity of PNKP, MCSZ cell lines would retain residual levels of PNKP activity (Reynolds *et al*., 2012). This could mean that, although MCSZ cells have elevated steady-state levels of unrepaired DNA damage due to the residual levels of repair, this may be sufficient to keep the levels of DNA damage below the threshold for apoptosis in post-mitotic neurons, which are not as sensitive as newly differentiated neurons in the developing brain.

Ataxia oculomotor apraxia 2

AOA2 is the second AOA disease lacking extra-neurological symptoms to be linked to inherited genetic mutations and is believed to be the second most common autosomal recessive cerebellar ataxia (Le Ber *et al*., 2004; Moreira *et al*., 2004; Anheim *et al*., 2009; Tazir *et al*., 2009). AOA2 is characterized by progressive cerebellar atrophy, oculomotor apraxia, early loss of reflexes, late peripheral neuropathy and slow disease progression. AOA2 is also associated with a mild-to-moderate increase in alpha-fetoprotein (AFP) level. This can be used in conjunction with the late age of onset and the lack of telangiectasia as a diagnostic aid to differentiate between AOA1, which is associated with normal levels of alpha-fetoprotein, and ataxia-telangiectasia, which often presents with a marked increase in alpha-fetoprotein levels. AOA2 is also associated with a progressively severe motor handicap, and ultimately, patients are confined to a wheelchair later in life. Similar to AOA1, the first symptoms to present are gait ataxia and dysarthria, although AOA2 typically has a later age of onset and occurs between 10 and 25 years of age. Although AOA2 has not currently been associated with defects in single strand break repair, the striking similarities between the clinical presentations of AOA1 and AOA2 means that useful insights into the underlying causes of neurodegeneration seen in these diseases could be gained from comparisons of their molecular defects.

AOA2 is caused by mutations in the gene senataxin (*SETX*) (Moreira *et al*., 2004). The majority of mutations result in small-to-large deletions or missense mutations leading to premature protein termination (Criscuolo *et al*., 2006; Arning *et al*., 2008; Anheim *et al*., 2009). In addition to AOA2, mutations in *SETX* have also been shown to lead to the autosomal dominant, juvenile-onset rare form of amyotrophic lateral sclerosis 4 (Chen *et al*., 2004). The presentation of amyotrophic lateral sclerosis 4 differs substantially from AOA2 and is characterized by a slow disease progression in which the progressive degeneration of motor neurons in the brain and spinal cord leads to limb weakness, muscle wastage and atrophy (Chen *et al*., 2004). In a situation similar to AOA1, there is a certain amount of phenotypic variability within AOA2, and patients frequently lack oculomotor apraxia (Airoldi *et al*., 2010). Additionally, a family has also been identified that combines the typical features of both AOA2 and amyotrophic lateral sclerosis 4 (Schols *et al*., 2008). It is not currently known how different mutations in the same gene can give rise to two very different clinical presentations, but it is possible that amyotrophic lateral sclerosis 4 may be associated with gain-of-function mutations, as all heterozygote carriers of AOA2 causing SETX mutations are clinically unaffected.

SETX encodes a 2677 amino acid protein, which comprises a putative N-terminal protein–protein interaction domain, a highly conserved C-terminal superfamily I DNA/RNA helicase domain and a nuclear localization signal at the extreme C-terminus (Gorbalenya *et al*., 1989; Chen *et al*., 2004, 2006; Moreira *et al*., 2004). It has been shown to be expressed throughout the mouse brain, with the highest expression levels present in the cerebellum and hippocampus (Chen *et al*., 2006). As SETX possesses a conserved DNA/RNA helicase domain, a role in DNA repair has been suggested, and several AOA2 cell lines have been reported to display a hypersensitivity to various DNA damaging agents, including hydrogen peroxide, camptothecin and the DNA crosslinker mitomycin C, which can be rescued with re-expression of the full length *SETX* complementary DNA (Suraweera *et al*., 2007). In addition, AOA2 cells display a defect in the rates of repair of hydrogen peroxide induced double strand breaks, which again can be complemented with wild-type SETX. From these data, it has been speculated that SETX has a role in DNA repair, and thus that the neuropathology seen in individuals with AOA2 is caused by the accumulation of unrepaired DNA damage (Suraweera *et al*., 2007). Indeed, this is similar to that proposed for AOA1 and could explain the overlapping clinical presentations. However, this interpretation has been challenged in another study that compared the sensitivities of two AOA2 cell lines possessing different homozygous SETX mutations: p.L147D, which leads to a single amino acid deletion, and p.R557X missense mutation that results in a premature stop codon and loss of full-length protein (Airoldi *et al*., 2010). In this study, the authors reported that the AOA2 line homozygous for p.L147Del was hypersensitive to hydrogen peroxide, camptothecin

and mitomycin C, as seen previously (Suraweera *et al*., 2007). However, the p.R557X cell line exhibited no sensitivity to these DNA damaging agents. Crucially, RNA interference mediated depletion of the p.L1467Del mutant protein rescued hypersensitivity of the AOA2 cell line to DNA damaging agents. Furthermore, a second study has also reported that several AOA2 lines possessing truncating SETX mutations exhibited normal sensitivity to DNA damaging agents (De Amicis *et al*., 2011). This raises the possibility that the sensitivity seen in AOA2 cell lines is a gain-of-function specifically related to the mutation present, and argues against a role of SETX in DNA repair. This suggests that a DNA repair defect may not be the underlying cause of AOA2.

The conserved domain structure of SETX may provide clues to its cellular functions. The DNA/RNA helicase domain shows a high degree of homology to the essential *S. cerevisiae* protein Sen1p (splicing endonuclease 1), a 5′–3′ helicase that has multiple roles within transcription and RNA processing (Ursic *et al*., 1997; Rasmussen and Culbertson, 1998; Kim *et al*., 1999; Chen *et al*., 2004; Moreira *et al*., 2004). Additionally, the C-terminal region of SETX also shows significant homology to two human proteins that possess superfamily I helicase domains, regulator of nonsense transcripts 1 (RENT1, now known as UPF1), a RNA helicase that promotes nonsense mediated decay of messenger RNA, and immunoglobulin mu binding protein 2, a putative RNA/DNA helicase proposed to be a component of the translation machinery (Grohmann *et al*., 2001; Medghalchi *et al*., 2001; Wang *et al*., 2001; Mendell *et al*., 2002; de Planell-Saguer *et al*., 2009). Together this suggests that SETX could also have roles within transcription as a DNA/RNA helicase, and that AOA2 may result from deficiencies in RNA processing. Intriguingly, mutations in immunoglobulin mu binding protein 2 cause a clinical disease similar to amyotrophic lateral sclerosis 4 that is characterized by dysfunction and progressive loss of motor neurons in the anterior horn of the spinal cord (Grohmann *et al*., 2001).

SETX has been demonstrated to interact with multiple nucleolar factors involved in transcriptional regulation and pre-messenger RNA processing, including RNA polymerase II (Chen *et al*., 2006; Suraweera *et al*., 2007). This is reminiscent of Sen1p, which resides at the centre of a network of protein–protein interactions (Ursic *et al*., 2004; Finkel *et al*., 2010). Importantly, a defect in the binding of RNA polymerase II to target genes and a corresponding decrease in levels of messenger RNA has been observed in AOA2- and SETX-depleted cells (Suraweera *et al*., 2009). In addition, SETX-depleted cells exhibited a deficiency in both normal splicing and alternate splicing events of various reporter constructs (Suraweera *et al*., 2009). All aspects of transcription, including initiation, elongation, termination and RNA processing, seem to be intimately connected, and it is probable that, like Sen1p, SETX performs numerous roles in the regulation of transcription. Indeed, SETX-depleted cells exhibit an increase in the levels of RNA read-through, as well as an enrichment of RNA polymerase II downstream of the polyadenylation signal, indicating a defect in transcription termination (Skourti-Stathaki *et al*., 2011). Additionally, SETX depletion also resulted in the accumulation of RNA/DNA hybrid loops downstream of the polyadenylation signal and defective recruitment of exoribonuclease 2 (Xrn2) to termination regions (Skourti-Stathaki *et al*., 2011). Taken together, it has been proposed that SETX has multiple roles in RNA transcription.

The clinical symptoms of AOA2 could therefore be explained by a decrease in efficiency of transcription and RNA processing. As AOA2 cells only display a decreased efficiency in transcription, rather than a more overt defect, this would explain why AOA2 cells are viable, and could explain why the disease phenotype is confined to the nervous system (Suraweera *et al*., 2009). As post-mitotic neurons are highly transcriptionally active, a decrease in efficiency in transcription could therefore have a larger impact than in other cell types (Flangas and Bowman, 1970; Sarkander and Uthoff, 1976). However, as SETX has also been proposed to perform other functions within the cell, it is possible that other factors also contribute to disease progression. It is possible that the existence of a DNA repair defect in some AOA2 cells exacerbates the disease by leading to an increase in the levels of stalled transcription forks. Additionally, it has been proposed that SETX also functions to promote telomere stability by regulating the transcription of non-coding RNA at telomeres (De Amicis *et al*., 2011). It was recently reported that SETX co-localizes to telomeres in a transcription-dependent manner, and that AOA2 lymphocytes possess significantly shortened telomeres, which is more evident following exposure to ionizing radiation and camptothecin (De Amicis *et al*., 2011). It is likely that the proposed role of SETX in telomere maintenance is intimately linked to its roles in transcription, and thus it is possible that the telomere instability seen in AOA2 cell lines could arise as the result of defects in transcription. Strikingly, the hypothesis that the neurodegeneration seen in individuals with AOA2 is caused by transcription defects is consistent with the proposed reasoning behind the neuropathologies of AOA1 and SCAN1. Therefore, it is possible that cerebellar degeneration is the common outcome of a reduction in global levels of transcription, whether this is due to the accumulation of unrepaired single strand breaks or due to defects in RNA processing.

Discussion

Why do different neuropathologies arise from defects within the same pathway?

It is striking that although mutations in *PNKP* result in a developmental defect resulting in severe microcephaly and mutations in *APTX* and *TDP1* result in progressive cerebellar degeneration, PNKP, APTX and TDP1 are all end processing factors involved in single strand break repair. As discussed in this review, the differences between these three disorders could be explained by the nature of the disease-causing mutations and the DNA lesions that are repaired by APTX, TDP1 and PNKP. Abortive ligation intermediates and abortive TOP1-single strand breaks are likely to arise only rarely compared with single strand breaks harbouring a 3′-phosphate, which is probably one of the most commonly occurring types of DNA lesion in a cell, especially in neuronal cells, which are exposed to a high oxidative load.

In AOA1 and SCAN1, in which certain types of single strand breaks cannot be repaired, it has been proposed that DNA damage would gradually accumulate over time to a level where

it would result in impaired transcription and neuronal cell death (Fig. 2) (Ahel *et al*., 2006; El-Khamisy and Caldecott, 2006; Brooks *et al*., 2008). Over time, increasing levels of neuronal cell death would lead to neurodegeneration. The presence of redundant repair mechanisms in the cycling neuroprogenitor cells that are absent in post-mitotic neurons would also prevent these lesions from accumulating during neurodevelopment (Fig. 3). In contrast, although the PNKP mutations in MCSZ are associated with reduced cellular protein levels of PNKP, and a corresponding reduction in rates of repair of oxidative DNA damage, they retain residual levels of 3′ phosphatase activity (Reynolds *et al*., 2012). In this case, MCSZ cells would possess higher steady-state levels of DNA damage than normal cells, rather than exhibit an accumulation of damage. Thus, due to the lower threshold of apoptosis in neurons in the ventricular and subventricular zone in the developing brain compared with post-mitotic neurons in the adult brain, a PNKP repair defect would have a greater impact during neurodevelopment (Fig. 2) (Gatz *et al*., 2011).

There are still other important differences in the presentations of AOA1 and SCAN1 compared with MCSZ. First, AOA1 and SCAN1 are associated with significant axonal neuropathy, whereas MCSZ is not. It is unclear why lower motor neurons in the spinal cord are more susceptible in individuals with AOA1 and SCAN1 than MCSZ, although the reasons behind the lack of neurodegeneration in individuals with MCSZ may also account for this difference. Second, individuals with MCSZ suffer from severe seizures from an early age. Again, it is not known why defects in PNKP-dependent single strand break repair would result in seizures. However, mice possessing a brain-specific deletion of *XRCC1* also suffer from seizures, suggesting that seizures could be a symptom common to disorders that are associated with severe single strand break repair defects (Lee *et al*., 2009).

Outstanding questions

Although the study of the molecular defects behind these three disorders has allowed significant progress to be made into the understanding of the neuropathologies that can arise in the presence of defective DNA repair, there are still important questions that remain to be addressed. The noted lack of cancer predisposition in individuals with AOA1, SCAN1 and MCSZ, a common feature of other human disorders associated with defective responses to DNA damage, is still somewhat puzzling. However, DNA repair pathways that operate solely in cycling cells, such as the error-free repair pathway homologous recombination, may be sufficient to protect cycling cells against increased genomic instability in the absence of APTX, PNKP or TDP1 (Fig. 3).

Additionally, it is not currently clear why neurons in the cerebellum, and specifically Purkinje neuronal cells, seem to be more sensitive to the loss of APTX and TDP1 than other neuronal cell types. It is possible that either the cerebellum is exposed to higher levels of DNA damage than other parts of the brain, or that it is more sensitive to unrepaired DNA breaks than other cell types. Alternatively, the cerebellar degeneration may be an indirect effect of the loss of structure or function of other types of cell in the brain. The majority of cells in the brain are glial cells that have important roles in the maintenance of the extracellular environment essential for neuronal functioning. It is conceivable that the loss of these supporting cells could exacerbate the progressive neurodegeneration seen in individuals with AOA1 and SCAN1. Indeed, a mouse model of the disease spinocerebellar ataxia 7 has shown that dysfunction of Bergmann glia, a type of glial cell that is associated with Purkinje cell dendrites and regulates Purkinje cell synaptic activity, is sufficient to drive neurodegeneration (Custer *et al*., 2006). Given that the loss of Purkinje cells is a common feature of DNA repair defective disorders, glial cell dysfunction could be a common cause of neurodegeneration.

Another outstanding question is whether defective repair of mitochondrial DNA contributes to the cerebellar degeneration seen in individuals with AOA1 and SCAN1. Both APTX and TDP1 have been shown to localize to the mitochondria and

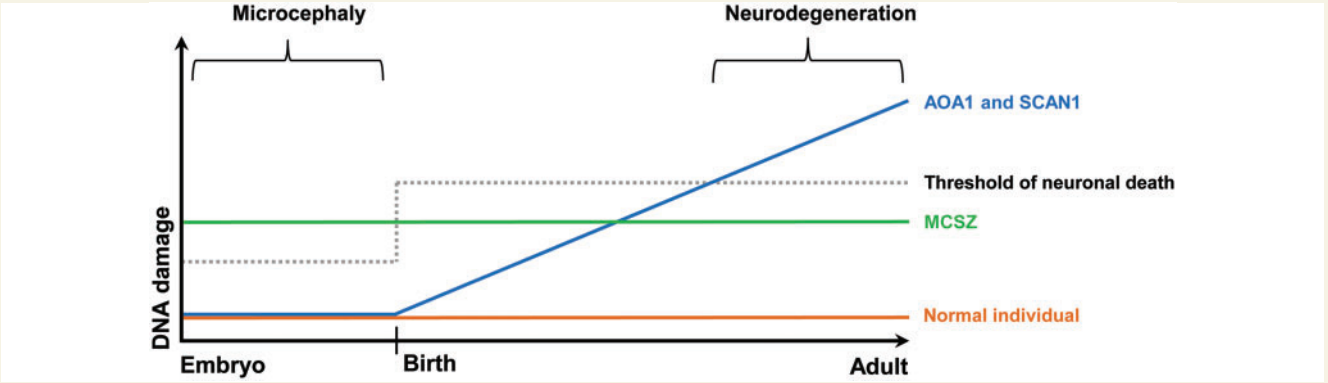

Figure 2 Model for the different neuropathologies exhibited by AOA1, SCAN1 and MCSZ. It has been proposed that the single strand break repair defects in AOA1 and SCAN1 result in a gradual accumulation of unrepaired DNA damage over time in post-mitotic neurons (blue line). In this hypothesis, neurodegeneration only occurs once the level of DNA damage is sufficient to cause neuronal cell death as a result of impaired transcription (dotted line). In contrast, the hypomorphic nature of the MCSZ mutations makes it more likely that an elevated steady-state level of DNA damage exists in MCSZ neurons (green line) compared with normal individuals (orange line), rather than an accumulation of DNA damage. As developing neurons are more sensitive to DNA damage than post-mitotic neurons, the elevated level of DNA damage in individuals with MCSZ would impact on neurodevelopment and result in microcephaly.

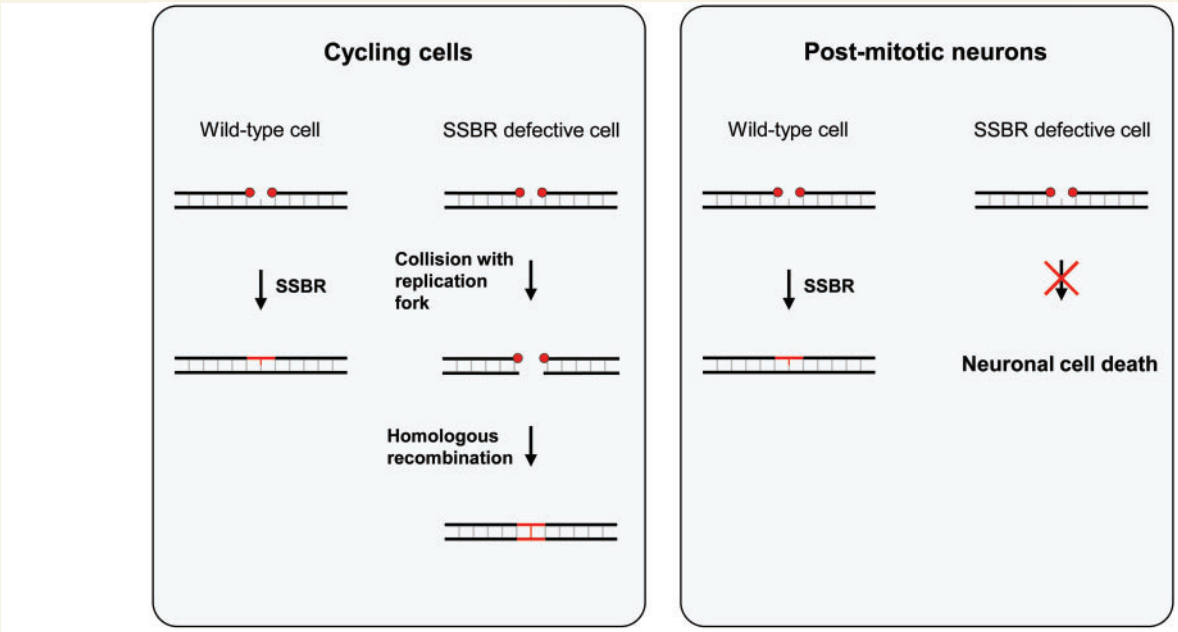

Figure 3 Model for the differential impact of DNA single strand breaks on the cycling cells and post-mitotic neurons. In wild-type cells, any single strand breaks, either in cycling cells or in post-mitotic neurons, are rapidly repaired by single strand break repair pathways (SSBR). In cycling cells possessing a single strand break repair defect, unrepaired single strand breaks are converted to a double strand break and are repaired by homologous recombination. These pathways are not operative in post-mitotic neurons, and in single strand break repair defective neurons, single strand breaks would remain unrepaired and eventually lead to neuronal cell death.

have been suggested to have a role within mitochondrial DNA repair (Das *et al*., 2010; Sykora *et al*., 2011). In this case, the increased cellular levels of reactive oxygen species associated with mitochondrial dysfunction could further exacerbate the disease (Armstrong *et al*., 2010). Finally, although this review has discussed the role of single strand break repair in the protection of the developing and adult brain, it is still not yet clear whether these neuropathologies are due to unrepaired single strand breaks or double strand breaks. APTX, TDP1 and PNKP have all been implicated in double strand break repair (Clements *et al*., 2004; Koch *et al*., 2004; Karimi-Busheri *et al*., 2007; Das *et al*., 2009; Becherel *et al*., 2010; Segal-Raz *et al*., 2011). It is also worth noting that the neuropathology of MCSZ is reminiscent of that exhibited by DNA ligase IV-deficient patients (O'Driscoll *et al*., 2001). It is possible that the microcephaly seen in individuals with MCSZ reflects a combination of defective PNKP-dependent single strand break repair and double strand break repair, whereas the neurodegeneration seen in individuals with AOA1 and TDP1 could reflect an accumulation of unrepaired single strand breaks and double strand breaks.

Conclusion and future perspectives

Significant headway has been made into understanding the defects behind these disorders using cell lines derived from patients. However, there are limits to this approach, and the answers to some outstanding questions may only become apparent through in-depth study of mouse models of these neurodegenerative diseases, although this may be complicated by the failure of many such models to phenocopy the associated human disorder. In addition, there are many human diseases associated with neurological dysfunction that have yet to be linked with a disease-causing mutation, and it is highly unlikely that the full complement of human diseases with defective DNA repair have been identified. As there are many end processing factors involved in single strand break repair other than PNKP, APTX and TDP1, it is possible that mutations in some of these factors are responsible for yet uncharacterized cerebellar ataxias or microcephalic disorders. The discovery of novel neuropathological disorders will allow a greater understanding of the mechanisms and pathways critical for the maintenance of neuronal homeostasis, and further study of both novel and existing disorders will hopefully yield future avenues for potential therapeutic strategies in the future.

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