Low levels of endogenous or X-ray-induced DNA double-strand breaks activate apoptosis in adult neural stem cells

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ABSTRACT

The embryonic neural stem cell compartment is characterised by rapid proliferation from embryonic day (E)11 to E16.5, high endogenous DNA double-strand break (DSB) formation and sensitive activation of apoptosis. Here, we ask whether DSBs arise in the adult neural stem cell compartments, the sub-ventricular zone (SVZ) of the lateral ventricles and the sub-granular zone (SGZ) of the hippocampal dentate gyrus, and whether they activate apoptosis. We used mice with a hypomorphic mutation in DNA ligase IV (Lig4Y288C), ataxia telangiectasia mutated (Atm−/−) and double mutant Atm−/−/Lig4Y288C mice. We demonstrate that, although DSBs do not arise at a high frequency in adult neural stem cells, the low numbers of DSBs that persist endogenously in Lig4Y288C mice or that are induced by low radiation doses can activate apoptosis. A temporal analysis shows that DSB levels in Lig4Y288C mice diminish gradually from the embryo to a steady state level in adult mice. The neonatal SVZ compartment of Lig4Y288C mice harbours diminished DSBs compared to its differentiated counterpart, suggesting a process selecting against unfit stem cells. Finally, we reveal high endogenous apoptosis in the developing SVZ of wild-type newborn mice.

KEY WORDS: Neural stem cell, DNA double-strand break repair, Apoptosis, Radiation sensitivity

INTRODUCTION

DNA double-strand breaks (DSBs) are severe lesions that can cause cell death and/or chromosomal rearrangements. They are the major lethal lesion induced by ionising radiation but can also arise from oxidative damage, following replication and during immune development. DNA non-homologous end-joining (NHEJ) is the main DSB repair pathway, with DNA ligase IV (LIG4) being the unique NHEJ ligase. DNA ligase IV knockout in mice is embryonically lethal owing to extensive neuronal apoptosis around embryonic day (E)13.5 (Barnes et al., 1998; Frank et al., 1998). In humans, hypomorphic mutations in DNA ligase IV confer LIG4 syndrome, which is characterised by immunodeficiency, microcephaly and developmental delay (O’Driscoll et al., 2001). A recent study has proposed that DNA ligase IV deficiency is a common cause of extreme growth failure and microcephaly (Murray et al., 2014).

Pioneering studies describing the structure of the embryonic neocortex have shown that the ventricular zone (VZ) and sub-ventricular zone (SVZ), hereafter VZ/SVZ, adjacent to the ventricles, contain the neural stem and intermediate progenitor cells, which replicate rapidly from E11 to E16.5 (Bayer et al., 1991). The intermediate zone (IZ), which lies above the VZ/SVZ, contains migrating cells and axons (Mitsuhashi and Takahashi, 2009; Pontious et al., 2008). At E14, post-mitotic neurons establish a layer known as the cortical plate (CP) between the IZ and the superficial marginal zone (Fig. 1A). Following ionising radiation at doses below 50 mGy, these embryonic neural stem and early progenitor cell compartments activate apoptosis, which we define hereafter as sensitive activation of apoptosis. Studies using DNA-ligase IV-null mice and a strain with a hypomorphic mutation in DNA ligase IV (Lig4Y288C), which arose from a random mutagenesis screen and causes a diminished rate of DSB repair, have revealed that there are elevated levels of endogenous DSBs and apoptosis in the neocortical VZ/SVZ and IZ (Gatz et al., 2011; Lee et al., 2001; Nijnik et al., 2007). Both the high level of DSBs observed in Lig4Y288C embryos and number of apoptotic cells diminish temporally following the cessation of rapid proliferation in the VZ/SVZ, suggesting a causal relationship. Collectively, these findings suggest that DSBs arise during neurogenesis and sensitively activate apoptosis in the neocortex. Ionising-radiation-induced apoptosis in the embryonic neocortex is largely dependent upon the damage response kinase ataxia telangiectasia mutated (ATM) (Gatz et al., 2011; Lee et al., 2001; Sekiguchi et al., 2001). In the adult brain, neurogenesis persists in two main regions – the SVZ, adjacent to the lateral ventricle, and the sub-granular zone (SGZ), located in the hippocampal dentate gyrus (Fig. 1B) (Lledo et al., 2006). The sensitivity of the response of the SVZ and SGZ to DNA damage has not been investigated.

Here, we examine whether the adult SVZ and SGZ incur endogenous DSBs and whether low levels of DSBs can activate apoptosis. We examined these endpoints in Lig4Y288C, Atm−/− and double mutant Atm−/−/Lig4Y288C mice. We observed similar DSB levels in the adult SVZ and SGZ of Lig4Y288C mice, and this level was also similar to that found in differentiated neuronal compartments, suggesting that, unlike the situation in embryos, DSBs do not arise at high frequency in the adult neural stem cells. However, apoptosis was sensitively activated by DSBs in the SVZ in a predominantly ATM-dependent manner. Thus, sensitive activation of apoptosis in neural stem cells is not a direct consequence of rapid replication but a feature of the compartment. These findings are important when considering the use of radiological procedures. To gain further insight into the generation of DSBs during development and the fate of cells with DSBs generated during embryogenesis, we undertook a temporal analysis in mice which revealed that the level of DSBs gradually decreased from late embryogenesis to shortly after birth, reaching a steady state level by 2 months. Such a temporal loss of DNA damage suggests that cells with DSBs generated during embryonic neurogenesis can progress into the neonatal mouse brain and undergo slow DSB repair. Additionally, the temporal analysis
revealed a defined postnatal stage of developmentally regulated and ATM-independent apoptosis that occurs during establishment of the adult SVZ. We provide evidence for reduced DSB levels in the stem cell compartment shortly after birth in Lig4<sup>Y288C</sup> mice, suggesting that there is selective loss of unfit stem cells.

RESULTS

Increased DSBs in neural stem and differentiated cells of adult Lig4<sup>Y288C</sup> mice

Our previous analysis of Lig4<sup>Y288C</sup> embryos, which repair DSBs with slow kinetics, has revealed that there is a high level of DSBs in the embryonic neocortex compared to other embryonic tissues (Gatz et al., 2011). First, we examined whether high levels of DSBs are also observed in the adult stem and early progenitor regions by quantifying 53BP1 foci, a DSB marker, in the SVZ and SGZ of wild-type (WT) and Lig4<sup>Y288C</sup> mice. To verify the system, we demonstrated that there was a dose-dependent induction of 53BP1 foci in the cerebellum of WT mice and impeded DSB repair in Lig4<sup>Y288C</sup> mice (Fig. 2A,B). We then quantified 53BP1 foci in various tissues from adult mice (2–3 months old). Given that we aimed subsequently to examine apoptosis, which is activated by ATM at DSBs, we examined 53BP1 foci in WT, Lig4<sup>Y288C</sup>, Atm<sup>−/−</sup>...
and double mutant $\text{Atm}^{-/-}/\text{Lig}^{4288C}$ mice. We observed a low level of endogenous 53BP1 foci in WT mice in all tissues examined, and a small, but significant, increase in the level of foci in $\text{Lig}^{4288C}$ mice (Fig. 3A, compare black and blue columns). The cerebellum and hippocampus, which are non-replicating, had similar DSB levels to that in the proliferating ileum. Thus, the steady state level of DSBs did not correlate with the proliferative status. In most tissues (except the kidney), 53BP1 foci numbers in $\text{Lig}^{4288C}$ mice were approximately half that obtained at 1.5 h after exposure of WT mice to 50 mGy X-rays, suggesting a level of DSBs similar to that induced by 15–20 mGy X-rays (Fig. 3A, compare blue and orange columns). $\text{Atm}^{-/-}$ mice harboured elevated numbers of 53BP1 foci compared to WT mice in the hippocampus and lung but not in the cerebellum, ileum or kidney (Fig. 3A, compare black and light grey columns). 53BP1 foci numbers were similar in the $\text{Atm}^{-/-}$ and $\text{Lig}^{4288C}$ hippocampus. Strikingly, $\text{Atm}^{-/-}/\text{Lig}^{4288C}$ mice had high numbers of 53BP1 foci in all tissues except the ileum (Fig. 3A,B).

Next, we quantified 53BP1 foci in the adult SVZ and SGZ. In rodents, the SVZ has more extensive germinal activity compared to the SGZ (Alvarez-Buylla et al., 2001; Sanai et al., 2004). The SVZ

![Image](https://www.jcs.biologists.org/content/128/10/3597.full)

**Fig. 3.** Similar levels of endogenous DSB formation in differentiated neuronal tissues and adult stem cell compartments. (A) Quantification of 53BP1 foci per cell in different tissues of untreated adult mice of the genotypes indicated and WT mice exposed to 50 or 100 mGy X-rays. (B) Representative images of untreated $\text{Atm}^{-/-}/\text{Lig}^{4288C}$ cerebellum stained for 53BP1 (green) and DAPI (blue). The lower image represents the boxed area shown in the upper image. Scale bars: 100 µm (top), 20 µm (bottom). (C,D) 53BP1 foci per cell in the adult SVZ and SGZ regions comparing proliferating (Ki67+) and non-proliferating (Ki67−) cells. Note that 53BP1 foci were not scored in the $\text{Atm}^{-/-}/\text{Lig}^{4288C}$ SGZ due to the low number of Ki67+ cells. (E) Representative images of the adult SVZ of untreated WT, $\text{Atm}^{-/-}$ and $\text{Lig}^{4288C}$ mice, and WT SVZ 1.5 h after 100 mGy X-rays stained for Ki67 (red), 53BP1 (green) and DAPI (blue). Scale bars: 150 µm (left), 20 µm (right). The position of the lateral ventricle (LV) is shown for orientation. Yellow arrowheads show 53BP1 foci. Data represent mean±s.e.m. (WT, n=4; $\text{Atm}^{-/-}$, n=3; $\text{Lig}^{4288C}$, n=5; $\text{Atm}^{-/-}/\text{Lig}^{4288C}$, n=3; WT 50 mGy 1.5 h, n=2; WT 100 mGy 1.5 h, n=3). ns, not significant; *$P \leq 0.05$; **$P \leq 0.01$ (unpaired Student’s t-test).
Temporal analysis reveals a gradual decrease in DSB levels in neuronal tissues from embryogenesis through to adulthood

This and previous analyses suggest that at E14.5, the Lig4Y288C neocortex has approximately one 53BP1 focus per cell, which is estimated to be equivalent to three DSBs per cell (Gatz et al., 2011). To gain insight into the fate of these cells and the origin of DSBs in adult neuronal tissues, we undertook a temporal analysis of DSB levels in neuronal tissues from E14.5 to adulthood (taken as 2–3 months post birth). In the embryonic neocortex at E14.5 and E17.5, we scored 53BP1 foci in the IZ and CP, the early progenitors of the VZ/SVZ, where foci numbers can be most accurately quantified (Gatz et al., 2011). After birth, we examined the isocortex and SVZ, tissues that originate from the embryonic VZ/SVZ (Alvarez-Buylla et al., 2001; Tramontin et al., 2003). Given that perinatally the majority of cells in the adult SVZ are Ki67+, we did not distinguish the Ki67 proliferative status in this analysis. Where possible, we examined all genotypes, but owing to the low numbers of living Atm−/−/Lig4Y288C mice born, this analysis was restricted.

For WT embryos, we observed an approximately twofold greater number of DSBs in the embryonic neocortex at E14.5 compared to E17.5, which we attribute to the fact that replication has substantially diminished by E17.5 and that DSBs arise as a consequence of rapid replication (Fig. 4A, C, black columns). Consistent with previous findings, the level of DSBs in Lig4Y288C embryos was similar to that induced by 120 mGy X-rays (Fig. 4A, blue columns) (Gatz et al., 2011). In Lig4Y288C embryos, DSB levels at E17.5 were elevated compared to control embryos, although they were significantly lower than at E14.5 (P ≤ 0.05) (Fig. 4A). We attribute this to a diminished rate of DSB formation, given that replication has ceased by E17.5, coupled with slow residual repair in Lig4Y288C embryos. Despite efficient repair in WT cells, a similar trend is observed, although DSB levels are lower than in Lig4Y288C embryos (Fig. 4C). Atm−/− embryos displayed a small increase in...
DSBs at E14.5, the level of which remained similar at E17.5, but the difference was not significant (Fig. 4A, compare black and light grey columns). Atm−/−/Lig4228/C embryos had a similar increased level of DSBs to Lig4228/C embryos at E14.5 suggesting that additional loss of ATM does not enhance DSB formation (E17.5 embryos were not available for analysis) (Fig. 4A, red columns). Taken together, these findings substantiate our previous observations that rapid proliferation from E11 to E16.5 results in high DSB formation, which diminishes when repopulation declines (i.e. by E17.5) (Gatz et al., 2011).

Examination of DSBs in the WT isocortex of newborn mice [postnatal day (P)5] revealed lower DSB levels compared to those in the embryo, and levels further decreased by 2–3 months (Fig. 4A,B, shown at a higher scale in Fig.4C for clarity). Lig4228/C mice showed a clear decrease in DSB levels from E17.5 to 2–3 months (Fig. 4B, blue columns). We did not detect any differences between 2-, 3- or 4-month-old mice suggesting that a steady state level is reached by 2 months (data not shown). At 2–3 months, DSB numbers in Lig4228/C mice were approximately one tenth of the level at E14.5 (Fig. 4A,B). Perhaps surprisingly, the level of DSBs in Atm−/− mice remained similar to the embryonic level, giving numbers slightly elevated compared to WT mice at P5, P15 and 2–3 months (Fig. 4A,B, light grey columns). Furthermore, the level of DSBs in the embryonic neocortex compared to that in the isocortex in Atm−/−/Lig4228/C mice also did not significantly decrease (Fig. 4A,B, red columns). Hence, DSB levels were similar at E14.5 and 2–3 months (approximately one 53BP1 focus per cell). Assuming that the induction of DSBs does not differ in these mice, these results suggest that ATM exerts an influence on the reparableity of DSBs generated during neurogenesis.

In summary, these findings reveal a gradual decline in DSB levels in WT and Lig4228/C mice from a high level during embryogenesis, when high DSB formation occurs, to a lower steady state level in adult mice. This decrease, particularly in the Lig4228/C mice, is consistent with the notion that DSBs generated in vivo in the neocortex can be transmitted to newborn neuronal tissue and undergo slow repair.

**DSBs in the SVZ are lower than in the isocortex**

Temporal analysis of DSBs in the SVZ revealed a pattern subtly distinct to that observed in the isocortex (Fig. 4B, compare SVZ and isocortex). For Lig4228/C mice, DSB levels were statistically significantly lower in the SVZ than in the isocortex. This difference was particularly marked in the Atm−/−/Lig4228/C mice, where numbers were lower than in the hippocampus or cerebellum (compare Fig. 4B and Fig. 3A). (Note that for Atm−/−/Lig4228/C mice, the analysis was carried out at P20 and not P15 and is based on a single mouse; P5 mice were not examined.) This trend was not apparent in Atm−/− mice. Taken together, these findings suggest that DSBs that arise in Lig4228/C embryos undergo slow repair but that neuronal cells with DSBs can progress into the newborn SVZ and isocortex. The fact that the embryonic neocortical cells have approximately one 53BP1 focus per cell but can give rise to viable mice, substantiates the notion that cells with DSBs can be transmitted to newborn mice. Moreover, DSBs generated in Atm−/−/Lig4228/C embryos either persist to adulthood or the neuronal tissue of such mice incurs further DSB formation. Most importantly, however, the results reveal that cells in the SVZ harbour fewer DSBs, raising the possibility that a process exists to diminish DSB levels in the SVZ, most noticeably at P5 and P15.

To assess whether this could be attributed to differences in the rate of repair between the two tissues, we assessed the rate of repair of DSBs induced by 100 mGy ionising radiation in the isocortex and SVZ of WT and Lig4228/C mice, a dose which induces a similar number of DSBs to that observed at E14.5 in Lig4228/C embryos (Fig. 2B, supplementary material Fig. S1A,B). For ethical considerations, the mice were only maintained for 15 h post ionising radiation. In WT and Lig4228/C mice, we observed a similar rate of DSB repair in the isocortex, the SVZ and the cerebellum, although the repair was much delayed in Lig4228/C mice compared to WT mice (Fig. 2B; supplementary material Fig. S1A). Thus, we conclude that the difference in DSB levels in the SVZ compared to the isocortex cannot be attributed to differences in the rate of repair of DSBs generated during neurogenesis.

To allow a comparison of the DSB repair kinetics with cultured cells and to assess the ATM dependency, we examined repair following 500 mGy ionising radiation in confluent-arrested primary mouse embryonic fibroblasts (MEFs) derived from WT and Lig4228/C mice, and in Lig4228/C MEFs treated with the ATM inhibitor (ATMi) KU55933 (Fig. 4D). Control experiments verified the linearity of the in vitro dose response following exposure to 50–500 mGy X-rays, and that 53BP1 and γH2AX foci numbers were identical (supplementary material Fig. S1C,D). 500 mGy induced ~15 53BP1 foci per cell (approximately tenfold greater than endogenously generated DSBs during embryogenesis) (Fig. 4D). These DSBs were almost completely repaired by 12 days in Lig4228/C MEFs (Fig. 4D, blue columns). Atm−/−/Mef showed elevated unrepaire DSBs at 6 h post ionising radiation, and a persistent fraction of unrepaired DSBs could be detected at 12 days post ionising radiation, which is consistent with previous findings (Fig. 4D; supplementary material Fig. S1C, light grey columns) (Riballo et al., 2001). These have been shown to represent DSBs at heterochromatin regions, which have an essential requirement for ATM for repair (Goodarzi et al., 2008). Strikingly, the rate of DSB repair in ATM-treated Lig4228/C cells was slower than in non-ATMi treated cells but, nonetheless, only a small subset of DSBs remained by 12 days post ionising radiation (Fig. 4D; supplementary material Fig. S1C). These findings revealed a similar rate of repair of radiation-induced DSBs in cultured cells and in vivo (supplementary material Fig. S1A). However, the rate of loss of 53BP1 foci arising endogenously during embryogenesis in Lig4228/C mice appeared slower than the repair of radiation-induced DSBs (compare Fig. 4A,B with supplementary material Fig. S1A). It is possible that in vivo cell division in the presence of DSBs impedes the rate of repair or that additional DSBs arise in vivo.

**Apoptosis is sensitively activated in the adult SVZ but not in other neuronal tissues**

Adult neural stem cells have been shown to activate apoptosis after a high dose of ionising radiation but the sensitivity of activation has not been examined (Lazarini et al., 2009). To assess this, we quantified the endogenous apoptotic level in Lig4228/C mice and the levels following exposure to a low dose of X-rays in WT mice, by measuring the total number of TUNEL+ cells in each brain region within the section. Using this approach, we were able to provide a correlation between apoptosis and DSB levels. No significant apoptosis was observed following ionising radiation exposure in WT mice or endogenously in the mutant strains in the adult hippocampus, cerebellum or isocortex (Fig. 5A, black columns). In marked contrast, substantial apoptosis was observed in the adult SVZ (Fig. 5A,B). Exposure of WT mice to ionising radiation gave a linear dose response with statistically significant apoptosis.
detectable after 50 mGy (Fig. 5C). Elevated endogenous apoptosis was also observed in the SVZ of Lig4Y288C adult mice, and following irradiation with 50, 100 or 500 mGy X-rays. The region scored in each section encompassed the entire area of the tissue under analysis. CTX, Isocortex; CA, Ammon’s Horn; CUL, culmen; CENT, central lobule. (B) Representative images of a portion of the ventral SVZ stained for Ki67 (red), TUNEL (green) and DAPI (blue). Scale bar: 25 µm. The upper panel shows untreated mice and the lower panel figures were from mice killed at 6 h following irradiation with the indicated doses. (C) Dose–response and linear fitting of radiation-induced apoptosis in WT adult SVZ. (D) Timecourse of radiation-induced apoptosis in WT and Lig4Y288C adult SVZ exposed to 100 mGy X-rays. Data represent mean±s.e.m. (WT 50 mGy 6 h, n=3; WT 100 mGy 6 h, n=5; WT 500 mGy 6 h, n=1; WT 100 mGy 15 h, n=2; Lig4Y288C 100 mGy 1.5 h, n=2; Lig4Y288C 100 mGy 6 h, n=2; Lig4Y288C 100 mGy 15 h, n=2). **P≤0.01 (unpaired Student’s t-test).

Consistent with previous findings, we observed a low level of endogenous apoptosis in the WT neocortex at E14.5 and substantially higher apoptosis in Lig4Y288C embryos, demonstrating the marked sensitivity of the embryonic neocortical VZ/SVZ to DSB-induced apoptosis (Fig. 6A, black and blue columns). Interestingly, although Atm−/− mice showed slightly higher apoptosis than WT mice, the levels were not further elevated in Atm−/−/Lig4Y288C mice despite high levels of DSBs, consistent with previous conclusions that apoptosis in the embryonic VZ/SVZ is predominantly ATM dependent (Fig. 6A, red columns) (Gatz et al., 2011). At E17.5, apoptosis had significantly decreased, as observed previously (Gatz et al., 2011), consistent with the reduction in DSB levels.

At P5, a low level of apoptosis was detected in the isocortex, which appeared similar in WT, Atm−/− and Lig4Y288C mice; by P15 and adulthood there was no detectable apoptosis (Fig. 6B). In contrast, we observed substantial levels of apoptosis in the SVZ in all mice at P5 and P15, which was greater than observed at E17.5. The level was similar in WT and Atm−/− mice and elevated in Lig4Y288C (Fig. 6B). To allow direct comparison, both the number of TUNEL+ cells per section and per mm² area were scored in the SVZ region, and they showed a similar profile (supplementary material Fig. S2B). This pattern persisted at P15 although the overall level of apoptosis was reduced. By 2–3 months, the level of apoptosis was much lower, although it continued to be elevated in Lig4Y288C mice (Fig. 6B,C). The number of Ki67+ cells in the SVZ diminished from
P5 to P15 to 2–3 months, suggesting that the ATM-independent process of apoptosis correlates with proliferation levels (Fig. 6D,E).

These findings provide the first evidence for ATM-independent apoptosis in the SVZ shortly after birth, and show that it is unrelated to the presence of DSBs because WT mice had low levels of DSBs at P5 compared to E17.5 yet a nearly tenfold increase in the level of apoptosis. Superimposed on this process, analysis of the Lig4Y288C mice also revealed the sensitive activation of DSB-induced ATM-dependent apoptosis in the SVZ. The slightly increased apoptosis at P5 in the isocortex could reflect either a similar process or the radial movement of some cells from the SVZ to the isocortex at this juvenile developmental stage.

**DISCUSSION**

**Sensitive activation of apoptosis in the adult neural stem cells**

The embryonic neocortex is characterised by high proliferation from E11 to E16.5, high DSB damage and by sensitivity to DSB-induced apoptosis (Bayer et al., 1991; Gatz et al., 2011; Pontious et al., 2008; Saha et al., 2014). Here, we find that cells in the adult SVZ do not incur high levels of DSBs but sensitively activate apoptosis (Fig. 7). The comparison of DSB formation and apoptosis of the embryonic neocortex and adult SVZ in WT and Lig4Y288C mice is summarised in supplementary material Table S1. Apoptosis was activated in a linear dose-dependent manner in the SVZ of WT mice with 50 mGy causing detectable apoptosis (Fig. 5C). Endogenous DSBs and apoptosis in the SVZ in Lig4Y288C mice was similar to that detected following exposure to 15–20 mGy X-rays, suggesting that apoptosis can be activated by approximately one DSB per cell (assuming an induction of ∼25 DSBs per Gy). Taking DSB levels into account, we conclude that there is a similar or even enhanced sensitivity to DSB-induced apoptosis in the adult SVZ compared to the embryonic neocortical VZ/SVZ, demonstrating that this is an intrinsic feature of these compartments rather than an indirect consequence of rapid replication. Endogenous apoptosis was not detected in the SGZ of Lig4Y288C mice although exposure of WT mice to 500 mGy enhanced apoptosis (Fig. 5A). The marked sensitivity to DSB-induced apoptosis in the adult SVZ is important given the increased usage of computerized tomography (CT) scanning, when doses of 1–10 mGy can be received (Brenner and Hall, 2007). The functional role of the SVZ in the adult human brain remains unclear, but it has been proposed to promote tissue regeneration and cognitive plasticity, especially during infancy (Yang et al., 2011). Thus, the sensitivity to activate apoptosis could be important in situations when radiological procedures are
in all the neuronal tissues. A steady state level seems to have been reached by 2–4 months of age. Currently, we are unable to distinguish whether these represent residual unrepaired DSBs that arose in the embryo or whether DSBs also arise in the adult brain. Further work is required to address the level of DSB formation in the embryonic precursor compartments for the cerebellum and hippocampus. Atm\(^{−/−}\) mice also displayed enhanced DSBs in some neuronal tissues. Atm\(^{−/−}\) mice accrue some unrepaired DSBs by E14.5 although less than Lig4\(^{−/−}\) mice. The level of DSBs at E14.5 and in all post birth stages appeared similar. A similar, but more striking and unexpected impact, was evident in the Atm\(^{−/−}/\)Lig4\(^{−/−}\) double mutant mice. Thus, although the DSBs generated in utero in Lig4\(^{−/−}\) embryos undergo slow repair post birth, this does not appear to happen in an ATM-deficient background. It is unlikely that the role of ATM in regulating apoptosis can fully account for this because many cells in Lig4\(^{−/−}\) mice can escape apoptosis, but DSBs still undergo slow repair. Another possibility is that because ATM also regulates checkpoint arrest, the passage of damaged cells through the cell cycle checkpoints with unrepaired DSBs renders them irreparable. Although we cannot eliminate the possibility that Atm\(^{−/−}\) and Atm\(^{−/−}/\)Lig4\(^{−/−}\) mice incur enhanced DSB formation during adulthood, the remarkable similarity in foci numbers in embryos and adult mice argues against this. Thus our findings raise the possibility that loss of ATM impedes the repair of DSBs generated during embryogenesis. Interestingly, despite enhanced embryonic lethality, the live Atm\(^{−/−}/\)Lig4\(^{−/−}\) mice, although small, do not show marked neurodegeneration even though they harbour 53BP1 foci.

**The developing SVZ compartment undergoes high endogenous apoptosis**

A new feature that we observed was a high level of apoptosis in the SVZ region at P5–P15. In WT mice, this process was ATM-independent, but the enhanced apoptosis at P15 in Lig4\(^{−/−}\) mice compared to in the double mutant mice suggests that an ATM-dependent process might also function (Fig. 7). The enhanced apoptosis is not simply due to the initiation of apoptosis in the embryo because the level is lower at E17.5 compared to P5. The SVZ region undergoes replication but diminishes in size during this period, suggesting the presence of a developmental process promoting cell loss; however, the precise mechanism has not been described (Sanai et al., 2011). Thus, we provide evidence that this stage of cell loss can proceed by apoptosis.

Interestingly, the Lig4\(^{−/−}\) neonates had fewer DSBs in the SVZ at P5 and P15 than observed in the corresponding isocortex. A similar and more evident relationship was seen in the single P15 and adult double mutant Atm\(^{−/−}/\)Lig4\(^{−/−}\) mice, although small, do not show marked neurodegeneration in any other neuronal tissue. Although this impact cannot be entirely attributable to apoptosis, we speculate that a competitive process promoting cell loss; however, the precise mechanism has not been described (Sanai et al., 2011). Thus, we provide evidence that this stage of cell loss can proceed by apoptosis.

**The impact of ATM deficiency on DSB levels in the adult brain**

The temporal analysis of DSBs and apoptosis in the mutant strains from E14.5 to 2–3 months post birth proved to be revealing. We compared DSBs and apoptosis in the embryonic neocortex with that in the postnatal isocortex and SVZ, tissues derived from the embryonic VZ/SVZ. Lig4\(^{−/−}\) and Atm\(^{−/−}/\)Lig4\(^{−/−}\) mice harboured high DSB levels in both tissues at P5 and P15. Although it is difficult to eliminate the possibility that DSB generation continues at a high level post birth and gradually diminishes, these findings are consistent with the notion that cells with DSBs generated in utero can progress into the newborn mouse, where the DSBs can undergo slow repair. In Lig4\(^{−/−}\) mice, the rate of repair appears to be slower than observed in non-dividing cultured cells. This could arise if cells in vivo undergo cell division after DSB formation, which likely impacts upon their rate of repair. Alternatively, new DSBs could arise in vivo. Despite ongoing repair, by 2–3 months Lig4\(^{−/−}\) mice still have enhanced DSBs used to assess neuronal damage and for scattered doses following cranial radiotherapy.

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is ongoing, as shown by the high Ki67 levels, it is likely that the ATM-independent process represents ATR-dependent apoptosis, which functions following replication. Collectively, based on these findings, we speculate that apoptosis and/or a competitive environment in the developing stem cell compartment and surrounding niche functions to reduce the number of damaged stem cells, providing a mechanism to select for the fittest stem cells.

In summary, we demonstrate that the adult neural SVZ is exquisitely sensitive to DSB-induced apoptosis, with a sensitivity similar to that observed in the embryonic SVZ. The adult SVZ, however, is less vigorously replicating and is less prone to endogenous DSB formation demonstrating that sensitivity to DSB-induced apoptosis and rapid replication can be uncoupled. Finally, we identify a further stage of high sensitivity to apoptosis post birth during the development of the SVZ, and provide evidence to suggest that this process can serve to diminish the level of DNA breakage by removing cells with DSBs that arose during embryogenesis.

**MATERIALS AND METHODS**

**Mice**

Lig4Y288C (C57BL/6 background) and Atm−/− (mixed 129/SV×C57BL/6 background) mice were as described previously (Barlow et al., 1996; Nijnik et al., 2007). Atm−/− mice were crossed with Lig4Y288C (Atm−/−/Lig4Y288C) and developed on a mixed 129/SV×C57BL/6 background. Atm−/−/Lig4Y288C mice were born at sub-Mendelian frequencies based on the number of living mice born (expected n=60; observed n=4).

For embryonic analysis, the date of appearance of a vaginal plug was considered as embryonic day 0.5 (E0.5) and the day of birth as postnatal day 0 (P0). Embryos were taken at E14.5 and E17.5. Mice were designated adult from P50 and examined at 2–4 months post birth. Controls (WT) were wild-type littersmates of either sex. All animal experiments were carried out in accordance with accepted standards of animal welfare approved by the United Kingdom Home Office and complied with the Animals (Scientific Procedures) Act 1986.

**Primary MEF isolation**

Primary MEFs were prepared from E13.5 embryonic mouse tissues and cultured as described previously (Nijnik et al., 2007).

**Mouse irradiation**

The whole body of the adult mice were exposed to X-rays using an AGO HS Mouse irradiation (TUNEL) staining was performed as described by the manufacturer (Roche, Basel, Switzerland). After primary and secondary antibody staining, the TUNEL reaction mixture was added and samples were incubated for 1 h at 37°C in a darkened humidified chamber. Slides were counterstained with DAPI and mounted with Vectashield.

**Image analysis and quantification**

The counts of 53BP1 foci per cell were scored in the IZ and CP region of the embryonic neocortex at E14.5 and E17.5. Quantification was performed as described previously (Saha et al., 2014). At least two sections were quantified per embryo with a minimum of 100 cells scored for each section. TUNEL quantification was carried out as TUNEL− cells per mm² in a defined area of the developing neocortex at E14.5 and E17.5 (VZ, SVZ, IZ and CP). Mutant or WT embryo forebrains were less than twofold different in size.

For counts of 53BP1 foci per cell in the postnatal and adult mouse tissues, 100 cells per region per section from at least two sections per mouse were scored. TUNEL quantification involved scoring the entire area of each brain region to quantify TUNEL− cells per section. To quantify proliferation in the SVZ of P5, P15 and 2–3-month-old mice, all Ki67+ cells in a 0.07 mm² area close to the ventral SVZ were scored and the ratio was related to the number of Ki67+ cells in the same area in 2–3-month-old WT mice. Images were acquired with a Zeiss Axioplan or a Nikon eclipse/E400 microscope and analysed using Simple PCI software and ImageJ.

**Statistical analysis**

At least two mice of each genotype and treatment were quantified (averaged from at least two sections per mouse). No mice were excluded. Bar and symbol plots represent mean values of independent replicates, and error bars represent s.e.m. Statistical analysis was carried out using a two-tailed unpaired Student’s t-test or a one-way or two-way ANOVA. Linear and exponential models were used for data fitting (Origin 8.6).

**Immunofluorescence staining**

Preparation and immunostaining of tissue sections were performed as described (Gatz et al., 2011). The primary antibodies used were against: 53BP1 (rabbit, 1:500; cat. no. A300-272A; Bethyl, Montgomery, TX), γH2AX (Ser139, mouse, 1:500; cat. no. 05-636; Merck Millipore, Billerica, MA), Ki67 (rat, 1:500; cat. no. 14-5698-82; eBioscience, San Diego, CA), lamin B (goat, 1:500; cat. no. sc-6216; Santa Cruz Biotechnology, Dallas, TX) and cleaved caspase-3 (rabbit, 1:200; cat. no. 95798; Cell Signaling, Danvers, MA). Appropriate secondary antibodies were used and were conjugated to FITC (donkey anti-rabbit-IgG, 1:200; cat. no. 711-095-152; Jackson ImmunoResearch, West Grove, PA), Alexa Fluor 488 (donkey anti-rabbit-IgG, 1:500; cat. no. A-21206; Life Technologies, Carlsbad, CA), Alexa Fluor 594 (donkey anti-rat-IgG, 1:500; cat. no. A-21209; Life Technologies) and DyLight 594 (sheep anti-rabbit-IgG; cat. no. C2306; Sigma).

**TUNEL assay**

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining was performed as described by the manufacturer (Roche, Basel, Switzerland). After primary and secondary antibody staining, the TUNEL reaction mixture was added and samples were incubated for 1 h at 37°C in a darkened humidified chamber. Slides were counterstained with DAPI and mounted with Vectashield.

**References**


Figure S1: In vitro and in vivo DSB repair kinetics after IR. (A) Kinetics of 53BP1 foci loss in adult WT and Lig4<sup>Y288C</sup> cerebellum, SVZ and isocortex following IR with 100 mGy X-rays (as presented in Fig. 2B). Data represent mean ± SEM. (n ≥ 2 for each genotype and treatment). (B) Enlarged version of the 53BP1 foci/cell level at early time points after IR with 100 mGy X-rays (as shown in panel A). (C) γH2AX foci repair analysis in MEFs after exposure to 500 mGy in the presence or absence of 10 µM ATMi KU55933. ut, untreated. (D) Quantification of γH2AX foci (left y-axis) and 53BP1 foci (right y-axis) at 0.5 h in WT MEF following exposure to 50-500 mGy X-rays.
Figure S2: Postnatal and adult apoptosis in the SVZ. (A) Apoptosis in the adult SVZ measured as TUNEL⁺ cells per section in untreated WT and Atm⁻/⁻ mice, and following irradiation with 50 and/or 100 mGy X-rays. (n as described in Fig. 5; Atm⁻/⁻ 100 mGy 6 h, n=3). (B) Apoptosis in the postnatal (P5 and P15) and adult (2/3 months) SVZ measured in TUNEL⁺ cells per mm² (as in Fig. 6B) to allow direct comparison with Fig. 6A. Data represent mean ± SEM. (n as described in Fig. 6). *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
Table S1: Comparison of endogenous and IR-induced 53BP1 foci and apoptosis in the E14.5 and E17.5 neocortex, and in the P5, P15 and adult SVZ. Quantification of 53BP1 foci/cell and apoptosis (measured as TUNEL+ cells per mm²) endogenously and after IR with 100 mGy in the WT and Lig4<sup>Y288C</sup> embryonic neocortex (at E14.5 and E17.5), in the postnatal (at P5 and P15) and adult SVZ compartment. Data represent mean ± SEM.

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<tr>
<th></th>
<th>Embryonic neocortex</th>
<th>Post-birth SVZ</th>
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<tr>
<td></td>
<td>E14.5</td>
<td>E17.5</td>
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<td>DSBs (53BP1 foci/cell)</td>
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<tr>
<td>WT</td>
<td>0.10 ± 0.01</td>
<td>0.05 ± 0.01</td>
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<td>Lig4&lt;sup&gt;Y288C&lt;/sup&gt;</td>
<td>1 ± 0.13</td>
<td>0.64 ± 0.02</td>
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<td>WT 100 mGy</td>
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<td>Apoptosis (TUNEL+ cells/mm²)</td>
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<tr>
<td>WT</td>
<td>31.7 ± 9.5</td>
<td>10.4 ± 3.3</td>
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<tr>
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<td>380.7 ± 31.8</td>
<td>138 ± 5.7</td>
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<td>WT 100 mGy 6 h</td>
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