Longitudinal assessment of neuronal 3D genomes in mouse prefrontal cortex

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Neuronal epigenomes, including chromosomal loopings moving distal cis-regulatory elements into proximity of target genes, could serve as molecular proxy linking present-day-behaviour to past exposures. However, longitudinal assessment of chromatin state is challenging, because conventional chromosome conformation capture assays essentially provide single snapshots at a given time point, thus reflecting genome organization at the time of brain harvest and therefore are non-informative about the past. Here we introduce 'NeuroDam' to assess epigenome status retrospectively. Short-term expression of the bacterial DNA adenine methyltransferase Dam, tethered to the Gad1 gene promoter in mouse prefrontal cortex neurons, results in stable G\textsuperscript{methyl}ATC tags at Gad1-bound chromosomal contacts. We show by NeuroDam that mice with defective cognition 4 months after pharmacological NMDA receptor blockade already were affected by disrupted chromosomal conformations shortly after drug exposure. Retrospective profiling of neuronal epigenomes is likely to illuminate epigenetic determinants of normal and diseased brain development in longitudinal context.
A large number of genetic and environmental factors impacting within the extended (prenatal to young adult) period of brain development result in cognitive and behavioural deficits only at much later periods. Unsurprisingly, therefore, considerable time and effort has been invested exploring long-term adaptations of neuronal and glial transcriptomes and epigenomes in a wide range of disease models. However, functional neurogenomics faces a key limitation, owing to the fact that, to date, even the most powerful genome-scale assays for chromatin modifications (chromatin immunoprecipitation (ChIP) sequencing), transcription (RNA sequencing) or chromosomal conformations (Hi-C) essentially provide only a single snapshot of genome organization and function at the time of tissue harvest. This often necessitates multiple subgroups of animals, to monitor, at different time points, long-term genomic adaptations in response to past exposure. What is missing from the field, therefore, is a molecular toolbox that would directly link past genome status to current brain function. Here we artificially tag neuronal genomes in mouse prefrontal cortex (PFC) with bacterial DNA adenine methyltransferase (Dam), then measure the animal’s behaviour at different points in time, followed by brain harvest and retrospective assessment of prefrontal genomes representing the exposure period months past. Specifically, we show that chronic deficits in cognition and working memory, and excessive anxiety after 21 days of disrupted NMDA (N-methyl-D-aspartate) receptor signalling in the juvenile and young adult period are associated with early emergence of long-lasting disruptions of intra-chromosomal conformations at the NMDA-sensitive Gad1 GABA synthesis gene locus (chr.2qC2). We predict that in vivo Dam-based retrospective tagging of neuronal genomes (hereafter referred to as ‘NeuroDam’) will provide an important longitudinal complement to conventional cross-sectional neuroepigenomic approaches currently available.

Results

NMDA antagonist-induced long term behavioural defects. Transient disruption of NMDA receptor signalling induces lasting impairments in neuronal signalling, cognition, social behaviours and emotion, and provides a frequently implied pharmacological model for schizophrenia and other psychosis spectrum disorder. Specifically, acute or subchronic (<21 days) exposure to MK-801 and other NMDA receptor antagonist drugs in the juvenile or young adult period is associated with disruptions of cortical function and cognition including associate and working memory, and increased anxiety. To recapitulate these findings and to explore potential long-term changes in behaviour, we exposed two age groups of C57Bl6/J mice (postnatal day P28 and P90) to daily treatments with the NMDA antagonist drug MK-801 (0.2 mg.kg⁻¹) or saline as control for a period of 3 weeks, followed by behavioural testing for spatial working memory (8-arm radial maze) and anxiety (open-field test) within 2 weeks (‘TIME A’ in Supplementary Fig. 1) or after 4 months (‘TIME B’ in Supplementary Fig. 1) after the last drug treatment. Indeed, MK-801-exposed mice showed significant deficits in working memory and increased anxiety when tested within 2 weeks post treatment (‘TIME A’ (Supplementary Fig. 1). These alterations continued to exist, in milder form, at the second, much later test period (‘TIME B’ (Supplementary Fig. 1). Although our findings are in broad agreement with studies using shorter time intervals between NMDA antagonist treatment and behavioural assessment, the results from our animals tested 4 months after MK-801 exposure indicate that such types of behavioural alterations do not remain static in the long term.

Dam-tagging of chromosomal contacts in longitudinal context. NMDA receptor blockade induces promoter-specific DNA methylation remodelling in corticoline circuits, but little is known about potential effects on higher-order chromatin, including chromosomal conformations bypassing linear genome to mobilise enhancers and other cis-regulatory elements into physical proximity to (NMDA sensitive) gene transcription start sites (TSSs). We wanted to chart, in the longitudinal context of the aforementioned drug-induced changes in behaviour, chromosomal loop-bound DNA sequences encompassing Gad1/Gad2, an activity-regulated gene highly sensitive to disruptions in NMDA receptor signalling. Given the protracted course of behavioural deficits after NMDA blockade, potentially emerging and extending over the course of multiple weeks, we reasoned that cross-sectional chromosome conformation capture (3C) approaches are less ideal to fully capture the dynamics of spatial genome architectures in susceptible neurons. We noted a report of bacterial adenine DNA methyltransferase (Dam)-based tagging of long-range chromosomal loopings at the Drosophila bithorax homeotic gene complex with chimeric Dam-GAL4 DNA binding domain constructs. Therefore, we asked whether it would be possible to artificially tag Gad1/Gad2 chromosomal contacts in mouse brain expressing chimeric protein comprising Dam fused to designer DNA-binding proteins targeting Gad1 promoter sequences. We hypothesized that such type of approach in vivo is well suited for long-term tagging of neuronal DNA because of postmitotic status, with the potential for a stable artificial DNA mark in the absence of the ‘diluting effect’ by cell division. Furthermore, vertebrate genomes essentially lack endogenous adenine methylation at G⁹ATC tetramers as the highly specific Dam target sequence.

To test this ‘NeuroDam’ approach, we first designed a herpes simplex vector (HSV) amplicon for simultaneous expression of two transcription cassettes, arranged in tandem nose-to-tail orientation, including cytomegalloivirus promoter driven mCherry (green fluorescent protein (GFP)) and HSV IE4/5 promoter-driven expression of Dam fused to a transcription activator-like effector (TALE) complementary DNA previously shown to specify bind to the predicted 14bp target sequence at the Glutamic acid decarboxylase (Gad1, chromosome 2qC2) promoter. We reasoned that HSV ampiclons are ideal vectors for the purposes of NeuroDam retrospective genomics, because expression is rapid starting 2–3h after transfection, but confined to a short period of several days before shutting down permanently. Indeed, immunohistochemical staining with NeuN neuronal antibody confirmed expression of our HSV TALEGad1/Dam/mCherry ampiclon in neuronal layers of the cerebral cortex at day 2 (Fig. 1a,c) but not day 10 post injection (Fig. 1b). Furthermore, when tested by quantitative reverse transcriptase-PCR with two independent primer pairs (‘Dam.1’ and ‘Dam.2’ in Fig. 1d), prefrontal Dam DNA was readily detectable in brains harvested 2 and 7, but not 10 days post injection. Furthermore, RNA levels of Gad1 and its parologue, Gad2, remained unaltered when tested 2, 7 and 10 days post injection (Fig. 1d and Supplementary Fig. 2). We conclude that HSV-mediated TALEGad1/Dam expression is transient and does not alter RNA levels of the target gene.

Mice, treated for 3 weeks with daily doses of saline or MK-801 as described above, received—an additional 2-week interval—bilateral PFC injections of HSV TALEGad1/Dam/mCherry (‘week 5’ on Fig. 2 timeline). Brains were harvested after another 2 weeks post injection, on completion of ‘TIME A’ behaviour testing (‘week 7’ in Fig. 2), or after 4 months post injection, on completion of ‘TIME B’ behavioural assays (Supplementary Fig. 1) (‘week 22’ on Fig. 2 timeline). We first explored whether artificial, Dam-mediated adenine methylation...
at GATC tetramers (GmATC) could identify some of the previous reported higher-order chromatin structures, based on conventional chromosome conformation capture assays (3C)\(^\text{19}\). These included loop contacts between the Gad1 promoter and regulatory sequences positioned 55 kb further upstream\(^\text{19}\). We extracted, then DpnII digested the prefrontal DNA, followed by PCR-based quantification of restriction-insensitive GmATC sequences indicative of Dam methylation activity\(^\text{20}\) (Fig. 3a,b).

Indeed, quantification of DpnII-resistant sequences within 100 kb from the Gad1 TALE target site identified in multiple experiments a sharp peak corresponding to the previously reported conformation\(^\text{19}\) (Fig. 3c). To further assess the sequence specificity of our TALE\(^\text{Gad1}\) Dam DNA-binding protein (which included a V5 epitope tag), we conducted chromatin immunoprecipitation with an anti-V5 antibody in additional PFC samples, harvested 2 days after HSV

![Image](image_url)
TALE\textsuperscript{Gad\textsubscript{1}}Dam injection. Indeed, ChIP-to-Input ratios were above background at the TALE target sequence and very low or not detectable in the surrounding sequences (Fig. 3d). Importantly, DamID–PCR yields from sequences comprising the Gad\textsubscript{1}-TSS\textsuperscript{(--55kb)} \textsuperscript{Loop} were readily detectable at comparable levels in PFC harvested 2 weeks ('TIME A') and 4 months ('TIME B') after injection of HSV TALE\textsuperscript{Gad\textsubscript{1}}Dam (Fig. 3e). Furthermore, these sequences were specifically G\textsuperscript{m}ATC-methylated in HSV-TALE\textsuperscript{Gad\textsubscript{1}}Dam-injected PFC, whereas no DamID–PCR products were produced in 3/3 PFC specimens injected with an HSV vector expressing Dam-Mef2c transcription factor fusion protein as negative control (Fig. 3b). We conclude that G\textsuperscript{m}ATC tetramer methylation, as an artificial epigenetic mark reflecting bacterial Dam methyltransferase activity, is suitable for chromosomal loop mappings in mouse brain in vivo and, moreover, the mark is maintained in cortical neurons for at least 4 months after transient (<10day) expression.

**Figure 3** | Chromosomal conformations tagged by TALE\textsuperscript{Gad\textsubscript{1}}Dam. (a) 150 kb of linear genome surrounding chromosome 2 TALE target sequence 5'--TATTGCAAGAGAGA--3' at ~1 kb position from Gad\textsubscript{1} TSS. Dotted arc marks loop formation mapped by 3' C' chromosome conformation capture\textsuperscript{19}. Position of Amplicon/primer pairs 1–8 (Supplementary Table 2) for DamID quantitative PCR assays from DpnII-resistant prefrontal DNA as indicated within chr2 position 70,304,636-70,455,066. (b) Dam-based 3D genome mapping, TALE\textsuperscript{Gad\textsubscript{1}}Dam methylates G\textsuperscript{m}ATC tetramers around Gad\textsubscript{1} TALE target sequence and at chromosomal contacts and loop formations within physical proximity to target. Methylated G\textsuperscript{m}ATC tetramers are selectively resistant to DpnII digest (in contrast to DpnII-sensitive non-methylated GATC). DamID–PCR products are detectable for 55 kb loop (primer pair 4), corresponding to previously reported loop formation by 3' C' and for sequences at TALE target sequence (primer pair 7) in HSV TALE\textsuperscript{Gad\textsubscript{1}}Dam-injected PFC samples PFC1, PFC2 and PFC3. The absence of DamID–PCR product in HSV\textsubscript{Mef2c-Dam}injected PFC4, PFC5 and PFC6 is noteworthy (see also Supplementary Fig. 6). (c) DamID quantitative PCR for G\textsuperscript{m}ATC quantification from prefrontal DNA, with primers within 100 kb from TALE\textsuperscript{Gad\textsubscript{1}} target sequence (see a), after normalization to control sequence on chromosome 18. The sharp peak at position 4, corresponding to ~55 kb promoter-enhancer loop\textsuperscript{19} and peak at position 7 at TALE target sequence are noteworthy. N = 3 per group. (d) ChIP with anti-V5 antibody to measure sequence-specific binding of TALE\textsuperscript{Gad\textsubscript{1}}Dam-V5 at Gad\textsubscript{1} locus. Notice robust binding at TALE target sequence (position 7, see a) but not at neighbouring positions 5, 6. N = 3 per group. (e) Quantitative comparison of Gad\textsubscript{1}-TSS\textsuperscript{(--55kb)} \textsuperscript{Loop} by gel densitometry from DamID–PCR products for TIME A and TIME B. N = 4–5 mice per group. Data in c-e shown as mean ± s.e.m.  

Dam-tagged Gad\textsubscript{1} long range chromosomal contacts. Next, we wanted to chart Dam G\textsuperscript{m}ATC-tagged sequences on a chromosome-wide scale in 'TIME A' and 'TIME B' PFC (Fig. 2). To this end, we prepared Dam-seq libraries from DpnII digested DNA to selectively ligate the adaptors at the methyl-G\textsuperscript{m}ATC cut sites and peak at position 7 at TALE target sequence (see a), after normalization to control sequence on chromosome 18. The sharp peak at position 4, corresponding to ~55 kb promoter-enhancer loop\textsuperscript{19} and peak at position 7 at TALE target sequence are noteworthy. N = 3 per group. (d) ChIP with anti-V5 antibody to measure sequence-specific binding of TALE\textsuperscript{Gad\textsubscript{1}}Dam-V5 at Gad\textsubscript{1} locus. Notice robust binding at TALE target sequence (position 7, see a) but not at neighbouring positions 5, 6. N = 3 per group. (e) Quantitative comparison of Gad\textsubscript{1}-TSS\textsuperscript{(--55kb)} \textsuperscript{Loop} by gel densitometry from DamID–PCR products for TIME A and TIME B. N = 4–5 mice per group. Data in c-e shown as mean ± s.e.m.
corrected for chromosomal length. We also included \( N = 4 \) primary neuronal cultures from embryonic day E15 cortex and hippocampus transfected 72 h before harvest with TALE\(^{Gad1}\)Dam plasmid. Two of four primary neuronal cultures were treated with KCl for 6 h before harvest, to upregulate neuronal depolarization and signalling and thereby provide a better model for active circuitry in adult cortex. Finally, untransfected naive E15 hippocampal cell culture served as a negative control.

Using a 50 kb sliding window, read counts were modelled using a negative binomial distribution (see Methods). We identified 276 chromosome 2 sliding windows that were Dam-tagged in TALE\(^{Gad1}\)Dam-exposed PFC samples, including 57 sliding windows tagged in at least 1/4 neuronal cultures transiently transfected with TALE\(^{Gad1}\)Dam (Supplementary Data 1). Filter criteria included zero background in the control (non-Dam exposed) culture and exclusion of ‘blacklisted’ sequences in modENCODE (model Encyclopedia of DNA Elements), owing non-informative enrichment in deep-sequencing data sets\(^{23}\). The filtered 276 Dam-tagged sliding windows, \( \text{in } \text{toto} \), represent \( \sim 7.5\% \) of 182 megabase (Mb) mappable chromosome 2 sequence. Of note, most genomic loci exhibit a non-zero probability to interact with almost any other locus in the genome\(^{24}\). Therefore, many of the 276 Dam-tagged ‘positions’ on chromosome 2 could reflect ‘random collisions’\(^{24}\) of the chromosomal material with the TALE\(^{Gad1}\) target sequence, resulting in G\(^{\text{M} \text{ATC}}\) methylation, while in physical proximity to Gad1 promoter-bound Dam enzyme. This scenario is plausible, given the longitudinal design of our \textit{in vivo} experiment with the G\(^{\text{M} \text{ATC}}\) adenine methylation activity of TALE\(^{Gad1}\) Dam chimeric protein extending over the course of 3 days (cell culture) and 1 week (\textit{in vivo} PFC). To reduce the pool of potential (Dam-tagged) intrachromosomal Gad1 contacts, we filtered for Dam-tagged sliding windows with robust \((> 25)\) normalized read counts, to be present in at least 3/4 TIME A plus 3/4 TIME B samples. We obtained 29/276 Dam-tagged positions that matched these criteria (Fig. 4a,b and Supplementary Data 2). Loop formations were tested by 3C for multiple candidate sequences, including formations bypassing 58 Mb to connect Gad1 with Myo3a intronic DNA positioned next to the Gad1 orthologue Gad2 (Fig. 4c), and a long-range contact, to connect Gad1 with neurodevelopmental risk genes including Phf21a\(^{5,26}\) and Kcn4 (ref. 27; Fig. 4d) and the chromatin regulator Bsh80 (refs 28,29). Additional 3C assays were conducted on an independent cohort of mice (Supplementary Fig. 3). As we verified with conventional 3C, altogether 3/4 or 75\% of chromosome 2 G\(^{\text{M} \text{ATC}}\)-tagged sequences as long-range Gad1-bound intrachromosomal loopings, we conclude that the TALE\(^{Gad1}\)Dam fusion protein indeed left G\(^{\text{M} \text{ATC}}\) mark ing at regulated Gad1-bound loop formations in prefrontal neurons (as opposed to ‘random collisions’\(^{24}\) of the chromosomal material or spurious methylation activity of Dam not bound to the Gad1 target).

**Gad1 loop alterations mapped retrospectively.** Importantly, although the \( N = 4 \) TIME A and \( N = 4 \) TIME B Dam-seq experiments, including DNA library and sequencing, were conducted in separate batches, G\(^{\text{M} \text{ATC}}\) profiles for the 29 Dam-tagged loci nonetheless showed a moderately strong correlation between TIME A and TIME B data sets \((R = 0.64, \ r^2 = 0.4,\ P < 0.0005)\). However, GenePattern-based cluster analysis using a larger set of Dam-tagged loci (Supplementary Data 1) showed that two of the altogether four TIME B Dam-seq libraries overall were poorly correlated with any other sample and therefore were not further considered (Supplementary Fig. 4A,B). Of note, the two remaining TIME B Dam-seq libraries showed robust correlations with each of the four Time A libraries (average \( R = 0.815 \)), which is only minimally different from the \( R \) between the two Time B libraries \((R = 0.824)\) (Supplementary Fig. 4A,B).

The aforementioned findings, including the comparisons between ‘TIME A’ and ‘TIME B’ samples by DamID–PCR quantification of local methyl-adenine tags upstream of the Gad1 target site (Fig. 3e) and Dam-seq for intrachromosomal long-range contacts ( Supplementary Fig. 4A,B) indicate that Dam-mediated G\(^{\text{M} \text{ATC}}\) profiles are maintained for at least 4 months in PFC neurons after transient Dam expression had ceased. Having shown that retrospective three-dimensional (3D) genome mapping in mouse PFC is feasible, we then wanted to explore whether our NeuroDam approach could link behavioural alterations (assessed in the ‘present’) to neuronal epigenome status dating back to an earlier time period in the past. To address this question, we studied our mice that were affected by changes in cognition and behaviour when tested up to 4 months after the last dose of subchronic NMDA antagonist regimen (Supplementary Fig. 1). We were particularly interested in the 58 Mb long-range loop interconnecting the Gad1 locus with Myo3a intronic sequences positioned 30 kb upstream of Gad2 (Fig. 4a,b). Interestingly, the Gad1 and Gad2 genes encode glutamic acid decarboxylase orthologues common to all vertebrate genomes after an ancient gene duplication event \( > 400\) MiMy 30\%. Remarkably, despite many megabases of linear genome interspersed between the Gad1 and Gad2 loci, the two genes show similar types of long-lasting adaptations in the adult mouse cortex after early life stress such as maternal immune activation. These include downregulated expression and promoter cytosine hypermethylation in context of cognitive and social impairments\(^ {31,32}\). Therefore, Gad1 and Gad2 expression could show similar types of changes in our MK-801 model, in conjunction with alterations in the 58 Mb Gad1–Gad2 loop. To explore this, we quantified in the ‘TIME B’ PFC specimens Gad1 and Gad2 RNA (quantitative reverse transcriptase–PCR, a cross-sectional assay to quantify transcript at the time of tissue harvest) and Gad1 loopings by DamID (reflecting chromatin status at week 7, which is 15 weeks before brain harvest. Indeed, both Gad1 and Gad2 transcripts were modestly decreased \((25–30\%)\) in PFC of MK-801-exposed ‘TIME B’ animals Fig. 4c). This was associated together with a significant increase in Dam-tagged sequences of the Gad1–Myo3a/Gad2 loop and weakening of the local, 55 kb Gad1 looping (Fig. 4c). Therefore, long-lasting defects in cognition after MK-801 exposure are associated with early emergence of abnormal Gad1 long-range chromosomal conformations. The MK-801-induced increase in Gad1–Myo3a/Gad2 contacts was specific, because DamID for a second type of Gad1 loop structure \((\text{Gad1-TSS}^{(−55\text{kb})} \text{ Loop})\) showed a subtle decrease in drug-exposed animals (Fig. 4c). To explore whether Gad1 higher-order chromatin alterations persist, we conducted 3C in an additional set of ‘TIME B’ PFC specimens \((N = 3\) per treatment). These 3C assays are cross-sectional, informing about the 3D genome at the time of tissue harvest or 4 months after MK-801 exposure. Indeed, 3C changes in the MK-801 group were similar to the DamID–PCR assays, which inform about 3D genome status 2–3 weeks after MK-801 exposure. Thus, our 3C–PCR studies showed a nonsignificant trend towards increased interaction frequencies for Gad1–Myo3a/Gad2 and a significant decrease in Gad1-TSS\(^{(−55\text{kb})} \text{ Loop}\) in PFC of ‘TIME B’ mice previously exposed to MK-801 (Fig. 4c). Next, we directly compared in PFC from additional ‘TIME A’ and ‘TIME B’ MK-801, and saline-treated mice, the levels of G\(^{\text{M} \text{ATC}}\) methylation at two disease-relevant genes, including the aforementioned Phf21a encoding a chromatin regulator associated with mental retardation and neurodevelopmental defects\(^ {25}\), and Kcn4 encoding a voltage-gated potassium channel broadly relevant for the regulation of neuronal excitability in the
context of epilepsy. Of note, PFC from MK-801-treated TIME A and TIME B animals showed a similar, two- to threefold increase in TALE Gad1 Dam-methylated Kcna4, compared with saline-treated TIME A and TIME B animals (Fig. 4d). These changes were specific, because GmATC levels at Phf21 showed only subtle and nonsignificant differences between treatment groups both in TIME A and TIME B animals (Fig. 4d). Unsurprisingly, expression both of Phf21 and Kcna4 was decreased in MK-801-exposed PFC (Fig. 4d), given the robust intra-chromosomal interactions of these gene sequences with the Gad1 and Gad2 loci, which, as mentioned above, undergo a coordinated transcriptional and epigenomic response after stress.

**Figure 4 | Dam-tagged long-range chromosomal loop formations in PFC neurons.** (a) Chromosome 2 linear map marking the positions of the 29 sliding windows consistently GmATC-tagged in TIME A and TIME B HSV TALE Gad1 Dam-injected PFC (Supplementary Data 1 and 2). (b) Browser view at Cacnb2, Myo3a/Gad2, Phf21a and Kcna4 chromosome 2 loci (positions marked in a), showing normalized Dam-seq profiles (top to bottom) for N = 4 TIME A and N = 4 TIME B HSV TALE Gad1 Dam-injected PFC, N = 2 KCL-treated TALE Gad1 Dam primary neuronal culture. NeuH, untransfected/untreated neuronal culture. Cerebral cortex CTCF tracks built from published data set. (c) Retrospective/longitudinal DamID and cross-sectional 3C loop assays, and quantitative reverse transcriptase–PCR (qRT–PCR) from HSV TALE Gad1 Dam-injected TIME B PFC from MK-801 and saline-treated mice for Myo3a/Gad2-Gad1 long-range loop and shorter range 55 kb Gad1 promoter loop. N = 3 per group for DamID–PCR and 3C–PCR, N = 6 per group for RNA. *Two-tailed t-test: P = 0.015 Myo3a/Gad2 and P = 0.049 Gad1-TSS (−55kb) Loop DamID–PCR; P = 0.058 Gad2 and P = 0.034 Gad1 RNA. (d) DamID–PCR and RNA from HSV TALE Gad1 Dam-injected TIME A and TIME B PFC, for Phf21a and Kcna4 sequences. DamID–PCR, N = 3 mice per group. qRT–PCR N = 6 per group. *Two-tailed t-test: P = 0.050 Kcna4 DamID–PCR, P = 0.016 Kcna4 and P = 0.027 Phf21a RNA.

**Dam-tagged loopings verified in additional models.** Having shown that neuronal 3D genomes in mouse PFC show long-lasting adaptations in response to NMDA antagonist drug treatment, we then tested whether chromosomal contacts discovered by Dam-seq in TALE Gad1 Dam PFC could play a role in
models of depression and posttraumatic stress disorder, in particular those that explore the genomic basis of inter-individual differences in resilience or vulnerability in the context of past exposures\cite{41,42}, are also likely to be fundamentally enriched by the retrospective genomics toolbox discussed here. Based on the findings presented here, 3D genome alterations in cortical neurons show disease-specific patterns, with a subset of chromosomal contacts, including the 58 Mb Gad1-Myo3a/Gad2 long range and the Gad1 (~55 kb) shorter range loop showing similar changes in two very different exposures (subchronic MK-801 versus SI). However, other types of interactions, including the 13 Mb Gad1-Phf21a loop, were sensitive to SI but not NMDA antagonist treatment. Therefore, it is likely that multiple, only partially interdependent regulatory networks regulate the neuronal 3D genome in locus- or sequence-specific manner.

The Dam-based tagging of chromosomal contacts in vivo, as shown here, will be a valuable complement to conventional restriction-ligation-based assays and microscopy-based approaches\cite{40} to study the regulation of the 3D genome in the brain\cite{43,44}. Furthermore, as the overwhelming majority of 3D genome contacts are intra-chromosomal, particularly in the larger chromosomes (incl. chr. 2 harbouring the Gad1 locus)\cite{22}, it remains to be determined whether NeuroDam will comprehensively inform about the 3D organization of smaller chromosomes, which tend to have a larger proportion of trans-chromosomal contacts. Moreover, Dam-based epigenomics in the living brain will not be confined to the study of chromosomal loopings and contacts. In non-neuronal cell cultures, Dam-based epigenomics was used to study the interactions of nuclear lamina proteins\cite{45}, distribution of linker histone subtypes\cite{46}, chromatin-remodelling complex and transcription factor occupancies\cite{47}. As an additional benefit, transgenes encoding Dam fusion proteins can easily be engineered to limit expression to a specific cell type and/or specific period in development, in context of Cre drivers and viral vectors. This is important given that many areas of the genome are epigenetically regulated in a manner specific for cell type and developmental stage. In this context, Dam-based neurogenomics will provide an important alternative to fluorescence-activated sorting of immunotagged nuclei\cite{48,49} or chromatin assays that rely on histone-fluorescent conjugates\cite{43,48}, or chimeric nuclear lamina proteins\cite{50}, to sort and enrich for specific cell (nuclei) types.

Of particular interest for in vivo studies in postmitotic cells is the apparent stability of the artificial G\textsuperscript{mATC} mark, which according to our findings is readily detectable even at 4 months and longer after Dam expression (Figs 3e and 4b–d). Nonetheless, the longitudinal persistence of the Dam signal in neurons will require additional investigations. For example, regulated DNA strand breaks and DNA repair mechanisms, which could potentially 'wash out' the G\textsuperscript{mATC} tag, affect neuronal genomes non-uniformly and in highly sequence-specific manner\cite{51,52}. The absence of the G\textsuperscript{mATC} tag in brain DNA not exposed to Dam (Figs 3b and 4b) could reflect the absence of adenine methylation machineries directed towards G\textsuperscript{mATC} tetramers in vertebrate genomes, limiting any residual levels of adenine methylation to sequence context other than non-G\textsuperscript{mATC}\cite{53}. Furthermore, Dam-based retrospective neurogenomics may even be applicable to the invertebrate nervous system, given that adenine methylation activity in invertebrates is directed almost exclusively towards non-GATC sequences\cite{53} conveying potentially heritable epigenetic information important for early development\cite{53,54}.

In summary, NeuroDam, as the retrospective genomics approach presented here, has the potential to illuminate the molecular bridges that link behavioural alterations in the present
to neuronal epigenome status from a distant past. Such type of approach is expected to provide a valuable alternative to conventional, cross-sectional studies that require parallel studies of multiple cohorts each harvested at a specific timepoint relative to the exposure period.

Methods

Animals. All animal work was approved by the Institutional Animal Care and Use Committee of the participating institutions (Icahn School of Medicine at Mount Sinai and University of Haifa).

MK-801 treatment. P28 and P90 mice housed at the Icahn School of Medicine at Mount Sinai maintained in transparent cages on a standard 12:12 h light:dark cycle with ad libitum access to food and water were used in the study. P28 C57BL/6 mice received an initial intraperitoneal (i.p.) injection of 1.0 mg drug per kg body weight MK-801 followed by 3 weeks of a 0.2 mg MK-801 per kg body weight. Controls included saline-treated mice. All animals received saline or MK-801 injection 5 days per week. P90 C57BL/6 mice received i.p. injections of 0.2 mg drug per kg body weight saline or MK-801 for 3 weeks. Both male and female mice were used for the experiments (M:F ratio = 102:28 and 10:4 for P90). Male and female mice were housed group housed with littermates (N = 3–5 per group).

Adolescent SI procedure. An independent cohort of mice, not associated with the Dam and MK-801 studies described above, was used to study the effects of SI stress. The SI protocol was performed as previously described. Briefly, group-housed C57BL/6 mice were randomly assigned to group housing (GH) or SI experimental conditions on P28. Mice in the GH condition were kept in the same cage, whereas mice in the SI condition were moved to individual cages and kept in isolation for 21 days. Re-grouping in the GH condition was avoided, to minimize intruder stress in GH mice. Likewise, experimenter interaction with mice from both groups was kept to a minimum (that is, cages were changed by the experimenter once a week and the experimenter was the only person in contact with the mice in both groups). All animals in the study were C57BL/6 mice and maintained in transparent cages on a standard 12:12 h light:dark cycle in the same room with ad libitum access to food and water. Mice were killed on P60 and the hippocampus was dissected out bilaterally and kept at −80 °C until further analysis by chromosomal conformation capture and RNA work.

Viral injection. HSV TALEGad1/Dam. A TALE targeted to the Gad1 TSS (5′-TAC TGGC AAGGAC-3′, chr2:7039932–70399346, mm9) was fused to Dam16,56. The HSV TALEGad1/DamCDNA included a V5 epitope sequence (5′-GKPINPGLLGDST-3′)56. The Gad1-TALE-Dam was packaged into a short-term herpes simplex virus 1 with an estimated 8 day expression peaking from day 3–5 post injection driven by the IE 4/5 promoter and co-expressed with mCherry (or GFP) driven by the cytomegalovirus promoter with 4 × 10^6 transducing units per ml17. HSV Mef2c-Dam was generated by fusing full-length mouse Mef2c cDNA to Dam, followed by virus packaging and preparation as described above. Animals received 2 µl of the HSV TALEGad1/Dam (or HSV Mef2c-Dam) virus over a period of 6 min using a syringe pump. Adult mice were anaesthetized using a ketamine/xylazine mixture in PBS. A rodent stereotaxic rig mounted with a micro pump (Stoelting) and Hamilton syringe was used to bilaterally inject viral vectors into the PFC (1 µl per hemisphere). Coordinates for injection were as follows: +1.5 mm anterior/posterior, ±0.5 mm medial/lateral and 1.5 mm dorsal/ventral. Virus was injected under heparin at 0.25 µl min^-1 and four additional minutes were allowed before syringe removal. Control animals received 2 µl of HSV-Mef2c-Dam virus using the same conditions as HSV- TALEGad1/Dam injections. Bilateral PFC injections (same coordinates as used for behaviour and molecular studies) in adult C57BL/6 wild-type mice were made with HSV-Gad1-GFP and the mice were perfused 48 h post injection (N = 4 animals). Brains were sectioned 40 µm and screened for fluorescence pattern in serial sections starting. Of note, from four animals subjected to bilateral injections, eight of eight injections extended along the rostro-caudal axis very similar to the example provided in the new (Fig. 1c).

Behaviour. All behavioural experiments were carried out in the light at the onset of the animals’ dark cycle by two experimenters not blinded to animal group condition.

Eight-arm radial maze. The maze consisted of eight arms (7.5 × 35 cm, 17.5 cm high walls) assembled radially around a circular starting platform. Mice were placed onto the starting platform and were free to enter the arms. Mice were tested until all eight arms were visited once. Each repeat entry in arm was counted as an error. Mice were tested on days 1 and 2, and tested on day 3.

Open field. The open-field chamber consisted of a white Plexiglas box (40 × 40 cm, 30 cm high), illuminated with bright white light (350lx). Mice were placed individually into the box for 5 min and the box was cleaned with bleach for 20 min. Total activity time spent in an imaginary centre square (15 × 15 cm) of the open field and stereotactic rearing activity counts were recorded using Fusion 5.0 Superflex system.

Immunohistochemistry and imaging. Mice were anaesthetized with a terminal i.p. injection of a ketamine/xylazine mixture (IP: 200 and 30 mg kg^-1, respectively) followed by heparin injection. Intravenous perfusion with 100% paraformaldehyde was perfusion with 100% paraformaldehyde infused with 200 ml of 4% paraformaldehyde in PBS. Brains were removed and placed in 4% paraformaldehyde overnight at 4 °C, followed by incubation in 30% sucrose until isotonic. Brains were cut on a freezing microtome (Leica SM2010 R) into coronal sections (60 µm) and permeabilized and blocked with 0.1% Triton X-100 and 1% goat serum (Southern Biotech), respectively. Sections were incubated with NeuN-488 (1:500, EMD Millipore, ABN78A4) for 2 h, followed by a wash with PBS. Mounting was done using 4:6-diamidino-2-phenylindole Fluoromount-G (SouthernBiotech, 0100-20). Images were taken with a Carl Zeiss CLSM780 microscope and processed using ImageJ (NIH).

DNA/RNA extraction from PFC. The PFC injection site harvested for the following assays: DamID–PCR and RNA expression from the same tissue samples, or DamID-seq, or conventional chromosome conformation capture (3C). DNA was isolated for DamID-seq and DamID–PCR. Tissue was homogenized in 1:1 of lysis buffer (10 mmol l^-1 Tris hydrogen chloride pH 8.0/10 mmol l^-1 sodium chloride/0.2% IPEGA CA-630 (Sigma-Aldrich)), 5 mg proteinase K (Invitrogen) was added for 30 min at 65 °C, an equal volume of phenol:chloform (pH 7.5) was added by genomic loci and sample using GenePattern (www.broadinstitute.org) pairwise average-linkage with mean-based row and column centring58. DamID–PCR. Samples were amplified using 1 × GoTaq Green master mix (Promega). PCR cycling conditions were 95°C for 5 min, 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, one cycle of 95°C for 30 s, 60°C for 30 s and 72°C for 5 min. Four independent final hold at 72°C. The final cycle was fixed. Geneseq and detected by visual inspection and confirmed by sequencing. DamID–PCR data were analysed in 50 kb sliding windows using a negative binomial (gammas) Poisson distribution. To normalize for within-group variability, samples were modelled by a dispersion parameter. Genes of similar expression levels were assumed to have similar dispersion and dispersion was estimated using maximum likelihood estimation for separate loci and smoothed cross-section was fit. Gene-wise distances were shrunken towards values predicted by the smooth curve using empirical Bayes57. In addition, sequences considered by the ENCODE and modENCODE consortia, via manual curation and automated heuristics, as prone to artefact signal in next-gen sequencing (ChiP sequencing, MNase sequencing, DNase sequencing and footprint-aided isolation of regulatory elements (FAIRE) sequencing) assays33 were removed from analyses. To compare Time A and Time B Dam-seq adenine methylation profiles, data were normalized using the negative binominal distribution. Loci with normalized read counts >10 (Supplementary Data 1) were used in Pearson’s hierarchical clustering (R Bioconductor, version 2.14.0) using genoNorm and sample using GenePattern (www.broadinstitute.org) pairwise average-linkage with mean-based row and column centring58. DamID–PCR. Samples were amplified using 1 × GoTaq Green master mix (Promega). PCR cycling conditions were 95°C for 5 min, 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, one cycle of 95°C for 30 s, 60°C for 30 s and 72°C for 5 min. Signal was measured via SYBR green-based quantitative PCR. Control PCR reactions, to ensure complete digestion at GATC tetramers, were designed with primers around DamID sites at the transcription start site of Cxxc5, a transcription factor on chromosome 18 with no Gadd interactions. All relevant data are available from the authors.

Chromosome conformation capture. Rostro-caudal cortex was homogenized and cross-linked for 10 min at 25 °C in 1% formaldehyde, 1 × protease inhibitor (Sigma) and 2 ml lysis buffer (10 mmol l^-1 Tris hydrogen chloride pH 8.0/10 mmol l^-1 sodium chloride/0.2% IPEGA CA-630 (Sigma Aldrich)). A final concentration of 0.125 mol l^-1 glycine was added for 10 min to stop cross-linking. The homogenate was incubated for another 25 min at 4 °C. Cells were lysed by pipetting and placed onto the starting platform for 20 min. Total activity time spent in an imaginary centre square (15 × 15 cm) of the open field and stereotactic rearing activity counts were recorded using Fusion 5.0 Superflex system.

Notes
50 μl aliquots. An additional 312 μl of 1 × NEB and 38 μl of 1% SDS were added to each aliquot and the samples were incubated at 65°C for 10 min. To quench the SDS, 10% of Triton X-100 was added to each sample and the samples were digested with HindIII-HF (NEB) at 37°C overnight with vigorous shaking. HindIII-HF was inactivated by the addition 86 μl of 10% SDS incubated for 30 min at 65°C.

Ligation mixture (7.61 ml) was added to each sample. The ligation mixture consisted of 745 μl of 10% Triton X-100, 745 μl of ligation buffer (1 M Tris HCl pH 7.5, 1.5 MgCl2, 1 M dithiothreitol (Bio-Rad)), 80 μl of 10 mg/ml BSA (NEB), 80 μl of 100 mM ATP (Sigma) and 9,560 μl of autoclaved water. Fifty microlitres of T4 DNA ligase (1 U ml⁻¹, Invitrogen) was added to three aliquots and one sample was used as a no ligase control. Ligation proceeded for 5 h at 16°C and samples were reverse cross-linked at 65°C overnight with 50 μl of 10 mg/ml proteinase K (Sigma). For improved ligated DNA recovery, another 50 μl of proteinase K was added and incubated at 65°C for 2 h. DNA was extracted with phenol (pH 8.0, Fisher) and phenol–chloroform (1:1) (pH 8, Fisher). DNA was precipitated with 1/10 the volume of 3 M sodium acetate (pH 5.4) and 2.5 the volume of ice-cold ethanol overnight. The samples were centrifuged at 8,000 r.p.m.

phenol (pH 8.0) respectively. 59. Ligase and no ligases libraries alone were run on a 2% agarose gel. 60, 61. After ligation, indicated by an upward shift on the gels. 62. Javitt, D. C., Zukin, S. R., Heresco-Levy, U. & Umbricht, D. Has an angel shown the way? Etiological and therapeutic implications of the PCP/NMDA model of schizophrenia. 63. Schizophr. Bull. 38, 958–966 (2012).

64. Li, J. T. et al. Persisting cognitive deficits induced by low-dose, subchronic treatment with MK-801 in adolescent rats. Eur. J. Pharmacol. 652, 65–72 (2011).

65. Paule, M. G. et al. Chronic exposure to NMDA receptor and sodium channel blockers during development in monkeys and rats: long-term effects on cognitive function. Ann. N. Y. Acad. Sci. 953, 116–122 discussion 121-114 (2003).

66. Li, J. T., Feng, Y., Su, Y. A., Wang, X. D. & Si, T. M. Enhanced interaction among ErbB4, PSD-95 and NMDAR by chronic MK-801 treatment is associated with behavioral abnormalities. Pharmacol. Biochem. Behav. 108, 44–53 (2013).

67. Manahan-Vaughan, D., von Haehl, D., Winter, C., Jackel, G. & Heinemann, U. A single application of MK-801 causes symptoms of acute psychosis, deficits in spatial memory, and impairment of synaptic plasticity in rats. Hippocampus 18, 125–134 (2008).

68. Lubin, F. D., Roth, T. L. & Sweatt, J. D. Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. J. Neurosci. 28, 10576–10586 (2008).

69. Belforte, J. E. et al. Postnatal NMDA receptor ablation in corticolimbic interneurons confers schizophrenia-like phenotypes. Nat. Neurosci. 13, 76–83 (2010).

70. Alkhabari, S. & Huang, H. S. Molecular and cellular mechanisms of altered GAD/GAD67 expression in schizophrenia and related disorders. Brain Res. Rev. 52, 293–304 (2006).

71. Cleard, F., Moskhan, Y., Karch, F. & Maeda, R. K. Probing long-distance regulatory interactions in the Drosophila melanogaster bithorax complex using Dam identification. Nat. Genet. 38, 931–935 (2006).

72. Koziol, M. J. et al. Identification of methylated deoxyadenosines in vertebrates reveals diversity in DNA modifications. Nat. Struct. Mol. Biol. 23, 24–30 (2016).

73. Konermann, S. et al. Optical control of mammalian endogenous transcription and epigenetic states. Nature 500, 472–476 (2013).

74. de Vries, G. et al. A new technique to quantitatively measure transcriptional activity. Gene 312, 145–153 (2003).

75. Neve, R. L., Neve, K. A., Nestler, E. J. & Carlezon, Jr. W. A. Use of herpes virus vectors to study the effects of chronic psychostimulants. Curr. Protoc. Neurosci. Chapter 4–12 (2012).

76. Bharadwaj, R. et al. Conserved chromosome 2q31 conformations are associated with transcriptional regulation of GAD1 GABA synthesis enzyme and altered cognitive function. Ann. N. Y. Acad. Sci. 1258, 251-259 (2011).
27. Winden, K. D., Bragin, A., Engel, J. & Geschwind, D. H. Molecular alterations in areas generating fast ripples in an animal model of temporal lobe epilepsy. *Neurobiol. Dis.* 78, 35–44 (2015).

28. Lan, F. et al. Recognition of unmethylated histone H3 lysine 4 links BHCG80 to LSD1-mediated gene repression. *Nature* 448, 718–722 (2007).

29. Iwase, S. A component of BRAF-HDAC complex, BHCG80, is required for neonatal survival in mice. *FEBS Lett.* 580, 3129–3135 (2006).

30. Bosma, P. T. et al. Multiplicity of glutamic acid decarboxylases (GAD) in vertebrates: molecular phylogeny and evidence for a new GAD paralog. *Mol. Biol. Evol.* 16, 397–404 (1999).

31. Labouesse, M. A., Dong, E., Grayson, D. R., Guidotti, A. & Meyer, U. Maternal immune activation induces GAD1 and GAD2 promoter remodeling in the offsetting prefrontal cortex. *Epigenetics* 10, 1143–1155 (2015).

32. Richetto, J., Calabrese, F., Riva, M. A. & Meyer, U. Prenatal immune activation induces maturation-dependent alterations in the prefrontal GABAergic transcriptome. *Schizophr. Bull.* 40, 351–361 (2014).

33. Jiang, Z., Rompala, G. R., Zhang, S., Cowell, R. M. & Nakazawa, K. Social isolation exacerbates schizophrenia-like phenotypes via oxidative stress in cortical interneurons. *Biol. Psychiatry* 73, 1024–1034 (2013).

34. Song, J. et al. Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision. *Nature* 489, 150–154 (2012).

35. Joo, J. Y., Schaukwit, K., Farbiak, L., Kilaru, G. & Kim, T. K. Stimulus-specific combinatorial functionality of neuronal c-fos enhancers. *Nat. Neurosci.* 19, 75–83 (2016).

36. Goff, D. C. Drug development in schizophrenia: are glutamatergic targets still worth aiming at? *Carr. Opin. Psychiatry* 28, 207–215 (2015).

37. Heckers, S. & Konradi, C. GABAergic mechanisms of hippocampal hyperactivity in schizophrenia. *Schizophr. Res.* 167, 4–11 (2015).

38. Hall, J., Trent, S., Thomas, K. L., O'Donovan, M. C. & Owen, M. J. Genetic risk of schizophrenia: convergence on synaptic pathways involved in plasticity. *Schizophr. Res.* 167, 868–878 (2015).

39. Heckers, S. & Konradi, C. GABAergic mechanisms of hippocampal hyperactivity in schizophrenia. *Schizophr. Res.* 167, 868–878 (2015).

40. Halder, R. et al. DNA methylation on N6-adenine in human brain. *Schizophr. Res.* 167, 868–878 (2015).

41. Feder, A., Nestler, E. J. & Charney, D. S. Psychobiology and molecular genetics of resilience. *Nat. Rev. Neurosci.* 10, 446–457 (2009).

42. Ito, S. et al. Loss of neuronal 3D chromatin organization causes transcriptional and behavioural deficits related to serotonergic dysfunction. *Nat. Commun.* 5, 4450 (2014).

43. Jiang, Y. et al. Setdb1 histone methyltransferase regulates mood-related behaviors and expression of the NMDA receptor subunit NR2B. *J. Neurosci.* 30, 7175–7176 (2010).

44. Guelen, L. et al. Domain organization of human chromosones revealed by mapping of nuclear lamina interactions. *Nature* 453, 948–951 (2008).

45. Izzo, A. et al. The genomic landscape of the somatic linker histone subtypes H1.1 to H1.5 in human cells. *Cell Rep.* 3, 2142–2154 (2013).

46. Shimbo, T. et al. MBD3 localizes at promoters, gene bodies and enhancers of active genes. *PLoS Genet.* 9, e1004028 (2013).

47. Jiang, Y., Matevosian, A., Huang, H. S., Strubhaar, J. & Akbarian, S. Isolation of neuronal chromatin from brain tissue. *BMC Neurosci.* 9, 42 (2008).

48. Haldor, R. et al. DNA methylation changes in plasticity genes accompany the formation and maintenance of memory. *Nat. Neurosci.* 19, 102–110 (2016).

49. Mo, A. et al. Epigenetic signatures of neuronal diversity in the mammalian brain. *Neuron* 86, 1369–1384 (2015).

50. Madabushi, R. et al. Activity-induced DNA breaks govern the expression of neuronal early-response genes. *Cell* 161, 1592–1605 (2015).

51. Chow, H. M. & Herrup, K. Genomic integrity and the ageing brain. *Nat. Rev. Neurosci.* 16, 672–684 (2015).

52. Greer, E. L. et al. DNA methylation on N6-adenine in *C. elegans*. *Cell* 161, 868–878 (2015).

53. Zhang, G. et al. N6-methyladenine DNA modification in *Drosophila*. *Cell* 161, 893–906 (2015).

54. Lopez, M. F., Doremus-Fitzwater, T. L. & Becker, H. C. Chronic social isolation and chronic variable stress during early development induce later elevated ethanol intake in adult C57BL/6 mice. *Alcohol* 45, 355–364 (2011).

55. van Steensel, B., Delrow, J. & Henikoff, S. Chromatin profiling using targeted DNA adenine methyltransferase. *Nat. Genet.* 27, 304–308 (2001).

56. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550 (2014).

57. Eisen, M. B., Spellman, P. T., Brown, P. O. & Botstein, D. Clustering analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* 95, 14863–14868 (1998).

58. Mitchell, A. C. et al. The genome in three dimensions: a new frontier in human brain research. *Biol. Psychiatry* 75, 961–969 (2014).

59. Dekker, J. The three ‘C’s of chromosome conformation capture controls, controls, controls. *Nat. Methods* 3, 17–21 (2006).

60. Dekker, J., Rippe, K., Dekker, M. & Kleckner, N. Capturing chromosome conformation. *Science* 295, 1306–1311 (2002).

61. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675 (2012).