Article

Neuronal enhancers are hotspots for DNA single-strand break repair

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Defects in DNA repair frequently lead to neurodevelopmental and neurodegenerative diseases, underscoring the particular importance of DNA repair in long-lived post-mitotic neurons[1,2]. The cellular genome is subjected to a constant barrage of endogenous DNA damage, but surprisingly little is known about the identity of the lesion(s) that accumulate in neurons and whether they accrue throughout the genome or at specific loci. Here we show that post-mitotic neurons accumulate unexpectedly high levels of DNA single-strand breaks (SSBs) at specific sites within the genome. Genome-wide mapping reveals that SSBs are located within enhancers at or near CpG dinucleotides and sites of DNA demethylation. These SSBs are repaired by PARP1 and XRCC1-dependent mechanisms. Notably, deficiencies in XRCC1-dependent short-patch repair increase DNA repair synthesis at neuronal enhancers, whereas defects in long-patch repair reduce synthesis. The high levels of SSB repair in neuronal enhancers are therefore likely to be sustained by both short-patch and long-patch processes. These data provide the first evidence of site- and cell-type-specific SSB repair, revealing unexpected levels of localized and continuous DNA breakage in neurons. In addition, they suggest an explanation for the neurodegenerative phenotypes that occur in patients with defective SSB repair.

POLB sg1, GCGCCGGAGGGAGATCCCCA; *POLB* sg2, GCCAGCTTGAAGG AGGTACC; *PNKP*, GCCAGGGCTTGCCCGTCCGA.

Puromycin selection for CRISPRi knockdown i^{3}Neurons

To perform CRISPRi knockdown, 1–4 million iPS cells were transduced with an aliquot of sgRNA-expressing virus immediately after an accutase split before cell attachment. The medium was changed the next day to remove residual virus. Two days after transduction, iPS cells were split into accutase and plated at low density in E8 + RI medium containing 10 μg/ml puromycin. The following morning, cells were washed with PBS and given fresh E8 or E8 + RI medium. Cells were then expanded for 1–2 days before inducing differentiation into neurons. sgRNA knockdown efficiency was tested at the iPS cell stage and confirmed in day 7 i^{3}Neurons via quantitative PCR.

Immunofluorescence and Microscopy

For imaging, i^{3}Neurons were plated onto 96-well plates (0.05×10^6 cells per well; Perkin Elmer, cat. no. 6055302) or on μ-Slide glass bottom IBIDI slides (0.2×10^6 cells per well; IBIDI). Before fixation, if indicated, cells were treated with 0.1 mg/ml MMS for 15 min, 10 μM PARG inhibitors (PARGi) for 20 min, or 50 μM etoposide (ETO) for 1 h. Cells were then washed with PBS and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were then washed three times in PBS, permeabilized in 0.5% Triton X-100 for 5 min at room temperature and blocked in 1% BSA/0.2% Triton in PBS for 1 h at room temperature before incubation with primary antibodies. Primary antibodies and dilutions used were as follows: anti-PAR (1:500, Sigma-Aldrich MABE1031), anti-53BP1 (1:1,000, Novus Biologicals, no. NB100-305), anti-phospho-histone H2AX (1:5,000, Millipore, no. JBW301), and anti-tubulin-β3 (TUBB3, 1:5,000, Biolegend no. 801201). Immunofluorescence was detected using fluorochrome-conjugated secondary antibodies as follows: rhodamine Red-X AffiniPure donkey anti-mouse IgG (1:1,000, Jackson Labs no. 715-295-151) for detection of TUBB3; Alexa Fluor 488 goat anti-rabbit (1:1,000, Invitrogen no. A11034) for detection of PAR or 53BP1; Alexa Fluor 555 goat anti-mouse (1:2,000, Invitrogen no. A21422) for detection of γH2AX. EdU was visualized using Click-iT Plus EdU Cell Proliferation Kit for Imaging, Alexa Fluor 488 dye (Life Technologies, cat. no. C10637), according to the manufacturer's instructions. Finally, DNA was counterstained with DAPI (2 μg/ml, Thermo Fisher Scientific no. 62248). Images were acquired on an inverted Nikon spinning-disk confocal microscope (Nikon Eclipse T1), using a 60× 1.40 NA oil-immersion objective.

Flow cytometry

For cell cycle profiling, cells were incubated with 10 μM 5-ethynyl-2′-deoxyuridine for 30 min at 37 °C and stained using the Click-IT EdU Alexa Fluor 488 or 647 Flow Cytometry Assay Kit (ThermoFisher) according to the manufacturer's instructions. DNA content was measured using DAPI (0.5 μg ml^{-1}). Data were analysed using FlowJo v.10 software.

Rat primary neuron cell culture

All animal procedures were conducted according to the NIH Guide for the Care and Use of Laboratory Animals, under Animal Study Proposal no. 19-011 approved by the NICHD Animal Care and Use Committee. Ten-to-eleven-week-old pregnant albino rats were delivered to our facility on day 17 of gestation from Envigo. They were housed under a 12-h light–dark cycle for 24 h with access to food and water ad libitum. On the next day, the rats were killed by carbon dioxide inhalation followed by decapitation before embryos were extracted and neurons prepared. Neurons from embryos of the same litter were pooled for each experiment.

Primary rat cortical neurons were prepared at embryonic day 18 (E18) as previously described[45]. Rats were killed at E18 by decapitation. The brain was collected and meninges were removed, after which cortices were isolated in sterile Hanks' medium (Hanks' balanced salt solution (HBSS), 20 mM HEPES, pH 7.5). Cortices were then collected and treated with 0.25% trypsin (Gibco), and 100 μg/ml DNase (Roche) for 15 min at 37 °C. One volume of adhesion medium (DMEM without phenol red, 4.5 g/l glucose, 25 mM HEPES, 10% heat-inactivated horse serum (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin) was added to stop trypsin enzymatic action. The tissue was then disrupted mechanically by pipetting it through a 10-ml serological pipette. Cells were then strained through a 70-μm nylon filter (Corning) and centrifuged at 700*g* for 10 min. The cell pellet was resuspended in 5 ml adhesion medium and cells were counted. Between 10 million and 25 million cells were plated on 10-cm culture dishes previously coated with poly-L-lysine (Sigma) and 5 μg/ml laminin (Roche). After 2 h, the neurons were adherent to the plate and the medium was changed to complete neurobasal medium (CNB) (neurobasal medium (Gibco), 1× B27 serum-free (Gibco), 4.5 g/l glucose, and 100 U/ml penicillin–streptomycin (Gibco)) and supplemented with 5 μM aphidicolin (Aph) to eliminate residual dividing cells. Primary neurons were then cultured for 7–8 days before being collected for CHiP–seq or SAR-seq.

SAR-seq

Neurons and iMuscle cells were incubated with 20 μM EdU for 18 h, unless otherwise noted. Cells were collected and fixed as follows. Cells were washed with PBS, incubated with accutase for 5–10 min, collected using a cell scrapper, pelleted at 200*g* for 5 min and resuspended in cold 0.1% BSA in PBS. Cold methanol was then added dropwise during slow vortexing to 80% final concentration. Samples were kept on ice for 20 min and then stored at −20 °C until processing.

Copper catalyses azide-alkyne click chemistry. For biotin labelling via Click-iT reaction, cells were first washed 1× in PBS, permeabilized with 0.2% Triton-X100/PBS for 10 min on ice, and then washed 1× in PBS. Then the following were added in order: 3 mM copper sulfate (Sigma), 50 μM biotin azide (ThermoFisher, cat. no. B10184), and 1× Click-iT additive (ThermoFisher, cat. no. C10424) for 2 h with shaking at room temperature. Cells were then washed 1× in PBS and lysed in 50 mM Tris pH 8.0 with 1% SDS and proteinase K overnight at 37 °C. DNA was extracted using UltraPure phenol:chloroform:isoamyl alcohol (25:24:1, v/v) (Invitrogen) according to the manufacturer's instructions, followed by 2.5:1 volume ethanol and 1:10 volume sodium acetate precipitation. DNA pellets were resuspended in TE buffer and sheared to 150–200-bp fragments using a Covaris S220 sonicator at 10% duty cycle, 175 peak incident power, 200 cycles per burst, for 240 s. DNA was again precipitated in 2.5:1 volume ethanol and 1:10 volume sodium acetate and resuspended in TE buffer. Biotin–EdU fragments were pulled down using MyOne Streptavidin C1 Beads (ThermoFisher, cat. no. 650-01). Before pulldown, 35 μl of Dynabeads was washed twice with 1 ml 1× wash and binding buffer (1× W&B; 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 M NaCl, 0.1% Tween20) on a DynaMag-2 magnetic separator (Invitrogen, cat. no. 12321D) and resuspended in 2× W&B (10 mM Tris-HCl pH8.0, 2 mM EDTA, 2 M NaCl, 0.2% Tween20). An equal volume of Dynabeads in 2× W&B was added to DNA in TE buffer and incubated at 24 °C with shaking in a ThermoMixer C at 800 rpm for 30 min. Dynabeads bound to biotin–EdU fragments were washed three times in 1 ml of 1× W&B, twice in 1 ml EB, and once in 1 ml 1× T4 DNA ligase buffer (NEB). Dynabeads were resuspended in 50 μl end-repair reaction mix (1X T4 DNA ligase buffer, 0.4 mM dNTPs, 2.7 U T4 DNA polymerase (NEB), 9 U T4 Polynucleotide Kinase (NEB), and 1 U Klenow fragment (NEB)) and incubated at 24 °C with shaking at 800 rpm for 30 min. Dynabeads were washed once in 1 ml 1× W&B, twice in 1 ml EB, and once in 1 ml NEBuffer 2 (NEB) and resuspended in 50 μl A-tailing reaction mix (1× NEB dA-tailing buffer and 20 U Klenow fragment exo- (NEB)), followed by incubation at 37 °C with shaking at 800 rpm for 30 min. Dynabeads were then washed again once in 1 ml NEBuffer 2 and resuspended in 115 μl ligation reaction mix (1× quick ligase buffer (NEB), 6,000 U quick ligase (NEB), 5 nM annealed TruSeq truncated adaptor) and incubated at 25 °C with shaking at 600 rpm for 20 min. The ligation reaction was

stopped by adding 50 mM EDTA, and Dynabeads were washed three times in 1 mL 1× W&B, three times in 1 ml EB, and finally resuspended in 8 µl EB+10 µl 2× Kapa HiFI HotStart Ready Mix (Kapa Biosciences). Primers (10 mM) 5′-CAAGCAGAAGACGGCATACGAGATXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3′ and 5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T-3′ (asterisk indicates a phosphothiorate bond and a NNNNNN TruSeq index sequence) were added with 37 µl PCR reaction mix (20 µl 2× Kapa HiFI HotStart Ready Mix, 17 µl H_2O) for a final volume of 60 µl. DNA was amplified using PCR programme: 98 °C, 45 s; 15 cycles of [98 °C, 15 s; 63 °C, 30 s; 72 °C, 30 s]; 72 °C, 5 min. PCR products were separated from DynaBeads and cleaned using 1.8× volume AMPure Beads XP. We isolated 150–200-bp bands on 2% agarose gel and purified them using QIA-quick Gel Extraction Kit (Qiagen). Before sequencing using Illumina NextSeq 550 (75 bp single read), library concentrations were calculated by KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems).

To specifically sequence only EdU-incorporated strands of DNA (Strand-Specific SAR-seq), SAR-seq was followed exactly as above with additional steps before PCR amplification. After washing three times in 1 ml 1× W&B and three times in 1 ml EB post-ligation, Dynabeads were washed in 50 µl 1× SSC buffer and resuspended in 20 µl 0.15 M NaOH for 10 min at room temperature to denature DNA strands. Beads were placed back on the DynaMag-2 magnetic separator and washed once with 20 µl 0.1 M NaOH, once with 1 ml 1× W&B, and twice with 1 ml EB, and then resuspended in 8 µl EB + 10 µl 2× Kapa HiFI HotStart Ready Mix. Primers and PCR reaction mix were added as above.

In the indicated experiments, i^{3}Neurons were treated with the following compounds: aphidicolin (Aph; 5 µM), etoposide (50 µM), olaparib (10 µM), velaparib (10 µM) or tazaparib (1 µM) were added along with EdU at 18 h before collection. The DNA polymerase-α inhibitor (POLαi) adarotene (1 µM) or Aph (50 µM) was added with EdU 14 h or 24 h before collection, respectively. Hydroxyurea (HU; 10 mM) powder was dissolved fresh into water to make a 1 M stock each time it was used. HU was added on day 3 of differentiation and again on day 6 at 18 h before collection along with EdU for a total of 4 days of treatment. For dideoxynucleoside (ddN) chain termination, 5 µM of each of ddA, ddT, ddG, and ddC chain-terminating nucleosides (20 µM total) were added to i^{3}Neuron culture for 18 h before cell collecting for END-seq and S1 END-seq experiments.

END-seq and S1 END-seq

To dissociate i^{3}Neurons for use in END-seq, we performed a modified papain dissociation protocol[46]. Papain (Worthington Biochemical Cat: LK003178) was dissolved into TrypLE Express Enzyme (1×) with no phenol red and warmed at 37 °C for 10 min. Then, day 7 i^{3}Neurons on a 15-cm plate were washed with PBS and treated with 5 ml papain/TrypLE for 1 min at 37 °C. Papain was removed with gentle pipetting and 5 ml trituration solution was added (30 ml i^{3}Neuron culture medium,10 µM ROCK inhibitor, and 1 vial freshly dissolved DNase). Cells were collected and gently pipetted 3–10 times in a conical tube using a wide-bore 10-ml pipette, being careful not to over-digest the sample. Cells were then washed with PBS, pelleted and resuspended in PBS containing 0.1% BSA and 0.5 mM EDTA, and kept on ice. Cells were processed for END-seq as previously described[47]. For S1 END-seq, cells were collected and embedded in 1% agarose plugs, lysed and digested with proteinase K (1 h at 50 °C, followed by 7 h at 37 °C), washed with TE buffer, and then treated with RNase A for 1 h at 37 °C. Plugs were then washed in EB and equilibrated in S1 nuclease buffer (40 mM sodium acetate pH 4.5, 300 mM NaCl, 2 mM $ZnSO_4$) for 30 min. We added 1.8 U S1 nuclease to 100 µl S1 nuclease buffer per plug and incubated them on ice for 15 min to allow the enzyme to diffuse into the plug. The reaction mix was then placed at 37 °C for 20 min before addition of EDTA (10 mM final concentration) to terminate the reaction. Finally, plugs were processed through the standard END-seq protocol.

AsiSI induction in pre-B cells

Abelson-transformed mouse pre-B cells[48] were retrovirally transduced with tetracycline-inducible ER-AsiSI[49]. Cells were arrested in G1 with 3 µM imatinib for 24 h, followed by addition of 3 µg/ml doxycycline for 24 h, and then further addition of 1 µM 4OHT for 18 h to induce AsiSI nuclear localization, as previously described[19].

Construction of the Dox-Cas9-D10A nickase

Dox-inducible Cas9-D10A was constructed using isothermal assembly[50]. In brief, a plasmid encoding Dox-inducible Cas9 nuclease was obtained from Addgene. pCW-Cas9-Blast was a gift from M. Babu (Addgene plasmid no. 83481; http://n2t.net/addgene:83481; RRID: Addgene_83481). This plasmid was digested with NheI/BamHI and assembled with 2 PCR fragments (Nickase-P1, Nickase-P2) and transformed into competent cells. Gel-extracted PCR fragments of Nickase-P1 and Nickase-P2 were generated using the Q5 HotStart 2× mastermix with the primers as follows using Addgene-83481 as template: Nickase-P1 forward primer, GTCAGATCGCCTGGAGAATTG; Nickase-P1 reverse primer, tgC CAGGC CGATG CTGTACTTCT; Nickase-P2 forward primer, AGAAGTACAG CATCGGCCTG Gca ATCGGCACCAACTCTGTGGG; Nickase-P2 reverse primer, TGCCTTGGAAAAGGCGCAAC.

MCF10A Cas9D10A-inducible cell line

To produce the MCF10A Cas9-D10A inducible cell line, we infected MCF10A cells (sourced from ATCC, not aunthenticated, and not mycoplasma tested) with lentivirus containing Dox-Cas9-D10A and cells were selected with 10 µg/ml blasticidin. Cas9-D10A expression was induced using 3 µg/ml doxycycline and confirmed by western blotting. Three guide RNAs (sequences: 5′-TGGGGCGTTTATCCGATGTC-3′; 5′-GCACTAGCCGGCCCGGACGT-3′; 5′- CCAGCCTGGTAGCGCCCCCA-3′) were cloned into the Lenti-Guide-NLS–GFP vector[51] and the MCF10A Cas9-D10A inducible cell line containing the three guide RNAs was selected using 2 µg/ml puromycin. To identify the nicks by S1 END-seq, cells were arrested in G1 for 48 h with 5 µM Palbociclib with doxycycline added during the last 24 h to induce Cas9D10A, followed by cell collection and S1 END-seq processing.

ChIP–seq and western blotting

Fifteen million i^{3}Neurons or rat neurons were fixed in 1% formaldehyde at 37 °C for 10 min. The fixation reaction was quenched with glycine at a final concentration of 125 mM. Cells were spun down and washed twice with chilled PBS, and pellets were then snap frozen on dry ice and finally stored at −80 °C until sonication. Sonication, immunoprecipitation, and library preparation steps were done as previously reported[17]. All antibodies were pre-conjugated to 40 µl magnetic protein A beads before immunoprecipitation: H3K4me1 (5 µg, Abcam no. 8895); MLL4 (antibody courtesy of K. Ge); H3K27ac (5 µg, Abcam no. 4729); H3K27me3 (5 µg, Millipore no. CS200603); H3K4me3 (6 µl, Abcam no. 8580); H3K9me3 (10 µg, Active Motif, cat. no. 39765); CTCF (6 µl, Millipore, cat. no. 07-729); RNA polymerase II (8 µg, Abcam no. 26721); H3K36me3 (5 µg, Abcam cat. no. ab9050); anti-PAR (5 µg, Millipore-Sigma MABE1016); XRCC1 (2.6 µg, Novus, cat. no. NBP1-87154). For western blotting, cells were collected and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5% Tween-20, 0.5% NP-40, 2 mM PMSF, 2.5 mM β-glycerophosphate (all from Sigma-Aldrich) and protease inhibitor cocktail tablet (complete Mini, Roche Diagnostics). Equal amounts of protein were loaded into precast mini-gels (Invitrogen) and resolved by SDS–PAGE. Proteins were blotted onto a nitrocellulose membrane, blocked with Intercept (TBS) blocking buffer (LI-COR Biosciences) and incubated with the corresponding primary and secondary antibodies: anti-DNA polymerase β (1:1,000, Millipore no. ABE1408), anti-tubulin (1:10,000, Sigma-Aldrich no. T5168), IRDye 800 CW goat anti-rabbit (1:15,000, Li-Cor no. 926-32211), and IRDye 680 RD goat anti-mouse (1:15,000, Li-Cor no. 926-68070).