



# Environmental Enrichment Induces Epigenomic and Genome Organization Changes Relevant for Cognition

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In early development, the environment triggers mnemonic epigenomic programs resulting in memory and learning experiences to confer cognitive phenotypes into adulthood. To uncover how environmental stimulation impacts the epigenome and genome organization, we used the paradigm of environmental enrichment (EE) in young mice constantly receiving novel stimulation. We profiled epigenome and chromatin architecture in whole cortex and sorted neurons by deep-sequencing techniques. Specifically, we studied chromatin accessibility, gene and protein regulation, and 3D genome conformation, combined with predicted enhancer and chromatin interactions. We identified increased chromatin accessibility, transcription factor binding including CTCF-mediated insulation, differential occupancy of H3K36me3 and H3K79me2, and changes in transcriptional programs required for neuronal development. EE stimuli led to local genome re-organization by inducing increased contacts between chromosomes 7 and 17 (*inter*-chromosomal). Our findings support the notion that EE-induced learning and memory processes are directly associated with the epigenome and genome organization.

**Keywords:** environmental enrichment, epigenetics, 3D genome organization, learning, postnatal development, chromatin accessibility, Hi-C, inter-chromosomal contacts

## INTRODUCTION

Exposure to environmental stimuli influences developmental programs of organisms by modulating gene regulatory networks. These programs direct early postnatal neuronal development, particularly during the “critical period” that is key to establish brain functions that are kept throughout the lifetime of an individual (Hübener and Bonhoeffer, 2014). Environmental enrichment (EE) is a commonly used paradigm to study the behavioral and electrophysiological

mechanisms of neuronal development (van Praag et al., 2000). Widely considered positively influencing cognition, it is a potential treatment application for a wide source of human traits (McDonald et al., 2018; Ball et al., 2019; Kempermann, 2019). On the counterpart, other studies found multiple sources of variability, confounds or even deleterious effects of EE (Berardo et al., 2016; Sparling et al., 2020). Generally, EE represents external factors, such as sensory, physical, cognitive, and social stimulation to provide and to maintain constant novelty and complexity, thereby laying a key foundation for future learning processes (Rountree-Harrison et al., 2018).

The coalescing mechanisms of gene regulation, epigenetics, and genome organization leading to learning and memory formation still remain largely unknown. Thus far, studies on how EE affects gene regulatory elements are sparse, but some findings point toward the involvement of epigenetic mechanisms, both at the level of DNA methylation and histone modifications and chromatin modifiers (Irier et al., 2014; Morse et al., 2015). Recently, advances in brain research indicated that three-dimensional (3D) genome organization can be causally involved in gene-regulatory networks and chromatin conformation dynamics that impact brain function, learning, and memory formation (Fernandez-Albert et al., 2019; Rajarajan et al., 2019; Yamada et al., 2019; Beagan et al., 2020; Peter et al., 2020). These findings imply that a comprehensive molecular analysis of the processes happening during EE is needed to understand how neuronal circuits are refined by environmental cues. To accomplish this aim, we leveraged multiple genomic techniques to identify regulatory changes leading to learning and memory formation by EE during early postnatal neuronal development. We assessed chromatin accessibility, chromatin modifications, transcriptomic and proteomic changes, as well as 3D genome conformation. Our results reveal for the first time a comprehensive genome-wide perspective of global and neuronal-specific regulatory epigenetic modifications under EE in whole cerebral cortex, followed by neuron-specific and pyramidal-neuron-specific profiling. Our present study demonstrates that EE-induced early learning experience changes the neuronal epigenome and causes altered genomic conformations, especially between different chromosomes.

## RESULTS

### Study Outline

EE significantly influences learning and memory and leads to cognitive improvement, as demonstrated by multiple studies (Ohline and Abraham, 2019). Our EE-protocol was successfully established and validated by behavioral testing (Morris water maze) in an earlier study (Pons-Espinal et al., 2013). Briefly it consisted in a constantly changing environmental stimulation (every 48 h) over the course of 1 week (postnatal day P28) or 1 month (P51), an important stage of the critical period (Sztainberg and Chen, 2010; Hübener and Bonhoeffer, 2014) (Method Details, **Figure 1A**). For detailed insights into cell heterogeneity in the context of EE and the cerebral cortex microenvironment,

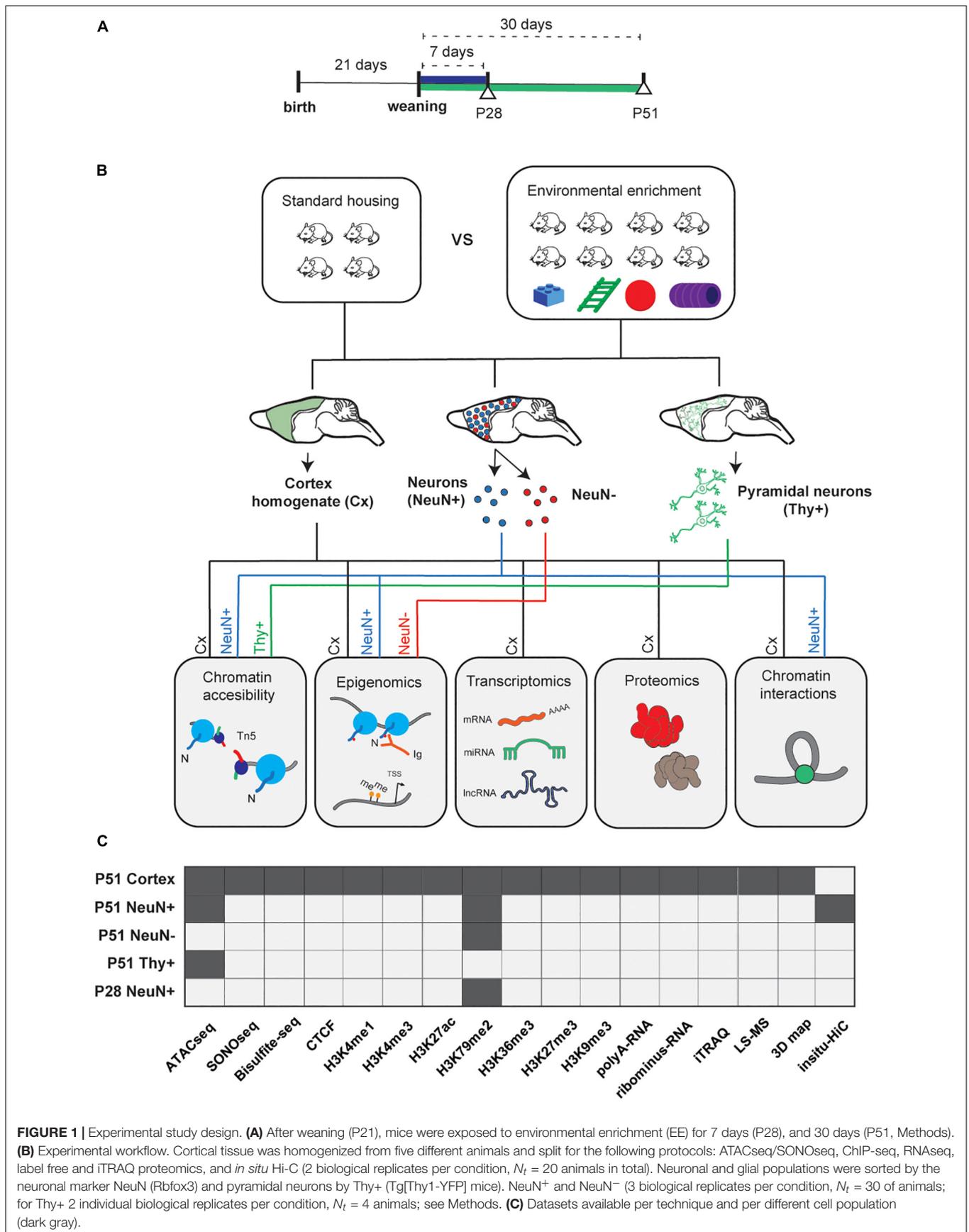
we used whole cerebral cortex, sorted neuronal, and non-neuronal cells and performed epigenomic, transcriptomic, and proteomic profiling, as well as capturing of genomic interactions to provide a communal resource (**Figures 1B,C**).

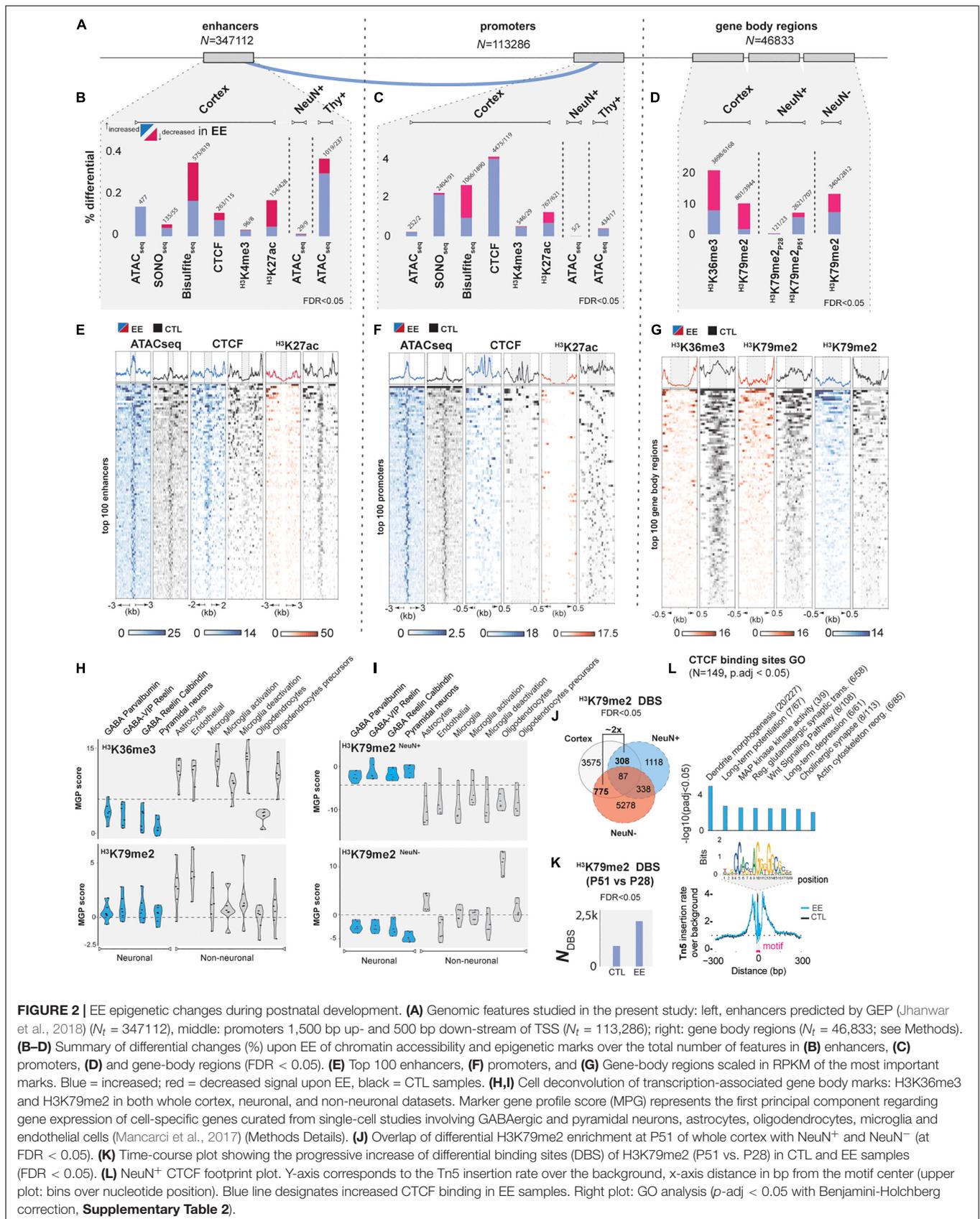
To analyze and intersect our multiple datasets, we devised a computational pipeline to determine activity and interplay of epigenomic marks in gene-regulatory regions, namely between enhancers predicted by the tool GEP (Jhanwar et al., 2018), and annotated promoters (Methods Details, **Figure 2A** and **Supplementary Table 1**).

### EE Induces Increased Chromatin Accessibility and Insulation Targeting Synaptic-Associated Genes in Cortical Tissue

EE is non-invasive in comparison to invasive neuronal stimulation which leads to increased chromatin accessibility in gene-regulatory regions to induce gene transcription (Su et al., 2017; Koberstein et al., 2018; Fernandez-Albert et al., 2019). Therefore, we asked if non-invasive EE could lead to quantifiable effects on gene and genome function during cortical cell postnatal development.

First, we studied EE in whole cortical tissue (Pons-Espinal et al., 2013). In ATAC-seq experiments investigating chromatin accessibility (Buenrostro et al., 2013), we observed that distinct ATAC-seq peaks (macs2, fseq) were increased genome-wide in EE samples compared to controls (CTLs), suggesting a global increase in chromatin accessibility after EE ( $FC_{\text{cortex}} 1.17X$ , **Supplementary Figure 1A**). To validate these findings, a differential analysis of enhancers and promoters further confirmed increased chromatin accessibility in a very specific set of 0.13% of enhancers and 0.22% of the total promoters ( $FDR < 0.05$ , **Figures 2B,C**). To link *intra*-chromosomal interactions of enhancers to their corresponding promoters, we used a modified version of EpiTensor (Zhu et al., 2016; **Supplementary Table 1**, Method details). We found regulatory regions showing increased accessibility specific to genes that could be linked to neurogenesis and differentiation (Speisman et al., 2013; Clemenson et al., 2015), angiogenesis (Yu et al., 2014), synapse organization (Ohline and Abraham, 2019), and pathways associated to memory and learning such as Wnt (He et al., 2018), and Rho signaling (Martino et al., 2013; **Supplementary Figures 1B,C** and **Supplementary Table 2**). To further confirm previous ATAC-seq results, we used SONO-seq (Auerbach et al., 2009), a method based solely on sonicated and sequenced chromatin. We validated 76 genes showing consistent increased accessibility in their enhancers and promoters ( $p\text{-adj} < 0.01$ , **Supplementary Figure 1E**). Additionally, SONO-seq showed differential accessibility on pathways that are important in neuronal function such as MAPK and JNK (Coffey, 2014), neural maturation BMP (Bond et al., 2012), synaptic plasticity PI3K-Akt (Tan et al., 2017), cellular aging prevention and telomere protective role of oxytocin (Faraji et al., 2018; Stevenson et al., 2019), and neurotransmission function by GPCR (Betke et al., 2012; **Supplementary Table 2**).





Accessible regions of chromatin regions encompass characteristic posttranslational modifications in surrounding histones (Fu et al., 2018). Due to the relationship between these histone marks and the increased chromatin accessibility in enhancers and promoters upon EE, we hypothesized that EE could also modulate posttranslational histone modifications and CTCF binding in gene-regulatory regions. We investigated a variety of histone marks from active (H3K27ac, H3K4me3, H3K4me1) to repressed regions (H3K27me3, H3K9me3), in addition to CTCF and DNA methylation. Interestingly, we detected relevant changes in H3K4me3, H3K27ac, DNA methylation, and CTCF upon EE (**Supplementary Table 3**). The regions represented about 0.2–0.4% of enhancers and 2–5% of the promoters depending on the mark being analyzed (FDR < 0.05, **Figures 2B,C,E,F** and **Supplementary Table 3**). With the exception of hypermethylated sites and weak changes in H3K27me3, the majority of changes occurred in activity-associated marks. We did not find significant changes in the heterochromatic mark H3K9me3, indicating that EE impacts the modulation of active gene sets rather than repressed regions. To investigate this in detail, we profiled transcription-associated marks such as H3K36me3 and H3K79me2 as potential readouts of gene expression (Huff et al., 2010), and determined a ~20 and 10% differential binding of gene body marks, respectively, confirming that EE impacts transcriptional programs (FDR < 0.05, **Figures 2D,G** and **Supplementary Table 3**). Remarkably, the modulation of transcription-associated marks post EE induction was also observed in ~13 and 8% of distal enhancers bearing H3K36me3 and H3K79me2, respectively, suggesting that enhancer-derived RNA genes are also differentially expressed (Kim et al., 2010) (FDR < 0.05, **Supplementary Figures 1F,G**). Genes associated to EE-induced cortical epigenetic marks changes were linked to the extracellular matrix (ECM) important for shaping synapses during postnatal development (Bikbaev et al., 2015; Ferrer-Ferrer and Dityatev, 2018), to circadian clock genes known to be required for proper healthy adult behavior (Brooks and Canal, 2013), and glutamatergic receptor complexes key for neuronal plasticity (Lüscher and Malenka, 2012; **Supplementary Figures 1H–N** and **Supplementary Table 3**).

Having determined that EE induced differential chromatin accessibility and modulation of histone modifications in postnatal cortical tissue, we next addressed potential cross-talk mechanisms. We explored the overlap across all differential epigenetically modified and accessible chromatin regions identified previously (**Supplementary Figure 1O**). The strongest overlap corresponded to increased CTCF binding (18.7% of total sites) co-occurring with a decrease of the gene body activity marks H3K79me2 and H3K36me3 (at FDR < 0.1, **Supplementary Figure 1P**). A highly relevant example of this priming state is the early-life stress gene *Met* (Heun-Johnson and Levitt, 2018), and the memory regulating phosphodiesterase *Pde8b* (Tsai et al., 2012), both showing increased chromatin accessibility of interacting enhancers upon EE, as well as increased CTCF insulation in promoters, but decreased occupancy of H3K79me2 and H3K36me3 when compared

to CTLs (**Supplementary Figures 2A,B**). This result could indicate a state where genes are poised to be transcribed, but are temporarily repressed by insulation, a specific state due to changes in chromatin architecture (Kim et al., 2015). But, it could also indicate mixed signals coming from the process of synaptic tuning, where some synapses gain strength meanwhile others are lost as consequence of the learning mechanism (Turriano, 2008).

## Molecular Phenotypic Changes by EE Mainly Target the Glutamatergic Synapse and Extracellular Matrix Elements

Next, we determined how the previously described epigenetic changes alter transcriptional (coding and non-coding) and translational landscapes upon EE. Expression analysis revealed a total of 473 differentially expressed genes (FDR < 0.05, **Supplementary Figure 2C** and **Supplementary Table 4**). Additional biological replicates and gene ontology analysis recapitulated previously described pathways and terms, such as: BMP, JNK, MAPK, AMPAR signaling, and elements of the ECM (**Supplementary Figures 2D–F**). Moreover, to corroborate these results we performed a comparative analysis with previous published RNA-seq studies under the influence of EE (Zhang et al., 2016, 2018; Grégoire et al., 2018; Wassouf et al., 2018). We found 41 differential expressed genes in our data (~10% of DEGs) that overlap with previous studies, showing consistent enrichment to ECM and elements from the postsynaptic density important for AMPA regulation such as *Arc* gene (Chowdhury et al., 2006; Shepherd et al., 2006; **Supplementary Figure 3**). By investigating the non-coding fraction of RNA, we identified 200 microRNAs and 52 long non-coding RNAs (lncRNAs) differentially expressed upon EE (**Supplementary Figures 4A–F** and **Supplementary Table 5**). Top microRNAs were validated in a new set of biological replicates (**Supplementary Figure 4B**). Using a multi-source microRNA target predictor (Friedman et al., 2010), we observed specificity for synaptic-associated mRNA targets in a gene ontology analysis ( $p$ -adj < 0.05, **Supplementary Figures 4C,D** and **Supplementary Table 5**). Of note, we found *Meg3* and *Rian* (*Meg8*) as downregulated lncRNAs upon EE ( $p$ -adj < 0.05, **Supplementary Figure 4E**). Both are known for their ability to regulate glutamatergic neurotransmission, potentially in collaboration with microRNAs (Tan et al., 2017). We then explored the potential interactions between both non-coding elements (microRNAs and lncRNAs) with LncBase (Paraskevopoulou et al., 2016), and observed that 20 of our differentially expressed microRNAs could interact with *Meg3* (**Supplementary Figure 4F**). Particularly interesting is the up-regulated miR125b-p5 reported to be involved in synaptic strength and *Grin2a* downregulation (Edbauer et al., 2010).

To recapitulate EE-induced changes by quantitative protein expression, we used iTRAQ and LCMS mass spectrometry, finding about 73 and 145 differential proteins, respectively ( $p$  < 0.05, **Supplementary Table 6**). Gene ontology analysis of intersected EE-induced transcriptomic and proteomic changes identified pathways highlighting the importance of the ECM and

neurotransmission receptor complexes, particularly involving the glutamatergic synapse (**Supplementary Figure 4G**).

## EE Stimulation in NeuN<sup>+</sup> Sorted Neurons

To further understand the poised state of genes observed in cortical tissue and to avoid cell bias composition, we decided to investigate EE-induced influence in a cell-specific manner. We performed a cell deconvolution analysis to specify which cell types are primarily responding to EE stimulation (Mancarci et al., 2017). Remarkably, we observed that H3K36me3 and H3K79me2 were enriched in non-neuronal populations in whole cortex data (**Figure 2H**). However, to address the neuronal extent of EE-induced epigenetic changes observed in whole cortex, we performed FACS sorting by nuclei immunostaining of the neuronal specific marker NeuN (Jiang et al., 2008) (Rbfox3, **Supplementary Figure 5A**). Another deconvolution of H3K79me2 in sorted populations demonstrated the neuronal-specific identification of EE-stimulatory effects (**Figure 2I**, **Supplementary Figure 5B**). We observed that differential analysis on H3K79me2 data between EE vs. CTLs showed two times more non-neuronal than neuronal H3K79me2 enrichment when compared to whole cortex, pointing the importance of non-neuronal for future studies (**Figure 2J**, FDR < 0.05). Neurons specifically, showed a total of 0.35% (P28) and 7.1% (P51) of genes with differential H3K79me2 gene-body occupation (FDR < 0.05, **Figure 2D** and **Supplementary Table 3**). Interestingly, we found that H3K79me2 occupancy changes from P28 to P51 in both CTL and EE samples (**Figure 2K**, FDR < 0.05, **Supplementary Table 3**). But the influence of EE amplifies this effect by affecting ~2× more genes that significantly enrich for neurodevelopmental terms (**Supplementary Figure 5C**). Between these two time points of postnatal development, H3K79me2 changes in control samples are associated to axon sprouting, mitochondrial activity, complement cascade and cerebral cortical regionalization with associated genes such as *Pcdh18*, identified as a potential candidate for intellectual disability (Kasnauskiene et al., 2012; **Supplementary Figure 5D**). In other hand, EE samples significantly enrich for learning processes, including both glutamatergic and GABAergic genes such as *Gabra2*, key modulator in multiple brain traits (Mulligan et al., 2019; **Supplementary Figure 5E**).

We revisited our previous findings in sorted neurons by addressing chromatin accessibility and gene-body epigenetic profiling (**Figures 2C,D**). Similar to whole cortex, we observed increased chromatin accessibility sites in enhancers and promoters after EE in NeuN<sup>+</sup> cells ( $FC_{\text{neurons}} = 1.61X$ , **Supplementary Figure 6A**). But differential sites represented around 0.01 and 0.006%, respectively, a lower rate when compared to whole cortex (FDR < 0.05, **Figures 2B,C** and **Supplementary Table 2**). As NeuN<sup>+</sup> marker is specific for a broad number of different neurons (**Figure 2I**), we extended our study to sorted pyramidal neurons overexpressing a yellow fluorescent protein (YFP) under the control of the *Thy1* gene promoter (Method Details, **Supplementary Figure 6B**). We found strongly increased chromatin accessibility upon EE (0.36% of enhancers, 0.39% of promoters), similar to the proportions

in whole cortex (at FDR < 0.05, **Figures 2B,C**, **Supplementary Figures 6C,D**, and **Supplementary Table 3**).

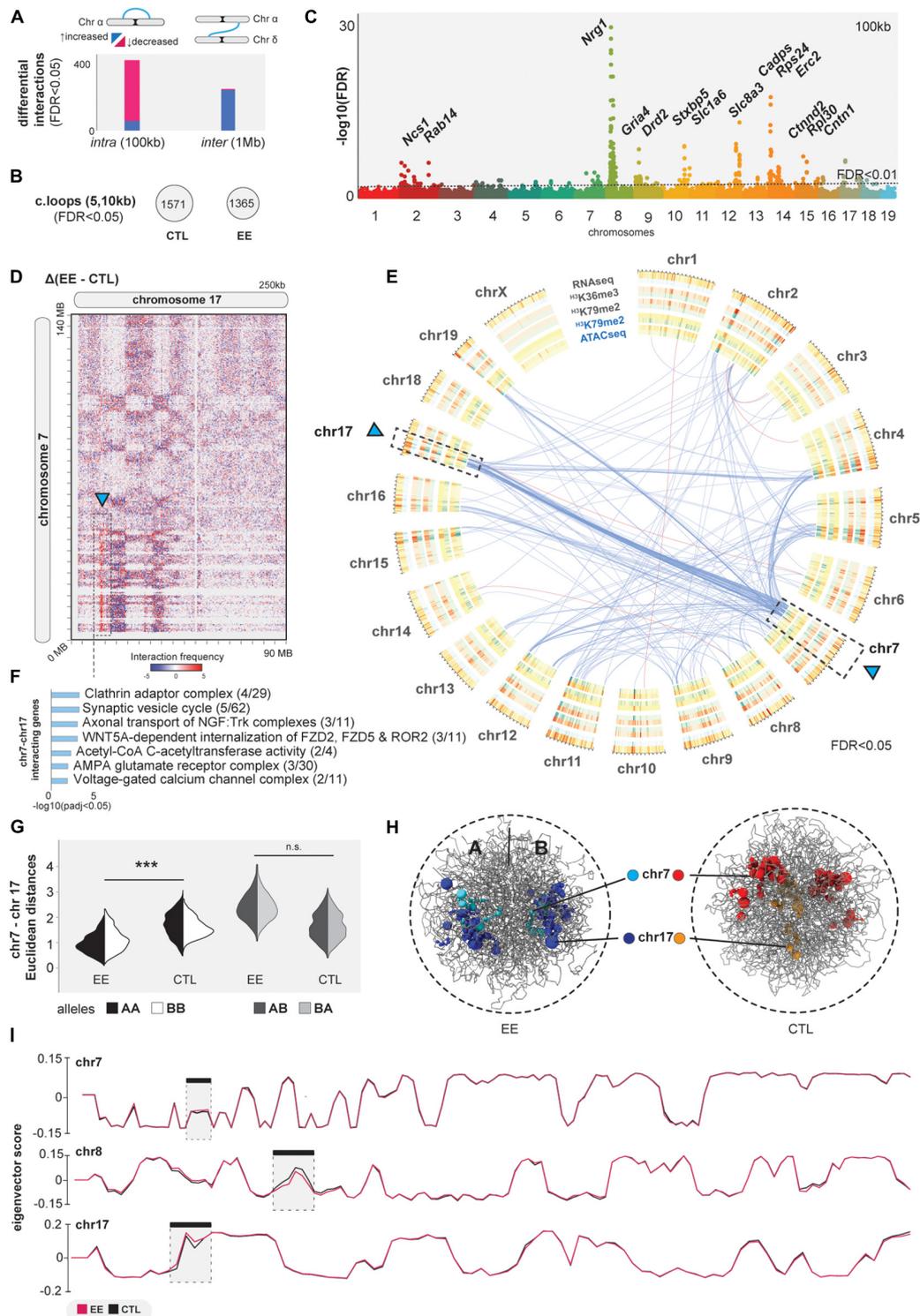
Our findings confirm that EE leads to increased chromatin accessibility in whole cortex, in NeuN<sup>+</sup> neurons, and more specifically in pyramidal neurons. We further confirm that these differential accessible enhancers are active forebrain enhancers at P0 and active in pyramidal neurons both in mouse and human (**Supplementary Figure 6E**). Particular genes could be linked to human cognition in the context of schizophrenia and autism, such as *Nrg3* and *Ank2*, respectively (Kao et al., 2010; Yang et al., 2019; **Supplementary Figure 6F**).

Because higher chromatin accessibility may allow increased TF binding, we ran a transcription factor binding site (TFBS) footprint analysis on whole cortex and NeuN<sup>+</sup> populations using Centipede which screens for all putative TFBS (Pique-Regi et al., 2011). We confirmed that more TFs were significantly bound in EE compared to CTLs, such as Lhx3, AP1, Nr5a2, and Phox2B (**Supplementary Figure 6G** and **Supplementary Table 2**). Interestingly, we observed that CTCF displayed one of the strongest instances bound in EE-stimulated neurons, similar to CTCF ChIPseq results ( $p\text{-adj} < 0.05$ , **Figure 2C**). This finding supports the idea that EE leads to increased genomic insulation and plays a role in genome organization during postnatal development.

Overall, we found that EE in neurons recapitulates cortical results inducing increased chromatin accessibility and CTCF binding. However in neurons, H3K79me2 increased upon EE which was not observed in the poised state of whole cortex. Noteworthy, GO terms of neuronal EE-induced changes show again genes associated with learning and memory targeting glutamatergic transmission predominantly, but also GABAergic and cholinergic transmission (**Figure 2L**, **Supplementary Figures 6I,J**, and **Supplementary Table 2**).

## EE Stimulation Prompts 3D Genome Changes

The described EE-related changes implicate that the epigenome plays an important role in 3D genome organization (Rao et al., 2014; Guo et al., 2015). The evidence of increased chromatin accessibility and CTCF binding may suggest that environmental stimuli impact higher-order genome organization. To further assess chromatin interactions in sorted neurons (NeuN<sup>+</sup>), we performed Hi-C experiments to explore the 3D genome organization upon EE. By comparing *intra*-chromosomal interactions at 100 kilobase (kb) resolution, we determined significant changes: 94 interactions increased and 544 decreased upon EE stimulation (FDR < 0.05, **Figure 3A** and **Supplementary Table 7**). A decrease of *intra*-chromosomal interactions was also observed when calculating the number of chromatin loops using HICCUPS between CTL and EE (FDR < 0.05, **Figure 3B**; Durand et al., 2016b). We found differential *intra*-chromosomal bins to be clustered in particular chromosomal regions, specially involving chromosomes 8 and 14 (**Figure 3C**). In these regions, we find genes such as the synaptic-linked gene *Ngr1* linked to cognitive function improvement (Chen et al., 2008;



**FIGURE 3** | 3D genome interaction changes upon EE. **(A)** Differential analysis of *intra* and *inter*-chromosomal interactions at 100 kb and 1 Mb, respectively (FDR < 0.05). **(B)** Significant chromatin loops computed with HiCCUPs at 5 and 10 kb resolution (FDR < 0.05). **(C)** Manhattan plot of differential *intra*-chromosomal interactions at 100 kb. **(D)** Juicebox heatmaps at 250 kb showing the extraction of EE vs. CTL of *inter*-chromosomal interactions. **(E)** Circos-plot of differential *inter*-chromosomal interactions (blue arcs-increased interactions, pink-decreased) together with concentric bedfiles representing the differential analysis of ATACseq, H3K79me2, H3K36me3 and RNAseq at 1 MB using Diffreps (increased regions upon EE = blue, decreased = red). **(F)** GO analysis of genes in the differential *inter*-chromosomal interactions at 1 MB upon EE stimulation ( $p\text{-adj} < 0.05$  Bonferroni-step down). **(G,H)** *In silico* chrom3D models for EE and CTL samples showing significant increase of *inter*-chromosomal interactions. **(I)** A/B compartmentalization measured by eigenvector scores in chromosomes 7, 8, and 17. \*\*\*denotes  $p \leq 0.001$ .

Xu et al., 2016); and the synaptic vesicle exocytosis regulator *Cadps* (Sadakata et al., 2007).

While these *intra*-chromosomal contacts were in the focus of chromatin biology in recent years, contacts between different chromosomes (*inter*-chromosomal) also occur and are involved in important biological functions (Quinodoz et al., 2018; Maass et al., 2019; Monahan et al., 2019), but they remain less studied. Therefore, we asked if EE is associated with large scale changes in genome organization by tracing chimeric *inter*-chromosomal Hi-C reads. Indeed, we identified 241 increased and 40 decreased interactions at 1 megabase (Mb) resolution (FDR < 0.05, **Figure 3A** and **Supplementary Table 7**). We determined a significant accumulation of *inter*-chromosomal contacts between chromosome 7 and 17 in EE vs. CTL (**Figures 3D–F**, arrow). We observed that these differential interactions form a clear genome architectural stripe, also termed Greek islands (Monahan et al., 2019; **Figure 3D**). Among bins involving these two chromosomes, we found relevant genes associated with the synaptic vesicle cycle, such as *Ap2a1* [2] being important for AMPAR endocytosis (DaSilva et al., 2016), *Acat2* [3] acetyl-CoA C-acetyltransferase playing a key role in neuronal metabolism (Ronowska et al., 2018), and *Cacng8* modulating AMPAR receptor complexes in the plasmatic membrane which are important for synaptic plasticity (Maher et al., 2016; **Figure 3F** and **Supplementary Table 7**). We validated our differential analysis by an independent method, called Chrom3D, and generated *in silico* models for pooled EE and CTL samples detecting significant proximity of chromosomes 7 and 17 (Paulsen et al., 2017; **Figures 3G,H**). We further corroborated the previous association of *inter*-chromosomal changes with gene-activity by studying chromatin compartmentalization (Lieberman-Aiden et al., 2009). As expected, A/B compartments do not change between CTL and EE, except for local changes in the strongest hubs of both *intra*- and *inter*-chromosomal contacts (chromosomes 7, 8, and 17), pointing to EE-related local chromatin changes in specific regions associated with active epigenetic modifications and gene expression changes (**Figure 3I**).

## EE Causes Coordinated Regulatory Changes That Cluster in Inter-Chromosomal Interactions Implicated in Memory-Related Functions

The multiple “omics” datasets to study the molecular basis of EE allowed us to conduct an intersection of all EE-induced changes determined in this study (**Figure 4A**). Interestingly, GO analysis revealed synapse organization as the strongest ranked term ( $p$ -adj < 0.05, **Supplementary Figure 7A**). We decided to explore this finding further using SynGO synaptic gene curator tool to identify overrepresented genes (hits > 4 showing intersection in different sets, **Figure 4B**; Koopmans et al., 2019). We found a significant enrichment of postsynaptic and presynaptic genes, particularly targeting the glutamatergic synapse (**Figures 4C,D**).

Further analysis of our merged data showed that about 60% of transcriptomic and 84% of proteomic changes are found in our other datasets, whilst 20% of changes were determined

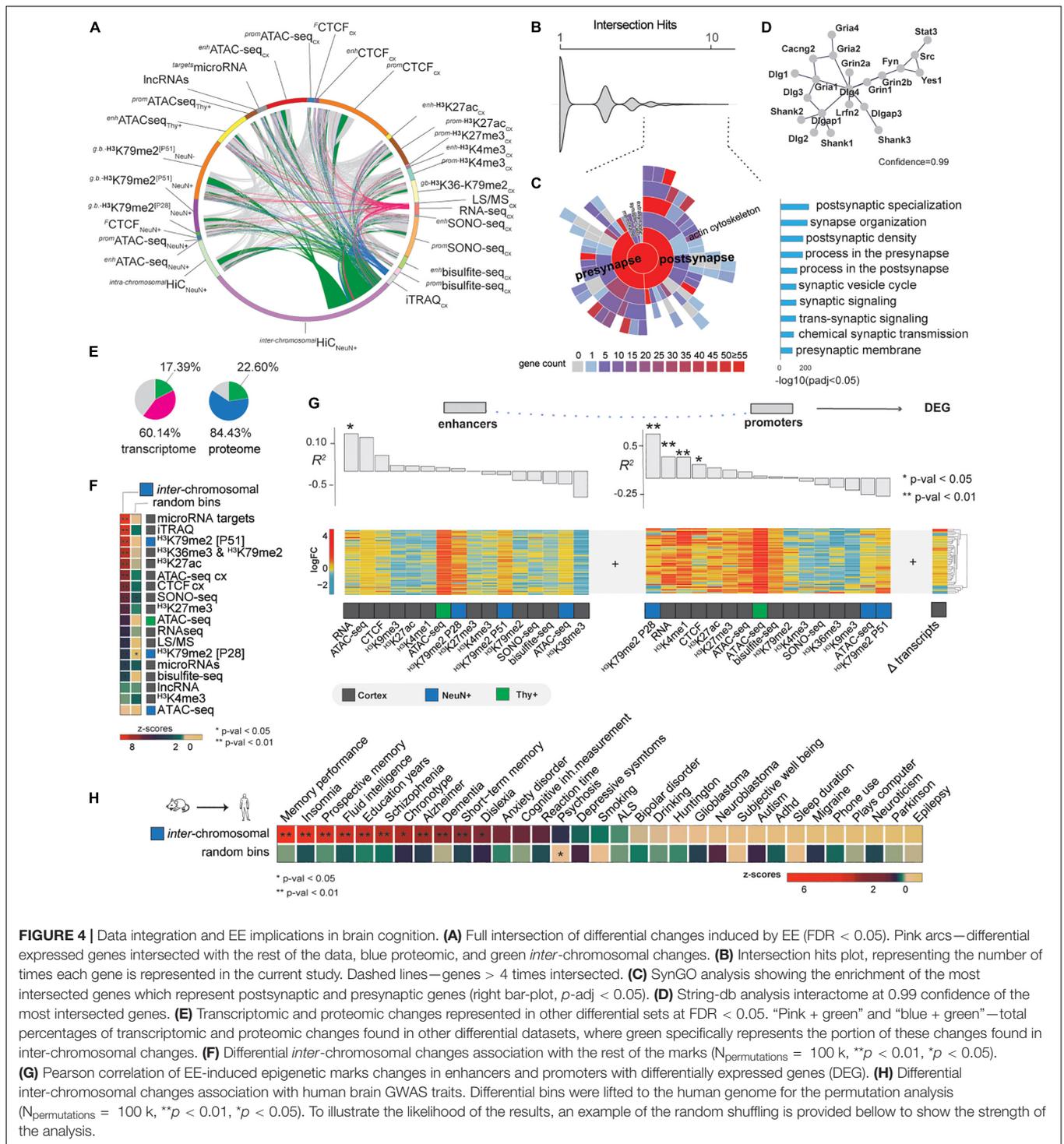
by EE-induced *inter*-chromosomal changes (**Figure 4E**). This enrichment together with the prior observation that differentially expressed genes tend to cluster in specific *inter*-chromosomal bins (**Figure 3E**), led us to the hypothesis that EE mainly induces changes locally in the genome, especially where active transcription occurs. To test this, we permuted the background genome at 1 Mb resolution 100 k times and calculated the likelihood of differential *inter*-chromosomal interactions to be associated with the differential epigenomic, transcriptomic, and proteomic changes found in the rest of the study. Strikingly, we observed that microRNA target genes, proteins (iTRAQ MS data), and gene-body associated histone marks were the strongest associated features within the specific *inter*-chromosomal hubs (**Figure 4F**). This finding confirms that EE orchestrates local changes of the nuclear architecture, especially *inter*-chromosomal communication.

Our intersection and permutation analysis indicated that chromatin conformation might connect the epigenome with the molecular phenotypes. We now asked how different marks influence others by estimating the linear dependency of EE-induced enhancers and promoters with transcriptomic and proteomic changes by Pearson and Spearman correlations (**Figure 4G** and **Supplementary Figure 7B**, Method details). We observed that enhancer RNA and promoter H3K79me2 activity drive transcriptomic and proteomic changes due EE. Particularly, we found H3K79me2 mark at P28 in promoters the most correlated modification indicating that transcripts and proteins observed at P51 are dependent on earlier stages of postnatal neuronal development, thereby underlining the temporal aspect of the critical period.

The cognitive and behavioral effects of EE could be a beneficial strategy for cognitive human disorders (Ball et al., 2019). Thus far, it is unclear if the molecular effects of EE that we found in mice can be retrieved in human. Therefore, we addressed the local changes in 3D genome organization in the human genome. Due our findings of clustered induced EE changes in differential *inter*-chromosomal bins, we performed a lift-over of these regions at 1 Mb from mouse to the human genome and ran a permutation analysis to test the association with 33 genome wide association studies (GWAS) relevant for human brain traits (**Supplementary Table 8**). We observed that the top associated traits were memory-related, such as memory performance ( $p$  < 0.01, **Figure 4H**). These findings not only validate our previous results, but point to conserved mechanisms between mouse and human that drive EE-related molecular effects by epigenomic, transcriptomic, and proteomic changes locally in specific regions of the genome that are important for both human and mouse cognition.

## DISCUSSION

We have characterized the regulatory response to EE by using omics both in whole cortex tissue and in two neuronal cell populations and provide a valuable resource for other scientists. We demonstrate that EE induces coordinated changes of gene-regulatory networks that involve epigenetics and genome



organization to adapt to constant cognitive stimulation and social interaction. EE induced increased enhancer and promoter chromatin accessibility in neurons, corroborating previous studies showing increased open chromatin upon invasive neuronal stimulation (Su et al., 2017; Koberstein et al., 2018; Fernandez-Albert et al., 2019). These studies also highlighted the importance of CTCF shaping the 3D genome during postnatal

development for memory formation (Sams et al., 2016; Kim et al., 2018). Here, we demonstrated that CTCF tends to bind preferentially to synaptic-associated genes upon EE, particularly glutamatergic associated pathways.

Our results also revealed, that gene body marks show differential activity in distal active enhancers upon EE, pointing to a potential role for these marks at transcriptionally active

enhancers (Zentner et al., 2011). This is conform with the finding some DNA methyltransferases depend on H3K36me3 to exert their function at enhancers (Rinaldi et al., 2016). We also observed that active transcription in regulatory regions during early stages of postnatal neuronal development may influence local transcriptomic and proteomic changes at later stages. Furthermore, studying gene body marks in sorted neuronal populations allowed us to identify the molecular effects during the postnatal critical period, reflected by a constant increase in H3K79me2 occupation. We found it exacerbated in an experience-dependent manner, with a greater number of increased binding sites in EE compared to CTL samples across time.

Despite the caveat of cell heterogeneity potentially skewing observations in whole tissue-related experiments, particularly involving epigenetic gene body marks, it has been shown that these marks can be anticorrelated with expressed genes during aging (Pu et al., 2015). Another conflicting example that could be influenced by cell composition was the observed results involving increased chromatin accessibility and decreased H3K27ac in whole cortex enhancers. Both marks are highly related with gene transcription and could share some degree of linear dependency due the influence of EE (Karlić et al., 2010; Klemm et al., 2019). However, a recent study might indicate the contrary, as changes in H3K27ac coverage do not influence ATAC-seq levels in the same genomic regions (Zhang et al., 2020). Moreover, we didn't observe overlap between the differential regions of both marks, supporting the hypothesis that changes due EE in both marks, could be totally independent. Even though, we cannot discard cell composition as a factor influencing those results. Future investigation using single cell/nuclei approaches is needed to better determine these epigenetic relationships due external factors such as EE. In fact, we have shown by cell-deconvolution the importance of other cell types which are often ignored in learning-memory studies. Additionally, EE-induced directional regulation particularly of epigenetic marks could reflect the discrepancy occurring upon cognitive stimulation, such as pruning and synaptic tuning, where both synaptic strength and loss are part of the learning process during postnatal development (Turrigiano, 2008; Stephan et al., 2012).

Furthermore, by applying Hi-C to neurons, we elucidated for the first time *intra-* and *inter-*chromosomal interactions sensitive to EE. Especially the mnemonic *inter-*chromosomal 3D conformation map with its major *inter-*chromosomal hub involving chromosomes 7 and 17 shows that the environmental stimulus EE affects local epigenomic regulation and chromatin interactions in a coordinated manner. EE-induced changes relate to both synapse strengthening and pruning genes, affecting cytoskeletal rearrangements and ECM associated genes (Wright and Harding, 2009; Smagin et al., 2018). These synaptic rearrangements need pathways, such as Rho, GPCR, and PKC/Akt signaling which we found enriched in our study, with special enrichment of Wnt signaling (Hu et al., 2013; Lichti et al., 2014; Tan et al., 2017).

Our results indicate that environmental cues in postnatal development, particularly stimulation provided by EE, modulates

epigenomic and 3D genome landscapes in a coordinated manner relevant for cognition.

## MATERIALS AND METHODS

### Experimental Procedures

#### Animals: Housing and Enrichment Conditions

All experimental procedures were approved by the local ethical committee (Procedure Code: ISA-11-1358). Wild type mice (C57BL/6J) and Tg(Thy1-YFP) [strain B6.Cg-Tg(Thy1-YFP)2Jrs/J No. 003782; The Jackson Laboratories] were kept and bred according to local (Catalan law 5/1995 and Decrees 214/97, 32/2007) and European regulations (EU directives 86/609 and 2001-486).

After weaning (21 days of age), mice were randomly reared under either non-enriched (NE) or enriched (EE) conditions for 30 days. In NE conditions, animals were reared in conventional Plexiglas cages (20 × 12 × 12 cm height) in groups of two to three animals. The EE group was reared in spacious (55 × 80 × 50 cm height) Plexiglas cages with toys, small houses, tunnels, and platforms. The arrangement was changed every 3 days to maintain the novelty of the environment. To stimulate social interactions, six to eight mice were housed in each EE cage. All groups of animals were maintained under the same 12 h (8:00 to 20:00) light-dark cycle in controlled environmental conditions of humidity (60%) and temperature (22°C), with free access to food and water. The experiments were conducted using only females, since male mice showed hierarchical behavior similar to that observed previously that may affect the outcome of EE (Martínez-Cué et al., 2002). To stimulate social interactions, six to eight mice were housed in each EE cage. The use of female mice allowed to mix different mice in the same cage from different litters.

Cortical data analysis derived from the same samples and EE protocol that was used by Pons-Espinal et al. (2013) and PhD thesis "Role of DYRK1A in hippocampal neuroplasticity: Implications for Down syndrome<sup>1</sup>," where behavioral studies (Morris water maze testing) showed successful EE treatment. At 5 (P28) or 8 weeks of age (P51), mice were euthanized by carbon dioxide, and the cerebral cortex was dissected within 1 min of death. The tissue was immediately flash frozen in liquid nitrogen. In the case of Tg(Thy1-YFP) animals, the tissue was immersed in Hank's Balanced Salt Solution (HBSS 1X, Gibco 14065-049) before proceeding with the sample preparation for the FAC-sorting (see section below). For each condition and replicate, we pooled the cortices of five mice with some exceptions: mice for *insitu*-HiC where single replicas as well as Thy-YFP mice for ATACseq ( $N_{\text{cortex}} = 20$  mice in 4 bioreplicates;  $N_{\text{sorted}} = 30$  mice in 6 bioreplicates,  $N_{\text{NeuN+}_\text{HiC}} = 4$  mice in 4 bioreplicates,  $N_{\text{Thy+}} = 4$  mice in 4 bioreplicates). The frozen cortices for pooled animals were ground together in a frozen mortar containing liquid nitrogen, to obtain a fine powder of pooled cortex tissue. The powder was aliquoted and stored at  $-80^\circ\text{C}$ .

<sup>1</sup>[www.tdx.cat/handle/10803/124485#page=1](http://www.tdx.cat/handle/10803/124485#page=1)

## Nucleic Acid Extraction

DNA was extracted using Phenol:chloroform:iso-amyl alcohol (25:24:1) according to manufacturer guidelines (Sigma 77617-500ml). RNA was extracted using Qiazol total RNA (Qiagen Cat No:79306) kit according to the manufacturer's instructions. The RNA was quantified by Qubit® 2.0 Fluorometer (Life Technologies) and the quality was assessed using a Nanodrop 2000c (Thermo Fisher Scientific) and a 2100 Bioanalyzer (Agilent Technologies, CA, United States).

## Nucleus Isolation

To obtain fresh nuclei, ground frozen tissue was resuspended in tissue lysis buffer (1x PBS containing 0.1% Triton X-100, 1x Complete Protease inhibitor cocktail tablette (cCOMPLETE mini EDTA-free, Roche Cat No. 11836170001) and 1 mg/ml AEBSF (Pefabloc, Roche Cat No. 11585916001) and dissociated by 60–90 strokes in a glass douncer (7 ml tissue grinder Tenbroek, Wheaton Cat No. 357424). Nuclei were counted using a hemacytometer and constantly checked under a microscope (Leica DM-IL).

## FAC-Sorting

Two different procedure were performed: (1) Sorting total neurons using NeuN (Rbfox3) marker and (2) Sorting pyramidal neurons in Tg(Thy1-YFP) mice. Briefly, after the nucleus preparation, nuclei were resuspended in 1 ml of PBS-PI 1X [1X-PBS, 1x Complete Protease inhibitor cocktail (cCOMPLETE mini EDTA-free, Roche Cat No. **11836170001**), 1 mg/ml AEBSF Pefabloc (Pefabloc, Roche Cat No. 11585916001) and 0.1 mg/ml of BSA (BSA, Molecular Biology Grade NEB, Cat No. B9000S)]. 1.5  $\mu$ l of anti-NeuN, clone A60, Alexa Fluor® 555 Conjugate (Merk Millipore Cat No. MAB377A5) was added to the solution and incubated at 4°C for 1.5 h protected from light. The sample was centrifuged for 10 min, 500 g at 4°C and washed with 1 ml of PBS-PI 1X. Next, 1  $\mu$ l of 4',6-Diamidino-2'-phenylindole dihydrochloride was added (DAPI, Roche Cat. No. 10236276001) and the sample was given immediately to the FACS-sorting Facility. Samples were sorted at 12PSI in cold condition in an INFLUX sorter (BD Biosciences INFLUX™). The sorted samples were centrifuged for 40 min at 700 g at 4°C to collect the nuclei before proceeding with the desired technique. For sorting pyramidal neurons Tg(Thy1-YFP), animals were dissected and tissue was immediately submerged in Hank's Balanced Salt Solution (HBSS 1X, Gibco 14065-049). Brain samples were dissociated using the Neural Dissociation Kit (P) (MACS Milteny Biotec Cat. No. 130-092-628; LS Columns Cat. No 130-042-401; Myelin removal beads Cat No. 130-096-731; MidiMACS™ separator Cat. No. 130-042-302), according to manufacturers' instructions. Cells were sorted using an INFLUX™ sorter (BD Biosciences INFLUX™). After sorting, samples were centrifuged for 40 min at 700 g at 4°C to collect the nuclei before proceeding with the desired technique.

## Whole Genome Bisulfite-Sequencing

WGBS was performed by CNAG Genome Facility on two independent sets of biological replicates. Briefly, genomic DNA (1–2  $\mu$ g) was spiked with unmethylated  $\lambda$  DNA (5 ng of  $\lambda$  DNA

per microgram of genomic DNA; Promega). DNA was sheared by sonication to 50–500 bp in size using a Covaris E220 sonicator, and fragments of 150–300 bp were selected using AMPure XP beads (Agencourt Bioscience). Genomic DNA libraries were constructed using the Illumina TruSeq Sample Preparation kit following Illumina's standard protocol. DNA was treated with sodium bisulfite after adaptor ligation, using the EpiTaxy Bisulfite kit (Qiagen), following the manufacturer's instructions for formalin-fixed, paraffin-embedded tissue samples. Two rounds of bisulfite conversion were performed to ensure a conversion rate of >99%. Enrichment for adaptor-ligated DNA was carried out through seven PCR cycles using PfuTurboC<sub>x</sub> Hot-Start DNA polymerase (Stratagene). Library quality was monitored using the Agilent 2100 Bioanalyzer, and the concentration was determined by quantitative PCR with the library quantification kit from Kapa Biosystems. Paired-end DNA sequencing (2  $\times$  100 bp) was then performed using the Illumina HiSeq 2000 platform.

## Chromatin Accessibility

Open chromatin studies were performed by ATAC-seq and SONO-seq procedures. Briefly, ATAC-seq was performed with minor modifications from Buenrostro et al. (2013). 100,000 nuclei were treated with 2.5  $\mu$ l Tn5 at 37°C for 30 min, followed by cleanup on a Qiagen Minelute column. Fragments >1 kb in size were removed using AmpureXP beads (Agencourt AMPure XP, Beckman Coulter Cat. No. A63881). DNA fragments were amplified by 11 cycles of PCR with custom adapter primers from Buenrostro et al. (2013). PCR reactions were cleaned up with AmpureXP beads, quantified by Qubit and quality controlled by Bioanalyzer (Agilent Technologies). SONO-seq consists of isolating and fragmenting crosslinked chromatin, before reversing crosslinks, purifying the DNA and preparing it for sequencing (Auerbach et al., 2009). Chromatin was fragmented by sonication using the same COVARIS specifications as for ChIP-seq (see above) to obtain a median fragment size of 200 bp. Sequencing libraries were prepared using the NEBNext Ultra (New England Cat. No. E7370L) kit according to the manufacturer's protocol. Both techniques were sequenced on a HiSeq2000 sequencer (Illumina).

## Chromatin Immunoprecipitation: ChIP-Seq

Nuclei obtained in section "Nucleus Isolation" were cross-linked with 0.5% formaldehyde (Sigma F8775-25ml) for 5 min at room temperature (RT). Residual formaldehyde was quenched by addition of glycine (MAGnify™ Glycine P/N 100006373) to a final concentration of 0.125 M and incubation for 5 min at RT. Nuclei were pelleted by centrifugation at 500 g during 10 min at 4°C and resuspended in 300  $\mu$ l lysis buffer (MAGnify™ Chromatin Immunoprecipitation System, Cat no. 49-2024). Chromatin was fragmented by sonication in a Covaris S2 [Duty Cycle: 20, Intensity: 8, Cycles per Burst:200, for 15 min (histone marks), for 25 min (FACS-sorted nuclei)] to a median size of 200 bp, aliquoted and stored at –80°C until further use. For non-histonic proteins such CTCF, no nuclei preparation was performed. Homogenized tissue was crosslinked with 0.5% formaldehyde for 10 min at RT, quenched and fragmented as above (Duty Cycle: 5, Intensity: 2, Cycles per Burst: 200, Time:

25 min). Chromatin immunoprecipitation was performed using antibodies against histone modifications (H3K27ac, H3K4me3, H3K4me1, H3K79me2, H3K36me3, H3K27me3, H3K9me2, and CTCF) with the MAGnify™ Chromatin Immunoprecipitation System (Invitrogen Cat no. 49-2024), according to manufacturer's instructions. For whole cortex and NeuN histonic ChIP-seq a total amount of 50 k nuclei was used per ChIP (~330 ng), 700 k nuclei (~4 µg) for non-histonic ChIP-seq. Recovered ChIP DNA was used to construct sequencing libraries, using the NEBNext Ultra (New England Cat. No. E7370L) kit according to the manufacturer's protocol, and sequenced on a HiSeq2000 sequencer (Illumina). The quality of the ChIP-seq was determined by qPCR, using positive and negative primers to detect the regions where the histones should be placed in the genome (**Supplementary Table 1**). Primers were diluted to a final concentration of 300 ng in Power SYBR Green PCR MM (Applied Biosystems Cat. No. 4367659). Samples were run in a Applied Biosystem qPCR system (7900 HT Fast Real-Time PCR System) as follows: 50°C/2 min, 95°C/10 min, 40 cycles of 95°C/15 s, 60°C/1 min, 95°C/15 s, 60°C-15 s, and 95–15 s.

### Transcriptomics

Transcriptome study involved both poly-A RNA, directional RNA and small RNA libraries. Poly-A RNA sequencing libraries were prepared from total RNA using the TruSeq™ RNA sample preparation kit (Illumina Inc., Cat. No. RS-122-2001) according to the manufacturer's protocol. Directional RNA libraries were prepared using the ScriptSeq™ Complete Gold Kit (Human/Mouse/Rat) (Epicentre Biotechnologies), according to the manufacturer's protocol. Briefly, 3 µg of total RNA were depleted of both cytoplasmic and mitochondrial rRNAs using the Ribo-Zero™ Gold rRNA Removal Reagents. The total rRNA depletion of the samples was confirmed on a 2100 Bioanalyzer RNA 6000 Pico Chip. For the library preparation we used 50 ng of Ribo-Zero-treated RNA as starting material for the ScriptSeq™ v2 RNA-Seq Library Preparation Kit, followed by amplification by 12 cycles of PCR, using the FailSafe™ PCR Enzyme Mix (Epicentre Biotechnologies) before purification with AMPure XP beads (Agencourt, Beckman Coulter Cat. No. A63881). Both the directional mRNA and the Poly-A RNA libraries were sequenced in paired end mode with read length 2 × 101 bp on a HiSeq2000 (Illumina, Inc.) following the manufacturer's protocol. After computational analysis, we validated 21 differential expressed genes in a new batch of biological replicates following the method of Schmittgen and Livak 2008 (see **Supplementary Table 4**). To validate the data generated in the RNA-seq analysis, 1 µg of the sequenced RNAs were used to prepare cDNA with SuperScriptIII (Invitrogen) according to manufacturer's instructions, cDNAs were normalized to 100 ng/µl. RT-PCRs for the alternative splicing events were performed using oligos annealing to the adjacent constitutive exons and performed under standard conditions; 2% agarose gels were used to resolve the different bands. Image J software was used for quantification of the observed bands and determination of the PSIs for each event. Small RNA libraries were generated using the TruSeq (Illumina, Cat. No. RS-122-2001) kit according to the manufacturer's protocol. The resulting 22 bp insert libraries were sequenced on

a HiSeq2000 sequencer (Illumina), yielding 15–20 million reads per sample. After the analysis, we validated 6 out of 10 miRNA in a second independent group of animals, following Chen et al. protocol with minor modifications: instead of using Taqman probes we designed our own RT-miRNA oligonucleotides and performed qPCRs with Power SYBR Green PCR MM (Applied Biosystems Cat. No. 4367659, see **Supplementary Table 5**; Chen et al., 2005).

### Proteomics

Samples were minced with RIPA-M buffer (1% NP40, 1% Sodium deoxycholate, 0.15 M NaCl, 0.001 M EDTA, 0.05 TrisHCl pH = 7.5, 1X cCOMPLETE Mini EDTA free, 0.01 M NaF, 0.01 M Sodium pyrophosphate, 0.005 M β-glycerolphosphate), sonicated with a Diagenode Bioruptor (cycles of 0.5 min ON 0.5 min OFF, medium intensity during 5 min). Samples were centrifuged during 10 min 16,000 rpm at 4°C and precipitated with acetone at -20°C for 1 hour. Samples were pelleted by centrifugation during 10 min 16,000 rpm at 4°C, dried and resuspended in Urea/200 mM ABC, sonicated again during 10 min (cycles of 0.5 min ON 0.5 min OFF, medium intensity) and quantified prior to mass spectrophotometry injection following isobaric tags for relative and absolute quantitation (iTRAQ) or Liquid Chromatography/Mass-Spectrophotometry (LC/MS).

### In situ-HiC

Cerebral cortex samples from individual C57BL/6J mice (2 bio-replicates per EE and CTL conditions) were sorted using NeuN<sup>+</sup> (Rbfox3+) as described above. After sorting, approximately 1 million of nuclei were used for in situ HiC following previous specifications (Rao et al., 2014). Libraries were sequenced on a HiSeq2000 (PE × 125 bp) yielding approximately 300 M of reads per sample.

## Quantification and Statistical Analysis

### In silico Identification of Active Enhancers

Active enhancers for EE and CTL cortex were predicted and annotated using a machine learning approach called Generalized Enhancer Predictor (GEP)<sup>2</sup> (Jhanwar et al., 2018). The method performed classification of epigenetic patterns coming from cortical ATAC-seq, H3K4me1, H3K4me3, H3K27ac, H3K36me3, and H3K27me3 using Random Forest (RF) and Support Vector Machine (SVM) classifiers to build predictive models for identification of active enhancers. The total amount of enhancers used in the present study corresponded to the consensus of GEP prediction for EE, control and ENCODE data, resulting in 347112 enhancers (**Supplementary Table 1**).

### In silico Prediction of Chromatin Interactions

Chromatin interactions were predicted from chromatin modifications using EpiTensor with minor modifications to adapt the script to the mouse genome (Zhu et al., 2016). We used epigenetic data from following tissues from the mouse ENCODE project (forebrain, heart, hindbrain, kidney, liver, lung, midbrain, stomach) (Yue et al., 2014). As well as in-house generated

<sup>2</sup><https://github.com/ShaluJhanwar/GEP>

data from cortex of animals with and without environmental enrichment. We used the following epigenetic marks: H3K9me3, H3K4me3, H3K4me1, H3K36me3, H3K27me3, H3K27ac, CTCF as well as RNA-seq coverage data. Chromatin accessibility was measured by DNase-seq for ENCODE tissues and ATAC-seq for in-house cortex samples. Promoter regions were defined using the *TxDb.Mmusculus.UCSC.mm10.knownGene* package in BioConductor as 1,500 up- and 500 down-stream of any possible TSS. Active enhancers in each of the input tissues were defined by training a machine learning classifier on a list of validated enhancers using core epigenetic mark intensities as features (DNase, H3K27ac, H3K27me3, H3K36me3, H4K4me1, H3K4me3 as well as intensity ratio between the last two marks). The trained classifier was then applied to the epigenetic data from ENCODE tissues and local cortex datasets. To limit computation load, possible interactions were limited to intra-TAD interactions, which were based on the set of mouse cortex TADs<sup>3</sup> (Dixon et al., 2012). Differential activity in enhancers was annotated using *annotate.enhancers.with.genes.sh* utility<sup>4</sup>.

### Whole Genome Bisulfite-Sequencing

Methylated CpGs were called from the raw reads using Bismark (Krueger and Andrews, 2011) following the user guide. Differential methylation analysis was carried out using bsseq (Hansen et al., 2012). Briefly, CpG methylation values were locally smoothed using the BSmooth function and CpGs that had a coverage of less than 5 reads in any of the samples were removed. We calculated the t statistic for the smoothed CpG values using the BSmooth.stat function with paired design in local correction mode and the dmrFinder function was used to identify differentially methylated regions (DMRs) that contained multiple CpGs with a t statistic below -4.5 or above 4.5. DMRs located inside or within 5 kb of a gene or enhancer were annotated accordingly. Additionally, DMRs that overlapped enhancers, were annotated with the enhancer's target gene according to the EpiTensor predictions.

### Chromatin Accessibility

ATAC-seq libraries were aligned to mm10 using bwa-mem with predefined parameters (Li and Durbin, 2010). Duplicate read pairs were marked using the *MarkDuplicates* command from the Picard software suite<sup>5</sup>. We used a peak-independent approach on the one hand using our predicted enhancers and promoters as defined above, and on the other hand a peak-dependent method using F-seq or MACS2 with default parameters with and without duplicates (Boyle et al., 2008; Zhang et al., 2008). We also used MACS2 with the shifted strategy with the following parameters: “-nomodel -shift -75 -extsize 150 -broad -keep-dup all.” Together with enhancer and promoter regions, each annotation was loaded into DiffBind (Ross-Innes et al., 2012) providing as background input SONO-seq chromatin (Ross-Innes et al., 2012). For SONO-seq libraries, the pipeline followed the same steps as the ChIP-seq (see below).

Footprinting analysis was done using the Centipede software using the core transcription factor binding motifs from the Jaspar database (version 2016-03-02) (Pique-Regi et al., 2011; Khan et al., 2018). Instances of each motif in the genome were kept if the PWM score was superior or equal to 13. We created and used a mm10 mappability file to filter out instances that are located in regions of the genome with low mappability (gem-mappability-retriever; Marco-Sola et al., 2012). A motif was considered bound if the *P*-value-Z-score-combined statistics was inferior to 0.05. A motif was considered differentially bound if the ANOVA *p*-value was inferior to 0.05. Individual instances of a motif were considered bound when their posterior probabilities were superior to 0.99.

### Chromatin Immunoprecipitation: ChIP-Seq

Samples were mapped to mouse mm10 (peak-independent) using Bowtie (Langmead et al., 2009) with “-quiet -sam -best -strata -m 1” parameters. Sam files were converted to bam files allowing only reads with mapping quality greater than 30 (“-q 30”). Files were visualized using bamCoverage utility from deepTools (Ramírez et al., 2016). We initially used a peak-dependent strategy as a benchmarking method and also as a tool to define our peak-independent approach (data not shown). The quality of ChIP-seq was assessed by the irreproducible discovery rate (IDR) (Li et al., 2011). The peak-independent method used in the present study consisted in the calculation of the coverage at defined regions as follows. The broad marks H3K36me3 and H3K79me2 were measured along the full gene body, defined as the distance from the TSS to the TES. Enhancer regions were provided by the GEP enhancer predictor in combination with ENCODE enhancers (Jhanwar et al., 2018; **Supplementary Table 1**). Promoter regions were defined as the interval from 1,500 bp upstream to 500bp downstream of the TSS. Reads were quantified by featurecounts (Liao et al., 2014) with the following parameters: “-ignoreDup -minReadOverlap 25 -Q 1 -O.” Counts tables were supplied to the batch effect corrector RUVseq for further differential analysis using edgeR (Robinson et al., 2010; Risso et al., 2014). A binned approach was used to determine differential changes in 1Mb bins to be associated with inter-chromosomal interactions hubs. This differential analysis was performed with Diffreps (Shen et al., 2013). Using the following parameters: “-pval 0.001 -frag 150 -window 1000.” Batch effects are a major issue in sequencing studies. In this study, we acknowledge the lack of bio-replicates in the cortical tissue by having a wide set of different techniques as well as a pooling strategy of animals per bio-replicate (5 individuals per sample). To minimize batch effects, we randomized the samples, applied standardized procedures and parallelized the experiments as much as possible. However, FAC-sorting experiments could not always be parallelized for different reasons (i.e., availability of the sorter). To remove batch effects, we decided to use the strategy of Russo et al. that was also used in data different from RNAseq (Risso et al., 2014; Koberstein et al., 2018). A principal component analysis was the main criterion to evaluate each dataset and the requirement for batch correction. If a PCA on uncorrected data could clearly separate the conditions, we ruled that there was no need for batch correction. However,

<sup>3</sup><http://chromosome.sdsc.edu/mouse/hi-c/download.html>

<sup>4</sup><https://github.com/ophiothrix/enhancer.annotator>

<sup>5</sup><http://broadinstitute.github.io/picard>

if required, we then selected the method(s) (among RUVg, RUVs, and/or RUVr) that were able to separate conditions and intersected the results with a FDR threshold of 0.1 to obtain a conservative final gene list of changes induced by EE. Coverage plots were produced using the function `bamCoverage` of `deepTools` to produce bigwig files (Ramírez et al., 2016) (v2.0 with Python 2.7.14). These files were supplied to the function `computeMatrix` using the “scale-regions” parameter that allows to plot the coverage profile using the `plotProfile` function along regions of interest. The cell-specificity of sorted populations was assessed by the tool `MakerGeneProfile` that estimates cell specificity using a curated single-cell mouse brain RNAseq database<sup>6</sup>. We transformed gene-body assigned reads of H3K79me2 NeuN<sup>+</sup> and NeuN<sup>-</sup> into RPKM values and ran `MakerGeneProfile` to compare neuronal and non-neuronal enrichments to oligodendrocytes, astrocytes and pyramidal neurons specific markers. MGP states for `MakerGeneProfile` and it is described as a correlate value of cell type proportions. Specifically, MGP scores consist in the summarized expression profiles into the first principal component to estimate cell ratios (Mancarci et al., 2017). Gene ontology term enrichment analysis was performed using the Cytoscape tools `clueGO` and `cluepedia`, `Metascape` and `SynGO` (Bindea et al., 2009; Koopmans et al., 2019; Zhou et al., 2019). We performed the analysis individually for each histone mark or together, supplying upregulated and downregulated DBS separately. We used Bonferroni step down or Benjamini-Hochberg with  $p$ -value thresholds of 0.05 or 0.01 depending of the amount of data provided. In general, larger datasets needed more stringency in the statistical correction (Benjamini and Hochberg, 1995).

## Transcriptomics

mRNA reads were aligned to mm10 using STAR with standard parameters (Dobin et al., 2013). Reads were counted using `featureCounts` by “gene\_name” (Liao et al., 2014), batch corrected by RUV-seq before differential analysis in `edgeR` (Robinson et al., 2010; Risso et al., 2014). We also provide a splicing analysis results compiled in **Supplementary Table 4**. To identify and quantify alternative splicing, we used *vast-tools v1.0.0* (Tapial et al., 2017). To increase effective read coverage at splice junctions, we pooled all replicates for polyA, respectively ribominus, samples using *vast-tools merge*. Differential splicing analysis was performed using *vast-tools compare*, comparing EE with Ctl samples in a paired manner, using the parameters `-min_dPSI 10 -min_range 5 -p_IR -noVLOW`. This resulted in, respectively, 40 and 53, cassette exons being up- or downregulated with EE (including 2 and 1 microexons; Irimia et al., 2014), 49 and 43 retained introns, 27 and 22 alternative 3'ss choices and 28 and 23 alternative 5'ss splice choices.

Regarding the lncRNA analysis, total RNA reads were aligned to mm10 using `Subjunc` splice-aware aligner with default settings (Liao et al., 2013), and reads overlapping exons were summarized at the gene-level to the corresponding genes using `featureCounts` (Liao et al., 2014). Read assignment to exons was carried out in a strand-aware manner, only fragments with both mates correctly aligned were considered and genomic regions with multiple

overlapping exons on the same strand were disregarded. The count matrix was further filtered to retain only GENCODE long non-coding RNA (GRCm38.p5\_M15). Reads with an average  $\log_2$  CPM < 0.9 across all samples were filtered out yielding 4,472 genes. Normalization factors were calculated using the TMM method from `edgeR` package (Robinson et al., 2010). Observational-level weights were calculated using `voom` (Law et al., 2014) and used to fit gene-wise linear models (Smyth, 2004). Multiple testing  $p$ -value adjustment was performed using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

Small RNA reads with homo-polymer and low PHRED scores were removed using `FASTQ-Toolkit` and a custom script. `SeqBuster` was used to remove adapters and align using `miraligner.jar` with mouse miRbase v18 annotation (Pantano et al., 2010). The small RNA dataset presented a strong batch effect which could not be corrected by RUVseq (Risso et al., 2014). We therefore devised an alternative strategy which consisted of filtering microRNAs for low read coverage (<50 counts), normalizing the libraries by read counts per million (rpm), before contrasting EE against CTL batch-wise and considering exclusively reproducible direction of change. Additionally, we calculated z-scores validating partially previous strategy (**Supplementary Table 5**).

## Proteomics

The analysis of iTRAQ data consisted in sorting the discrepancy ( $\delta = \varphi/\beta$ ) between both biological contrasts ( $\varphi = EE1/CTL1$  and  $\beta = EE2/CTL2$ ), following the premise that consistent results will be indicated by:

$$\lim \delta = 1$$

$$\varphi \leftrightarrow \beta$$

Discrepant results then will be considered as values far from  $\delta = 1$ . Based on this consideration we select all the proteins that follow the condition and we established a threshold of  $\pm 0.1$ . The protein expression values from the LC/MS were  $\log_2$ -transformed and loess normalized using the *normalize.ExpressionSet.loess* function from the BioConductor package `AffyPML`. Differential expression analysis was conducted with a standard `limma` (BioConductor) pipeline by calculating sample weights, fitting a linear model for each gene across all samples and calculating moderated F-statistics. Unadjusted  $p$ -values were used to rank the proteins (Smyth, 2004).

## In situ Hi-C

For quality check, sequencing reads were mapped to the mouse reference genome assembly (mm10), artifacts were filtered, and library was ICED normalized using the Hi-C-Pro (Servant et al., 2015) (v2.9.0) (**Supplementary Table 7**). For visualization we used the *hicpro2juicebox.sh* utility to convert the previous into a \*.hic format to visualize heatmap interaction matrices in Juicebox (Durand et al., 2016a). `Juicer_tools` were used to calculate A/B compartments using eigenvector utility (Durand et al., 2016b). We called chromatin loops with HICUP at 5 and 10 kb of resolution (Durand et al., 2016a). For differential analysis, resulting interactions from Hi-C-Pro were splitted into intra and interchromosomal interactions, sex chromosomes and self-interacting bins were removed. Then piped into the `edgeR`

<sup>6</sup><https://github.com/PavlidisLab/markerGeneProfile>

wrapper RUVseq were RUVr for *intra* and RUVg for *inter* were used to normalized batch effects.

Required EE and CTL track file to run chrom3D (Paulsen et al., 2017) was produced using the chrom3D wrapper `automat_chrom3D` utility<sup>7</sup>. Domains were called using Arrowhead (Juicer tools 1.7.6; Durand et al., 2016b). “`-ignore_sparsity`” parameter was used, and calls could be only produced at not lower than 10 kb. For the present study, we finally selected 5 M iterations including the parameter “`-nucleus`” to force the beads to remain confined inside the designed radius: “`-r 3.0`.” Domain coloring was produced by `automat_color`<sup>8</sup> that allows to color any region of interest in the model. Both *in silico* models are available **Supplementary Table 7**.

## Data Integration

We used Metascape to intersect the totality of the data. Some of the results were clumped as the maximum amount of sets allowed is 30 (Zhou et al., 2019). Transcriptomic and proteomic changes percentages explain by the rest of the data were calculated out of the evidences table reported as Metascape result (**Supplementary Table 8**).

Linear dependency test was performed by using Pearson and Spearman correlations to test how enhancer and promoter activity of different marks influence differential changes observed in the transcriptome and proteome. Briefly, normalized counts by RPKM mapping into enhancers and promoters were first averaged by target gene name they interact with using EpiTensor (Zhu et al., 2016). Then a fold change was calculated of EE vs. CTL samples. These values then are correlated with the corresponding fold changes found in differential RNA-seq and proteomic analysis. We used Spearman for proteomic changes as the scale of the fold changes were considerably different coming directly from iTRAQ data and the method is less sensitive for these outliers.

In order to test the association of differential regions induced by EE with *inter*-chromosomal interactions we run a permutation analysis using the package *regionR*. Both permutations shown in the study were performed with a total number of 100,000 iterations. GWAS brain trait studies are collected in detail in **Supplementary Table 8**.

## DATA AVAILABILITY STATEMENT

Datasets are currently accessible in the SRA repository: <https://www.ncbi.nlm.nih.gov/sra/SRP154319>. Processed data and other resources can be found in the dedicated website: [www.mousecortexregulome.org](http://www.mousecortexregulome.org).

## ETHICS STATEMENT

The animal study was reviewed and approved by Comité Ético de Experimentación Animal del PRBB (Procedure Code: ISA-11-1358).

<sup>7</sup>[https://github.com/sespesogil/automat\\_chrom3D](https://github.com/sespesogil/automat_chrom3D)

<sup>8</sup>[https://github.com/sespesogil/automat\\_chrom3D\\_colors](https://github.com/sespesogil/automat_chrom3D_colors)

## AUTHOR CONTRIBUTIONS

SE-G performed most of the experimental procedures (sorted ATAC-seq samples, all ChIP-seq, RNA-seq and miRNA validations, *in situ* HiC, and proteome preparation for LS/MS), carried out most of the analysis (ATAC-seq, ChIP-seq, RNA-seq, miRNA, proteome, and *in situ* HiC), and wrote the manuscript. CNH performed part of the experimental procedures (bisulfite-seq, cortex homogenate ATAC-seq, RNA-seq preparation, and proteome preparation for iTRAQ). AH performed the bisulfite-seq analysis, differential lncRNAs analysis, and run EpiTensor. SB and RP-R performed the footprint analysis. SJ run the GEP enhancer predictor analysis. SC performed Diffreps and epigenetic relationship analysis with HiC datasets. JA-R and MP-E performed animal environmental enrichment procedures, breedings, and dissections. MM performed library preparations. MI carried out the splicing analysis. JP did the validation. MF effectuated the miRNA mapping. SA, MD, PM, CNH, and SO worked in the elaboration of the manuscript and funded the study. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnmol.2021.664912/full#supplementary-material>

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