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DNA double-strand breaks as drivers of neural genomic change, function, and disease

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Abstract

Early work from about two decades ago implicated DNA double-strand break (DSB) formation and repair in neuronal development. Findings emerging from recent studies of DSBs in proliferating neural progenitors and in mature, non-dividing neurons suggest important roles of DSBs in brain physiology, aging, cancer, psychiatric and neurodegenerative disorders. We provide an overview of some findings and speculate on what may lie ahead.

Keywords

DNA repair; non-homologous end joining; DNA double-strand breaks; neural stem and progenitor cells; neurons; somatic mosaicism; exon shuffling; genomic instability; DNA replication; transcription; neurodevelopment; copy number variations

1. Introduction

Overall stability of the genome is important for normal cellular function and disease prevention, yet it is becoming increasingly clear that the genome is subject to sequence alterations in the context of various cellular processes. Beyond endogenous sources of DNA damage, such as DNA replication, transcription, and chromosome segregation, chemical mutagens and radiation can cause DNA damage-mediated genomic alterations [1]. Depending on cellular context, genomic alterations can drive cancer development and have been implicated in aging, and neurodevelopmental and neurodegenerative disorders [1]. Strikingly, despite widespread expression of DNA repair factors in many cells types, the central nervous system is the predominantly affected organ when DNA repair is impaired

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Conflict of interest

The authors declare no conflicts of interest.

[2]. How genomic alterations are formed and how cells repair DNA damage in various tissues continue to be important fundamental questions with many implications for human health and disease.

Although genomic alterations can be mediated by various forms of DNA damage, in this article we focus on DNA double-strand breaks (DSBs), which arise when both DNA strands are broken at a given genomic position. When DSBs are not joined back together in the original configuration—and instead fuse with DSBs in other genomic locations—they give rise to DNA rearrangements. Such rearrangements can occur within a given chromosome or can fuse different chromosomes together to form of inter-chromosomal translocations.

Beyond any potentially negative outcomes, DSBs provide the opportunity to functionally modify genomic information. For example, during meiosis, DSBs mediate the exchange of genomic sequences between homologous chromosomes [3]. DSB-mediated genomic alterations are critical for the development and function of the adaptive immune system. V(D)J recombination in lymphocyte progenitors utilizes DSBs to generate a diverse repertoire of antigen receptors, and immunoglobulin heavy (IgH) chain class switch recombination in mature B lymphocytes relies on DSBs to alter the effector functions of a given antibody [4]. Based on cellular context and aspects such as cell cycle phase, mammalian cells use several different mechanisms to recognize and repair chromosomal DSBs, including homology-directed repair [5] and end-joining repair pathways requiring minimal or no homology of the DSB ends [6].

Classical non-homologous end joining (C-NHEJ) requires the evolutionarily conserved “core” NHEJ factors Ku70, Ku80, Xrcc4, and DNA Ligase 4 (Lig4), and is considered a major DSB repair pathway in mammalian cells due to its ability to operate in all phases of the cell cycle [4, 6, 7]. Although C-NHEJ is sometimes referred to as a “bad” form of DNA repair, it actually functions as a major protector of genomic integrity, rapidly repairing DSBs, frequently with no or only minimal loss of genomic sequence. C-NHEJ can, however, also catalyze the formation of gross chromosomal alterations in form of inter- and intra-chromosomal translocations [6, 8]. Notably, “alternative” end-joining pathways, which can be defined as any form of end-joining not requiring the core C-NHEJ factors [7], also have been implicated in the formation of genomic aberrations [9].

Early studies of C-NHEJ factors in mice revealed that C-NHEJ is required for immune system development and neurodevelopment [10–13]. Recent findings, in significant parts based on studies of C-NHEJ-deficient cells, have identified recurrent DSB clusters in dividing neural progenitor cells [14, 15]. Further, DSB formation and repair have been implicated in the function of post-mitotic neurons [16, 17]. In the following sections, we will give an overview of recent findings about DSB formation and repair in neural cells in the contexts of neurodevelopment and the mature brain.

2. DNA double-strand breaks in dividing neural progenitor cells

Multiple lines of evidence suggest that somatic cells, including neural cells, are subject to much more extensive genomic alterations than previously anticipated. Variations in coding

and non-coding regions of the genome may confer differences amongst individuals in terms of brain function and susceptibility to brain disorders. Indeed, mature brain cells have been shown to contain frequent genomic alterations proposed to promote neuronal diversity [18–20]. There is now great interest in somatic mutations that form post-zygotically – as opposed to inherited germline mutations – in neural cells and thus give rise to somatic brain mosaicism [19]. Such genomic alterations, which may arise in dividing neural progenitors, may underlie the diversity of neuronal cell types and have a role in neuronal physiology [19]. They may also be involved in the etiology of neurodevelopmental and psychiatric disorders [19].

Potential mechanisms causing genomic alterations in brain cells remain mostly unexplored. Early work performed shortly after the identification of C-NHEJ pathway factors [21, 22] that involved genetic inactivation of these factors in mice [10, 13] revealed critical roles in lymphocyte development and found abrogation of neuronal development because of unrepaired DSBs in neuronal progenitors [11, 12]. These findings suggested the possibility that neuronal development may involve genomic alterations and, perhaps, diversification of genomic information functionally related to the process of antigen receptor rearrangement via V(D)J recombination [12, 23, 24].

V(D)J recombination involves RAG endonuclease-mediated DSB formation [25] at the ends of antigen receptor gene segments known as V, D, and J segments. Subsequent processing and joining results in V(D)J variable region exons [26]. C-NHEJ inactivation in mice blocks progenitor B and T lymphocyte development because functional antibody and T-cell receptor genes required for development into mature B and T cells cannot be assembled in the absence of C-NHEJ-mediated repair of RAG-mediated DSBs [4]. Specifically, RAG-mediated DSBs are introduced in the G1 phase of the cell cycle and persistent DSBs in C-NHEJ-deficient B and T lymphocyte progenitors undergo p53 G1 checkpoint-mediated apoptosis [27–29]. p53 inactivation rescues the embryonic lethality of XRCC4- or Lig4-deficient mice but does not restore lymphocyte development due to persisting inability to complete V(D)J recombination. However, p53 inactivation allows XRCC4- or Lig4-deficient lymphocyte progenitors with persistent RAG-mediated DSBs to divide [27, 28], which contributes to development of pro-B cell lymphomas with recurrent translocations between the *IgH* locus and *c-Myc* [29–31]. These pro-B cell lymphomas develop rapidly and cause early death in mice. Many of these mice also exhibit *in-situ* medulloblastomas, a primary tumor of the cerebellum [29], further suggesting that developing neural cells undergo abundant DSBs.

Neuronal progenitor cells in C-NHEJ-deficient mice undergo apoptosis at developmental time points associated with differentiation into post-mitotic, mature neurons [12]. This checkpoint-mediated apoptosis of newly post-mitotic neurons with persisting DSBs may be a mechanism to prevent incorporation of such neurons into the nervous system. Inactivation of p53 prevents neuronal death in XRCC4- or Lig4-deficient mice [13, 27, 28] but due to rapid death from pro-B cell lymphomas potential implications of unrepaired, developmental DSBs for neuronal function could not be assessed.

Several fundamental questions related to neural DSBs remained unanswered for many years. What are the causes, genomic locations, and implications of abundant DSBs in developing neural cells? Initial answers came from studies involving conditional *Xrcc4* inactivation in neural stem and progenitor cells in p53-deficient mice [32]. These mice developed medulloblastomas with recurrent chromosomal translocations, including translocations involving the *N-myc* and *c-Myc* oncogenes [32], further suggesting intriguing parallels between DSB formation and repair in lymphocyte and neural progenitor cells. However, because of a lack of appropriate technology, comprehensive identification of DSBs in developing neural cells was not possible at that time.

To comprehensively elucidate DSB biology in various contexts, we developed high-throughput, genome-wide, translocation sequencing (HTGTS), an approach to rapidly identify DSBs genome-wide with high sensitivity and specificity [33–35]. HTGTS is based on the translocation of endogenous DSBs in any region of the genome to bait DSBs introduced in defined locations via endonucleases such as Cas9 [30, 33, 34, 36]. Further, recurrent endogenous DSBs such as those occurring during IgH class switch recombination (CSR) [37] or V(D)J recombination [38–40] can be used as bait DSBs. HTGTS studies provided several fundamental insights into how DSBs are formed and repaired. For example, they revealed that several classes of DSBs join preferentially to DSBs within the same topological domain because of proximity effects caused by spatial genome organization [4, 39, 41].

Two random DSBs only rarely occur within close proximity of each other. Thus, the effect of spatial proximity is most pronounced in the context of two recurrent DSBs occurring within kilobase- to megabase-sized topological domains. In this regard, the physiological process of IgH CSR in mature B cells takes advantage of the preferential joining of high-frequency DSBs within topological domains to achieve high levels of IgH isotype switching via joining of IgH switch-region DSBs [37, 41, 42]. Moreover, topological domains not only promote effective joining of antigen receptor gene segment DSBs during V(D)J recombination but can also control joining directionality within a domain [38, 43].

To address long-standing, fundamental questions about the nature of neurodevelopmental DSBs, we adapted HTGTS to dividing, primary mouse neural stem/progenitor cells (NSPC). We initially introduced bait DSBs on three chromosomes to reveal any potential endogenous, recurrent DSB clusters in the genome based on their ability to join to bait DSBs on multiple chromosomes [14]. This approach revealed 27 recurrent DSB clusters (RDCs) and implicated additional candidate RDCs throughout the NSPC genome [14]. All of the 27 RDCs were enhanced by mild replication stress caused by the polymerase inhibitor aphidicolin and, strikingly, were found to lie within genes encoding proteins with roles in synaptogenesis and neural functions [14]. Genomic alterations of most of the 27 RDC-genes have been associated with psychiatric disorders such as autism and schizophrenia and several are altered in brain cancers including medulloblastoma [44, 45] and glioblastoma [46]. In the latter context, RDCs may potentially contribute to recurrent genomic alterations that contribute to gene amplifications, deletions, and translocations in medulloblastoma [32] and other cancers [14].

Subsequent work involving introduction of CRISPR/Cas9-mediated bait DSBs into each of the 19 autosomes and the X chromosome of mouse NSPCs confirmed all 27 RDC genes and identified many additional RDCs [15]. All additional RDCs localize to genes or gene clusters and many were detected in the absence of induced replication stress, indicating that replication stress enhances an endogenous process present in NSPCs [14, 15]. NSPC RDCs can be assigned to three groups based on features including genomic length, organization, transcription rate, and replication timing [15]. Group 1 RDCs, which includes a majority (~80%) of the most robust RDCs, comprise a single, generally long gene; group 2 RDCs comprise several genes, including no less than one long (>80 kb) gene; and group 3 RDCs feature clusters of several small (<20 kb) genes [15]. Genomic lengths and transcription rates of group 1 and 2 RDCs are similar, yet group 3 RDCs are substantially shorter and display higher transcription rates [15].

DSBs in RDC genes in NSPCs could contribute to the formation of genomic copy number variations (CNVs) detected in normal neurons of the frontal cortex in humans [18]. In this regard, 30 RDCs map to regions reported to contain CNVs based on single-cell sequencing of human neurons [14, 15, 18] although the significance of these findings is not yet clear due to the current resolution limits of single-cell sequencing.

Much still remains to be elucidated with respect to the mechanisms causing RDC gene fragility in NSPCs and this is an area of active investigation. Replication timing and transcriptional activity of RDC genes suggests that they are prone to collisions between the replication and transcription machinery [14]. In this regard, the identification of RDCs provides a potential mechanistic model for many common fragile sites and CNVs that have been proposed to result from transcription/replication collisions [14, 15, 47, 48]. Indeed, some RDCs incur CNVs in embryonic stem cells and fibroblasts [14, 15, 47, 48]. How transcription/replication collisions cause DSBs in RDC is an important open question. Notably, a subset of RDCs fall into genomic regions reported to contain early replicating fragile sites in B cells [49], suggesting further potential mechanistic implications that will need to be explored [15].

The most robust RDC genes are generally large, occupying up to 2 Mb of genomic sequence, and lie within their own topological domains [14, 15]. Despite their large size, these RDC genes contain small exons and encode relatively short transcripts. DSBs occur across the RDC gene body, which means that most DSB occur in the long introns characteristic of these genes. Notably, because HTGTS can only detect endogenous DSBs that join to a bait DSB, it is likely that the DSB frequency within RDC genes is actually higher than the minimal frequency of 12 RDC translocations per NSPC that we estimated based on HTGTS [14]. These observations suggest an intriguing parallel to a major DSB-mediated, physiologic process in B lymphocytes: although of lower density, RDC DSBs approach the frequency of DSBs mediating IgH CSR in activated B lymphoyctes [14].

As most long RDC genes occupy replication domains [14] that correspond to single topological domains or sub-domains [50], two DSBs within an RDC gene would be predicted to undergo preferential joining. As indicated above, preferential DSB joining promoted by topological domains drives IgH CSR [37, 38], which involves joining of two

DSBs over genomic distances of 100 kb or more, and deletion of the intervening DNA sequence [4]. Based on the estimated high frequency of DSBs in RDC-genes upon replication stress [14], we propose that separate, intronic DSBs within long RDC-genes are frequently joined to each other, thus potentially leading to gene diversification in NSPCs [51] (Figure 1A).

Many RDC genes, especially the neurexins, produce large numbers of isoforms via alternative splicing [52, 53]. Novel exon combinations can also potentially be generated by recombination between intronic regions at the DNA level via “exon shuffling” [54]. We propose that modification of RDC genes via DSB-mediated, intronic recombination might cause such exon shuffling in NSPCs, thus altering the repertoire of possible transcripts in neural progeny, including mature neurons. In this context, introns can split the reading frame between codons (phase 0 intron), or within codons (phase 1 and 2 introns) [55] (Figure 1B). Exons flanked by introns of the same phase can be joined without altering the downstream reading frame. Notably, analyses of intron phases revealed that introns in many RDC genes tend to form compatible clusters (B. Schwer, unpublished observations) (Figure 1C), suggesting that domains could be altered by DSB-mediated intron-intron joining at the level of genomic DNA.

The proposed exon shuffling via intronic DSBs would be somewhat akin to IgH CSR, which also generates different isoforms of a protein rather than new exons as during V(D)J recombination. In this regard, the structure of long neural genes, with their small exons and very large introns [56], theoretically could have evolved to support replication stress-associated, DSB-mediated gene diversification. This poses important questions, including how such DSB-mediated gene diversification is prevented in the germline, highlighting the importance of investigating the extent of RDC-gene fragility in other cell and tissue types.

Beyond alterations caused by joining of DSBs in separate introns, RDC DSBs may also cause altered splicing patterns via localized DSB-induced splice site mutations and subsequent intron retention, which may impact transcript and protein abundance [57], or more limited intronic deletions that could affect gene function by altering regulatory elements. Thus, future work is required to determine whether increased replication stress during neurodevelopment promotes brain disorders via RDC formation in neural progenitors.

3. Neuronal activity and transcription-induced DNA double-strand breaks in neural cells

Recent work has implicated DSB formation and repair in the function of non-replicating, mature neurons [16, 17, 58]. The need to maintain mature neurons over the entire lifespan of an individual highlights the particular importance of repair and maintenance pathways in this cell type. Indeed, neurons are thought to incur frequent DNA lesions [2]. Whereas replication is likely a major source of DSBs in dividing neural progenitors, major sources of DSBs in non-dividing, mature neurons include oxidative stress and transcription. Notably, neurons undergo DSB formation in response to various forms of neuronal stimulation, including optogenetic activation, or physiological neurobehavioral tasks [16, 17, 58].

In mature neurons, activity-induced DSBs form in early response gene promoters and have a role in induction of gene expression [16]. Most early response genes encode transcription factors with roles in learning and memory [59, 60]. Neuronal activity-induced DSBs have been proposed to be mediated by topoisomerase IIβ (Top2β) [16]. Decreased Top2β levels reduce DSB formation and early response gene induction, whereas targeted CRISPR/Cas9-mediated DSBs in early response gene promoters are sufficient for gene induction [16], suggesting a direct role of DSBs in the regulation of neuronal activity.

Full evaluation of the implications of Top2β-mediated DSBs for neuronal function will require to determine if Top2β-mediated DSBs are routinely formed during early response gene activation–i.e., in all or most promoters–or only in a subset of early response gene promoters under specific circumstances. In this regard, Top2β mediates release of torsional stress during transcription [61] and Top2β-mediated transcription start site breaks are proportional to the rate of transcription in non-neural cells [62, 63]. If early response gene promoters in neurons are subject to a higher DSB burden over lifespan than other promoters, it would follow that they may incur higher rates of genomic alterations due to an expected rate of imperfect DSB repair over time. Such alterations could impact brain functions with age, given the important role of early response genes in cognitive function, and thus highlight a potential role of DSB repair in the prevention of aging-associated cognitive decline.

The mechanisms by which neuronal activity-induced DSBs promote gene activation are not well understood. The enrichment of Top2β-mediated DSBs at sites bound by CCCTC-binding factor (CTCF) in neurons and other cell types suggests a potential role of DSBs in the topological remodeling of enhancer-promoter interactions [16, 64, 65]. Overall, high transcription activity appears to recruit Top2β to transcription start sites [66]. Findings showing that Top2β inhibition causes increased RNA polymerase II pausing at early response genes, suggest that Top2β-mediated DSBs may have a role in gene expression induction via release of paused RNA polymerase II [67]. Beyond transcription-associated DSBs in non-dividing neurons, early response genes have also been shown to undergo activation-induced Top2β-mediated DSBs in dividing, non-neural cells [67]. Indeed, Top2β-mediated DSBs mediate stimulation-induced gene expression in various non-neural cell types [68–70]. Specifically, DSBs around transcription start sites have been implicated in nuclear hormone receptor gene activation in tumor cell lines [68, 71]. DSBs were also found enriched around active transcription start sites in activated B cells [33, 72] and NSPCs [73], although it is unclear if they were caused by Top2β activity. It should be pointed out that Top2β also has a role in the formation of transcription-independent DSBs at chromosome loop anchors bound by CTCF and cohesin [64].

It is possible that factors other than Top2β contribute to the formation of neuronal activity-induced DSBs. Although the implications are not yet clear, Spo11, the endonuclease that generates DSBs during meiosis, has been speculated to contribute to the formation of activity-induced DSBs in neurons of the hippocampus [17].

The finding that neuronal activity-induced DSBs are marked by the DSB response factors γH2AX [16, 17, 58] and 53BP1 [17] suggests that they are recognized by the DSB repair

machinery. However, how precisely these breaks are resolved in neurons is currently unclear. Experiments involving chemical inhibition of DNA-PK suggest that neuronal activity-induced DSBs are likely repaired via NHEJ [16]. Recent studies have further proposed a role of the BRCA1 factor in neuronal DSB repair [74]. Overall, these intriguing, recent findings suggest a role for DSB repair in cognition and neurodegenerative disorders such as Alzheimer's disease [74] and highlight the importance of further studies of DSB formation and repair in neurons.

4. Conclusions

Emerging evidence points to potential roles of DSB formation and repair in neurodevelopment, somatic brain somaicism, and neuronal function. The field is beginning to address the causes and implications of DSB break formation and repair in various neural cell types. Identification of the underlying mechanisms will likely yield insights relevant for both brain physiology and disorders. In addition to gaining further insights into roles of DSB formation and repair in pathologic conditions such as cancer and aging-associated functional decline, we speculate that future studies of neural DSBs may reveal roles of DSBs and repair factors in physiologic processes beyond meiosis, V(D)J recombination, and immunoglobulin class switch recombination.

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Abbreviations

DSB	DNA double-strand break
IgH	immunoglobulin heavy
C-NHEJ	classical non-homologous end joining
XRCC4	x-ray repair cross complementing 4
Lig4	DNA ligase 4
RAG	recombination activating gene
HTGTS	high-throughput, genome-wide, translocation sequencing
CSR	class switch recombination
NSPC	neural stem/progenitor cell
RDC	recurrent DSB cluster
CRISPR	clustered regularly interspaced short palindromic repeats

Cas9	CRISPR-associated protein 9
CNV	copy number variation
Mb	megabase
kb	kilobase
Top2β	topoisomerase Iiβ
CTCF	CCCTC-binding factor
γH2AX	serine 139-phosphorylated histone H2AX
53BP1	tumor suppressor p53-binding protein 1
DNA-PK	DNA-dependent protein kinase
BRCA1	breast cancer 1 gene
Ctnna2	catenin, alpha-2
Cadm2	cell adhesion molecule 2

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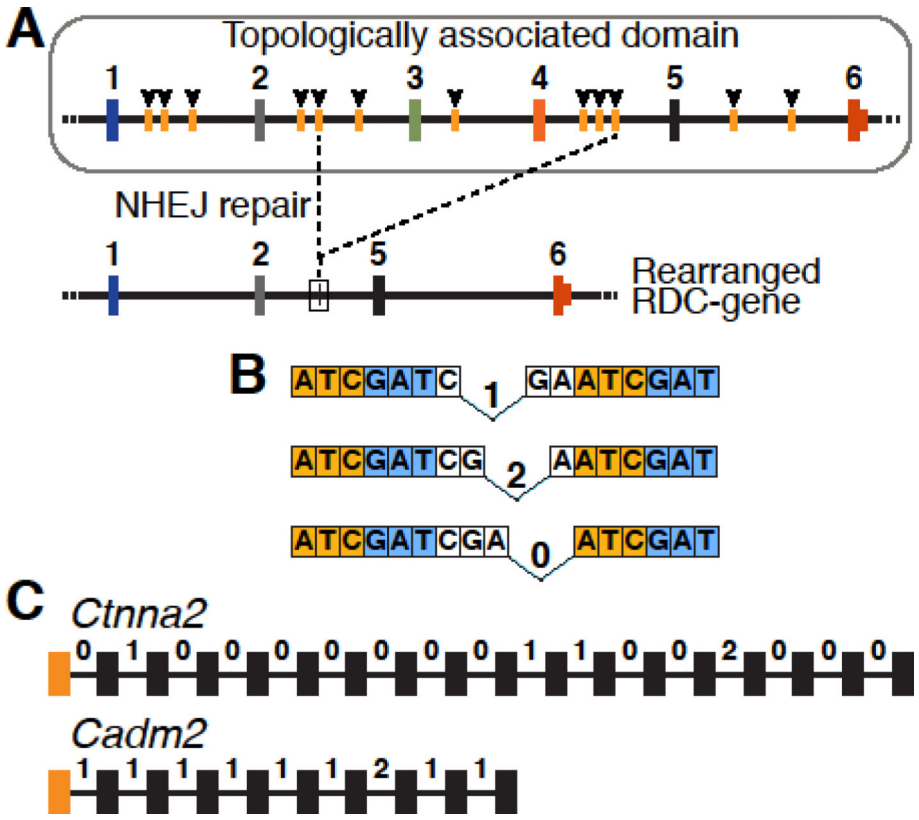

Figure 1. Hypothetical model of replication stress-induced, DSB-mediated RDCgene rearrangements in neural stem/progenitor cells.

(**A)** RDC-genes often contain very large introns and small exons (not drawn to scale). Joining of separate, intronic DSBs (indicated by black arrowheads, top) would result in exon deletions and RDC-gene diversification (bottom). In the model shown, exons 3 and 4 are deleted via end-joining of two intronic DSBs (dotted lines), with the resulting breakpoint junction indicated by a dashed box. Intragenic joining of DSBs is expected to be promoted by location of RDC-genes within topologically associated domains. Adapted from [51]. **(B)** Illustration of intron phases. Adapted from [55]. The three intron phases (1,2,0) are shown. Exons flanked by introns of the same phase (0–0, 1–1, 2–2) can be joined without shifting the reading frame. **(C)** Annotation of intron phases in RDC-genes *Ctnna2* and *Cadm2*. Black rectangles indicate exons. First coding exon is shown in orange.