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Letter to the Editor

DNA repair in post-mitotic neurons: a gene-trapping strategy

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Dear Editor,

DNA repair is essential for maintaining the integrity of the genome. Although mutation often occurs during meiosis and mitosis, the DNA of post-mitotic neurons is also under risk of damage, for example, from free radicals. For humans, given that the lifespan of post-mitotic neurons is many decades, competent DNA repair could be a critical factor in slowing ageing and the progression of some pathologies. Double-strand breaks (DSBs) are considered the most lethal form of DNA damage which, if left unrepaired, can cause cell death.[1,2] Mammalian cells repair DSBs by two pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ).[1,2] Although *in vitro* assays support the idea that the mature brain can repair DNA,[3–5] repair of DSBs has not been directly demonstrated in living post-mitotic neurons. The aim of this study was to test directly the possibility of DSB repair in post-mitotic neurons by using a promoter-less gene-trapping (GT) vector.

The promoter-less GT vector had a 6 kb $GABA_A$ receptor $\alpha6$ subunit gene fragment containing part of exon 4 through to the middle of intron 8.[6] The GT vector carried a splice acceptor site (SA), followed by stop codons in all three reading frames and an internal ribosome entry site (IRES) element linked to either β-galactosidase (lacZ) or green fluorescent protein (GFP). The IRES-lacZ or IRES-EGFP was inserted into exon 8 downstream of the SA of the $\alpha6$ gene.[6] Following a strand break in chromosomal DNA, the linear gene trap vector can be ligated into the gap. If this happens in the correct orientation and the gene is expressed in neurons, the result is a trapped gene which expresses GFP or β-galactosidase.

By using the biolistic in-chamber technique,[7] we transfected the GT vector, containing either GFP or lacZ, into cerebellar granule cell cultures and, after 48 h, we scored cells expressing the reporter gene. The expression of the marker present in the promoter-less GT vector would require the integration and re-ligation of its free ends into an actively transcribed genomic region. As expected, the frequency of integration was low. For the GFP reporter, just seven recombinant cells were observed on 29 coverslips (frequency of 7×10^{-7} – Figure 1b), whereas for lacZ, there were five cells in 27 coverslips (a frequency of 5×10^{-7}; Figure 1d). On the contrary, transfection with both CMV-EGFP and RSV-lacZ-positive control plasmids resulted in a large number of GFP- and β-galactosidase-expressing cells (Figure 1a and c).

We then looked if DNA ligation can happen in a wider range of neurons by transfecting organotypic cerebellar slices. Transfected organotypic cerebellar slices with the CMV-EGFP-positive control plasmid produced GFP expression in numerous cells (Figure 1h). With the promoter-less gene trap constructs, some cells of various morphologies in the slices also expressed the reporter genes after transfection (Figure 1i). However, the frequency was low: in 35 transfected slices, there were only seven GFP-positive cells. Relative to the observed expression of the CMV-EGFP construct, this represents a relative frequency of 2.6×10^{-3}.

The incorporation of the gene trap vector into the neuronal genome depends on the vector having free ends. In fact, for a number of transfections comparable to those with linearized plasmid ($n = 30$), cultures transfected with supercoiled (uncut) gene trap vectors had no positive cells (data not shown).

If post-mitotic neurons have the ability to repair DNA, this repair activity should be enhanced following the induction of DSBs and so the frequency of GT events should increase. By switching the culture medium to minimal essential medium containing 5 mM K^+/serum-free (K5/S^-) and no insulin, cerebellar granule cells start apoptosis and undergo DNA fragmentation.[8] To investigate the effect of induced DNA damage on GT frequency, cerebellar granule cells were switched to K5/S^- for 4 h and then transfected. The K5/S^- medium was then replaced with the previously saved culture-conditioned medium and the dishes were returned to the incubator for 48 h, when they were fixed. Cells remained viable and survived the crisis (Figure 1f and g). We found a strong increase in DNA recombination frequency, which increased from virtually undetectable to 4.75 ± 0.95 neurons/field (microscopic field of $800 \times 500\,\mu m$) with GFP construct (Figure 1e).

The vector used in the current study could become trapped by enzymatic activities in the process of repairing DSBs arising from environmental DNA damage as an attempt to reverse it. To investigate if the increment in GT frequency observed by inducing DNA damage correlates with DNA repair machinery activation, we analysed changes in expression of genes involved in DSBs repair mechanism by real-time PCR, 4 and 8 h after apoptotic stimulus.

After giving granule cells apoptotic stimulus, we first analysed RNA levels of the histone proteins (H1, H2A, H2B, H3 and H4) since chromatin modification has been documented during DSB repair as an early step in the cellular response,[9,10] and they also indicate the occurrence of DNA damage. We indeed found, 4 h after the apoptotic stimulus, two-fold increase in H3, an approx. five-fold increase in H1 and H2A, 11-fold increase in H2B and 15-fold increase in H4

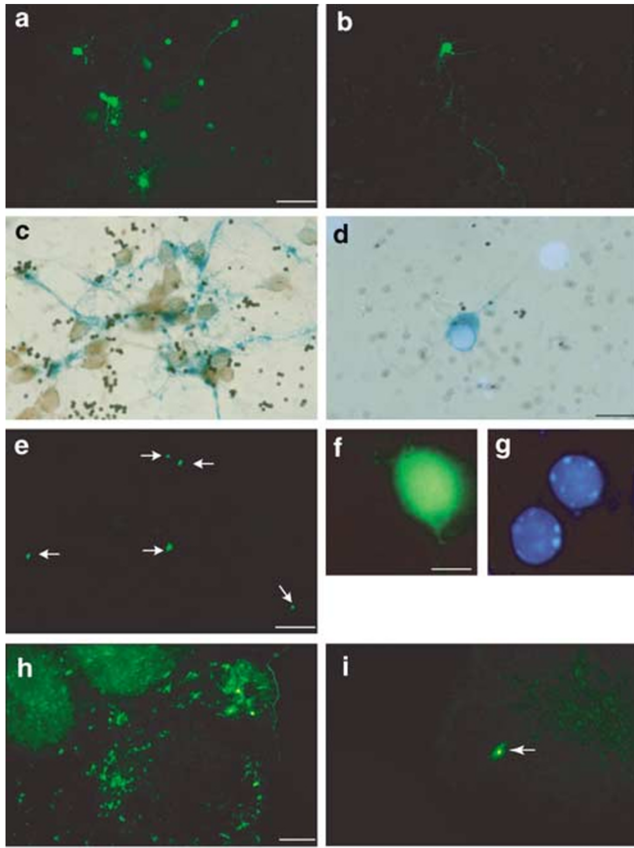

Figure 1 (**a**) Granule cells cultured in complete medium give numerous GFP-positive cells after transfection with the control CMV-EGFP plasmid (scale bar = 50 μm) or with RSV-lacZ vector (**c**) (scale bar = 10 μm). Recombinant GFP or lacZ-positive cells are rare, but detectable, after transfection with GT-GFP vector (**b**) (scale bar = 50 μm) and GT lacZ vector (**d**) (scale bar = 10 μm). Examples of typical cells are shown. There are no differences in the numbers of positive cells obtained between the two conditions. The black dots in (**c**) are the gold particles shot by the gene gun. (**e**) Granule cell cultures transfected with GT-GFP 4 h after switching to K5/S- and then back in complete medium for 48 h contain many GFP-positive granule cells (arrowed), note 5 in this field of 800 $\times$ 500 μm. (**f**) High power view ($\times$ 100, oil immersion) of gene-trapped granule cell which retained normal Hoechst profile presented in (**g**) (scale bar = 5 μm). (**h**) Transfecting organotypic slice cultures of mouse cerebellum with CMV-EGFP plasmid produce many GFP-positive cells. (**i**) Transfection with GT-EGFP vector gives some positive cells (arrow), but these are rare (just one on the slice shown)

expression. The increment in expression of histone genes remained above the basal level for at least 8 h. We then studied changes in expression of different genes coding for both HR and NHEJ repair factors and found a significant increment in most of the key transcripts involved in both repair pathways in granule cell cultures switched to K5/S$^-$ for 4 h. In particular, the expression levels of Ku80, DNA-PKcs, XRCC4, Mre11 and Rad50 were increased by approx. 70–90%, while Rad 54 showed a 400% increment. Interestingly, in neurons which underwent an 8 h apoptotic stimulus, the values of the repair factors returned to basal levels, suggesting the presence of a threshold condition over which the pathways to repair DNA damage are not active anymore.[11]

If the apparatus needed for DNA repair is active in the adult brain, neurons from mice with demonstrated DSBs repair deficiency should not be able to reverse mild DNA damage and should be more susceptible to apoptotic stimuli. To confirm this hypothesis, we studied neuronal survival in cerebellar granule cell cultures prepared from mice deficient in DNA-PKcs whose activity is involved in V(D)J recombination and DSBs repair (*Scid* mice).[12]

We performed terminal transferase-mediated dUTP-biotin nick end-labeling (TUNEL) in granule cell cultures from control mice and *scid* mice following an apoptotic stimulus (Figure 2A). The morphology of the neurons and the extension of the neuritic tree in cells from control mice were preserved after 4 h of apoptotic stimulus induced by switching to K5/S$^-$. However, there was a low percentage of TUNEL-positive nuclei (Figure 2A, panel c, and Figure 2B). This percentage remained constant 24 h after recovery, indicating that the DNA repair mechanism was activated (Figure 2A, panel e, and Figure 2B). Under the same experimental conditions, neurons from *scid* mice showed a percentage of fragmented nuclei comparable to neurons from control mice, but neuritis were fragmented and their density had decreased (Figure 2A, panel d, and Figure 2B). On the contrary, by 24 h of recovery, the percentage of dead cells in *scid* mice almost doubled, compared with control mice (Figure 2B). Furthermore, cellular fragmentation was extensive, cells had begun detaching from the substrate and neuritis were completely fragmented (Figure 2A, panel f). Analysis of DNA from control and *scid* cells confirmed these observations. K5/S$^-$ treatment for 4 h did not lead to cleavage of DNA into oligonucleosomal-sized fragments in both types of cell cultures (Figure 2C, lanes 1 and 3). On the contrary, based on the intensity of the ethidium bromide staining, we observed extensive DNA fragmentation 24 h after recovery only in *scid* cells (Figure 2C, compare lanes 2 and 4). This finding indicates that neurons defective in a key factor of DNA repair, by accumulating DNA damage, are more sensitive to programmed cell death.

In summary, we found that granule cell primary cultures and cerebellar organotypic slices can integrate and express a promoter-less GT vector, thus revealing DNA repair activity in post-mitotic neurons. Furthermore, we showed that this activity increases after a mild apoptotic stimulus and correlates with an increased expression of both HR and NHEJ DNA repair factors.

At present, experiments showing DNA repair activity in adult brain have been exclusively performed *in vitro* using brain extracts.[3–5] A GT strategy represents a good way of monitoring DNA repair events *in vivo*. In normal conditions, as expected, GT vector integration occurred at low frequencies for several reasons: most of the genome is noncoding; the vector has to incorporate into active genes in the correct orientation and this is bound to be a low probability (even when they do hit a gene, 50% of vector insertions will be back-to-front). Furthermore, DSBs that provide sites for ligation are rare events.

The increment of levels of the factors linked to DNA-damage-response pathways indicate that DSBs lead to the activation of a complex regulatory response. Indeed, both HR and NHEJ components were upregulated 4 h after the apoptotic stimulus. Little is known about the relative contribution of NHEJ and HR to the repair of DNA damage that is not genetically programmed, but there is evidence that both pathways have access to the same DSBs.[13,14] Our findings

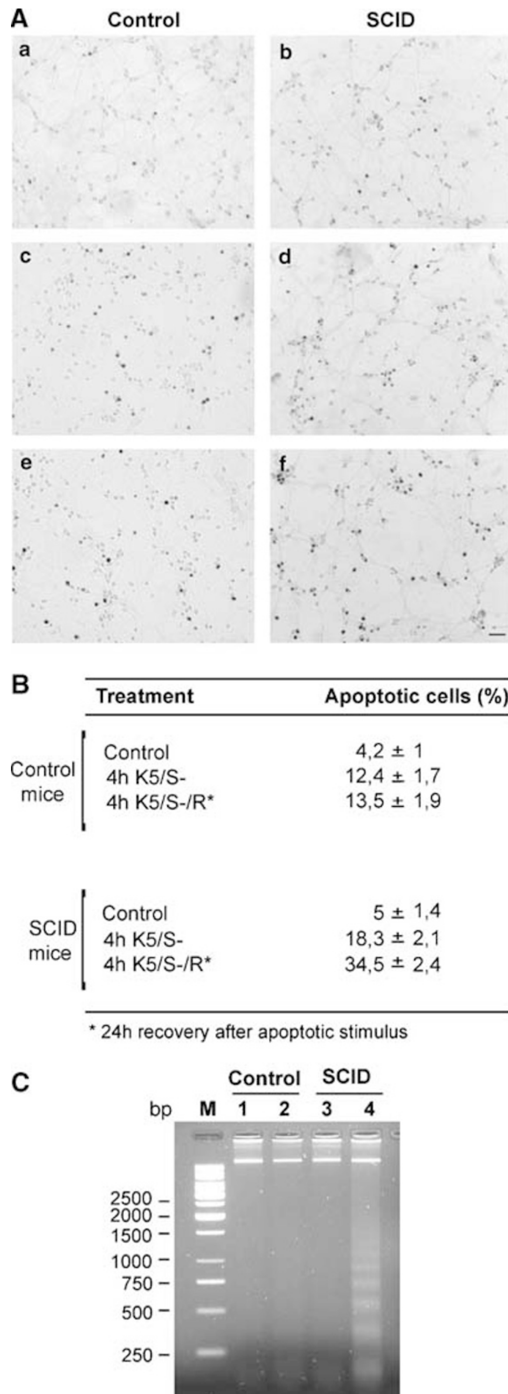

Figure 2 (**A**) TUNEL shows an increased apoptotic rate in cerebellar granule cell cultures from scid mice (**b**, **d**, **f**) compared to control mice (**a**, **c**, **e**). (**a**, **b**) Control; (**c**, **d**) neurons were switched to K5/S- for 4 h and then fixed; (**e**, **f**) cells were maintained in K5/S- for 4 h and then allowed to recover in complete medium for 24 h. Scale bar = 100 μm. (**B**) TUNEL-positive cells were counted randomly choosing five microscopic fields to reach a total of at least 900 cells per coverslip. Two coverslips were scored for each condition. The percentage of apoptotic cells was calculated as the number of TUNEL-positive cells divided by the total cell number. Data shown are means ± S.E.M. ($n = 3$). $P < 0.05$. (**C**) Extensive DNA fragmentation after 24 h of recovery occurs only in *scid* cells (lane 4). Lanes 1 and 3: 4 h of K5/S-; lanes 2 and 4: 4 h of K5/S-, followed by 24 h of recovery in complete medium

are consistent with the hypothesis of cooperation of the two DSB repair pathways for genomic integrity.

Interestingly, we also found that most of the DNA repair factors returned to basal level 8 h after the induction of DNA damage, indicating that the activation of the repair machine is operable at the early stage of DNA damage and that, after a condition in which the insult is stronger, a death-signalling cascade that abolishes the repair pathways initiates. This view is supported by the findings obtained by using cultured neurons from mice with DSB repair deficiency; s*cid* mice, lacking DNA-PKcs activity, may accumulate DNA damage, which could reach critical levels and trigger the apoptotic cascade.

Thus, it seems likely that DNA repair mechanisms contribute essential housekeeping functions, maintaining the neuronal genome in a healthy state as precursors divide in development and during the long life of mammalian neurons.

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