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EARLY-LIFE STRESS ALTERS POSTNATAL CHROMATIN ARCHITECTURE AND DEVELOPMENT IN A BRAIN REWARD REGION

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Princeton, NJ : Princeton University

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EARLY-LIFE STRESS ALTERS POSTNATAL CHROMATIN ARCHITECTURE AND DEVELOPMENT IN A BRAIN REWARD REGION

REBEKAH L. RASHFORD

A DISSERTATION

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ABSTRACT

This thesis presents work investigating how early-life stress (ELS) affects chromatin architecture and development. ELS sensitizes individuals to subsequent stressors, which increases lifetime risk for psychiatric disorders. ELS includes negative experiences such as abuse, neglect, loss of a caregiver, and survival of traumatic events such as exposure to violence or natural disaster. ELS impacts cellular and transcriptomic response to additional stress within the brain's reward circuitry. How early experiences encode such long-lasting effects is poorly understood but implicates the epigenetic response process, particularly at the level of long-lasting global chromatin architecture remodeling. Here, I describe how epigenetic memory processes could be influenced by ELS, present empirical work showing how chromatin architecture and development change after ELS, and briefly explore potential methods for determining the role individual chromatin regions may play in the ELS susceptibility phenomenon.

The empirical work focuses on the effect of ELS on the nucleus accumbens (NAc) — a key limbic brain region, with some discussion of other regions implicated in the reward pathway. Using leading-edge techniques, we find that early-life stress initially opens chromatin in stress-activated cells, suggesting epigenetic priming as a mechanism of stress sensitization. By adulthood, we observe chromatin remodeling throughout the NAc, indicating that the effects of stress are long-lasting and propagate across development into the broader cell population. We also find that deposition of H3K4me1 in NAc during early postnatal development is sufficient to mimic early-life stress and prime hypersensitivity to subsequent stress. We conclude that a

molecular memory of early-life stress is encoded at an epigenetic level through changes in chromatin architecture.

To complement the initial genome-wide manipulation studies, I have set the groundwork to target specific genomic loci for epigenetic suppression or priming by CRISPR dSaCas9-mediated epigenetic editing, *in vitro*. This approach was designed to utilize dSaCas9-effector complexes to target enhancers and other regulatory elements to impact chromatin architecture or gene expression, ultimately giving rise to resilience or susceptibility to future stress. While additional work is needed to optimize these systems, this approach has the potential to functionally test whether suppressing or priming specific genomic enhancers during development can promote resilience or sensitivity, respectively, to later stress.

Together, this work generates fundamental insights into how stress during key developmental periods increases sensitivity to future stress at an epigenetic level in the brain.

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There are many to whom I owe my deepest gratitude. Listing every person and how they've supported me would probably fill the length of this dissertation, so I will attempt my best to be brief.

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To my thesis committee, Drs. Lisa Boulanger, Liz Gould, and Sarah Kocher: being able to meet with and receive honest feedback from such brilliant people has shaped the direction of this thesis and I owe you thanks a million times over for taking the time to invest in my research and career goals for the past half decade. (Also, an all woman committee? Amazing.)

To my lab: I nearly don't have the words to describe this group of people who have consistently challenged me to be better, made me laugh harder, or supported me more enthusiastically on this journey. Each and every one of you means so much to me, and I can't wait to see what good is in store for you all. I must name Laura Warman Hirschfield, though, whose humor and support specifically got me through the last big push of experiments. I doubt I'll ever meet someone who can so perfectly bald a baby mouse with Nair cream as long as I live.

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Also: thank you to the mice that were sacrificed for this research.

"Give justice to the weak and the fatherless;

maintain the right of the afflicted and the destitute."

Psalm 82:3, Holy Bible, ESV

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CHAPTER 1: Epigenetic memory of ELS underlies risk for hypersensitivity to future stress

***Note:** This chapter will be adapted into a review article. As such, it functions not only in introducing the specific topics found in the empirical manuscript provided in Chapter 2, but also to describe and comment on a wider scope of this research area. Therefore, there are some references to and citations of the work shown in Chapter 2 throughout my discussions of recent and relevant literature.*

1.1 Introduction

Negative stress experiences have been linked to the onset of adverse psychological disorders, such as depression, anxiety, addiction, and more[1–3]. Early-life stress (ELS) during critical developmental periods has been extensively shown to increase risk for developing psychological disorders in adulthood upon experiencing further stress[1–3]. ELS is defined here as sustaining intense or chronic experiences including but not limited to physical, sexual, or emotional abuse, neglect, and/or loss of a caregiver. According to the United States Census Bureau, 1 in 6 children satisfy the definition of having experienced early life stress according to the census bureau[4]. Many studies over the last few decades have tried to understand the mechanism behind this increased risk of susceptibility to adult stress driven by ELS. Foundational work in this field has made strides in understanding how the HPA axis and the brain develop and are altered through studying behavior and gene expression. Over the last few years, however, the role of epigenetics and gene regulatory mechanism response networks has been of growing interest. Using rodent models of ELS and adult stress to study these molecular

underpinnings has provided better understanding of how increased likelihood of negative emotional and behavioral outcomes develop[2,3,5–7].

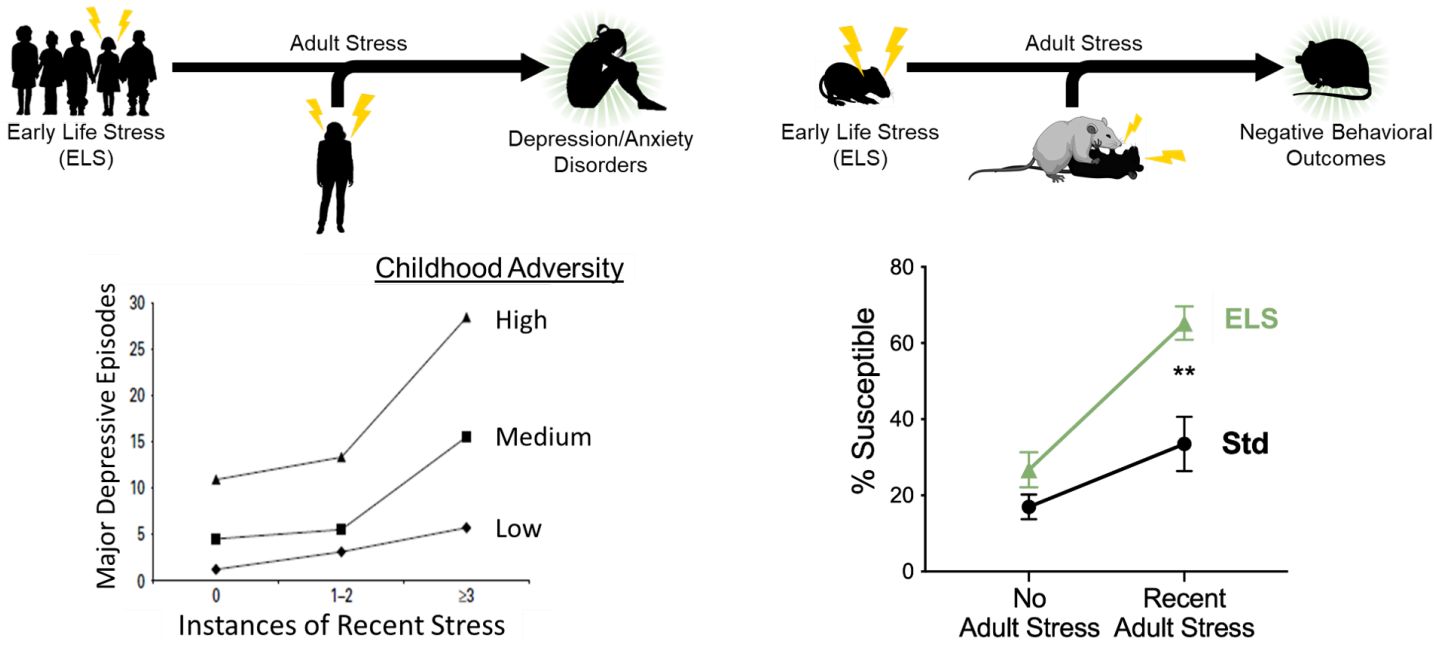


Figure 1 | ELS is associated with increased risk of mood disorder development in mice, mirroring human susceptibility. (Left) Adapted from McLaughlin et al (2010). Human data showing highest number of major depressive episodes impacted by combination of increasing recent stress exposure and childhood adversity experiences. (Right) Adapted from Peña et al (2019). Aggregate mouse behavioral data showing significant increase in behavioral susceptibility [measured with behavioral tests for presence of anxiety-like and depressive-like behaviors] with both ELS and recent (adult) stress exposure.

In the context of ELS, the use of rodent species to model and study its impact on the brain and other biological systems has become standard practice[2,3,8–10]. Data showing increases in behaviors characterized as avoidant and decreases in those characterized as social point to behavioral parallels mirroring the human profiles describing mood disorders (Figure 1)[1,11]. For both rats and mice, animals experiencing only ELS do not necessarily exhibit anxious- or depressive-like behaviors. Rather, it is when a second hit of stress (even a mild one) is sustained in adulthood that these negative behavioral outcomes are observed. This is referred to as the "two-hit" stress paradigm[2]. There are various experimental environments used to model ELS.

Peña et al showed that the use of maternal separation for at least one week during the critical developmental period of the first two weeks of life, along with limited nesting/bedding (with or without dam stressors to promote disorganized pup care) was the most effective at eliciting a susceptible behavioral profile[2]. Having animal models of this phenomena allows for in-depth investigation into the molecular mechanisms in the brain underlying the effect of ELS on predicting susceptibility to future stress.

This chapter focuses on the molecular changes concerning the epigenetic landscape after ELS that work to stabilize the underlying increased risk for psychological and behavioral disorders after additional stress. The ability for epigenetics to influence holistic response to future stress is founded in the concept of epigenetic memory. Epigenetic memory, as defined by D'Urso and Brickner in 2017, is the ability of an organism to more quickly adapt or respond to a repeated stimulus at a molecular level[12]. D'Urso and Bricker eloquently posited that the duration and epigenetic inheritance of memory requires transcription factors (TFs), changes in chromatin structure, and recognition or modulation of chromatin state by writer, reader, and eraser proteins[12].

The ultimate functional aspect of epigenetic memory is to modulate gene expression. This is impossible without chromatin dynamics. Chromatin is a term describing the complex formed by DNA and eight histone proteins, functioning to compactly package genetic material within a cell; it is often described as looking like "beads on a string"[13]. The genetic material found within regions of DNA become available for transcription based on the 3-dimensional conformations of chromatin at specific locations and physical accessibility to those locations by transcriptional machinery. Chromatin canonically takes on three architectural states: (1) closed,

which describes DNA and histones so tightly packed as to restrict gene expression (also known as “heterochromatin”), (2) open and active, describing loosely packed DNA and histones, which provides sites of active gene expression (also known as “euchromatin”), and (3) open chromatin, describing loosely packed DNA and histones, providing sites of *potential* gene expression, but not necessarily active (also known as “primed”). Primed chromatin is understood to be a signal-dependent state of chromatin[14]. This is key to the central idea of epigenetic priming. Initial stimuli functions to rearrange chromatin, inviting binding of specific transcription factors, coactivators/corepressors (enzymes responsible for histone modifications), and/or RNA PolII at enhancers and/or promoters. These elements may institute “pausing”, retaining the epigenetic landscape formed by the initial stimulus, leaving the cell vulnerable to further external stimuli[14]. Upon additional stimulus of the same type, the primed regions of chromatin are able to respond quickly to produce a transcriptional profile in response to the environment.

Overall, priming epigenetic response mechanisms through changes in TFs and histone modifiers to alter chromatin architecture and elicit aberrant gene expression in the brain is key to be able to correlate and predict hyperreactive responses when additional stress is experienced later in life. Here, primary literature is reviewed, highlighting these key and related aspects of epigenetic memory, specifically within the context of ELS. I start with transcription factors and histone modifications, as they are two key aspects of epigenetic control that influence the main focus of this thesis: the global chromatin state caused by ELS.

1.2 Histone modifications and modifying enzymes affected by ELS

Histone modifications and modifying enzymes can control accessibility of chromatin and therefore gene expression. As such, they likely play a role in both initial and repeated response to

stress and other stimuli. Chromatin state can be altered by addition or removal of methyl and acetyl groups, among other modifications (phosphorylation, etc). These reactions are catalyzed by histone methyltransferases (HMTs), histone demethylases (HDMs), histone acetyltransferases (HATs), and histone deacetylases (HDACs)[15].

The impact of ELS on histone modifications seems to generate a unique expression profile of various modifiers to either increase resilience or susceptibility[15]. There are a few notable specific modification states among these, however, that are probable drivers for modulation of behavioral outcomes. These are histone H4 acetylated at lysine residue K12 (H4K12ac), histone H3 di-methylated at lysine residue K27 (H3K27me2), and histone H3 monomethylated at lysine residue K4 (H3K4me1); all of which are impacted by ELS in lasting ways[5,6,15,16]. These are interesting modifications to highlight as they are functional in developmental pathways (H4K12ac), notably implicated in ELS in the reward pathway (H3K27me2), and specifically mark primed chromatin loci (H3K4me1). Out of all the histone proteins comprising the octamer, H3 and H4 are focal points for looking at functional affect to nucleosome positioning because they are the two proteins that have long protruding tails available for modification[15,17].

Concerning H4K12ac, Levine et al found that ELS (modeled by maternal separation) induced biphasic expression of acetylated H4K12 in the mPFC of Balb/c mice[5]. They reported an initial decrease in the modification at postnatal day (P) 21, but an increase by P60[5]. The same trend of initial decrease and later increase of H4K12ac has been confirmed in the NAc of C57BL/6J mice by mass spectrometry[6]. The biphasic expression trajectory in two regions of the reward pathway begets an interesting potential mechanism for response and susceptibility to

future stress after ELS. This modification is an important player in developmental pathways and storing molecular memories across the lifespan[5,18]. The initial decrease in H4K12ac could drive closing of developmental pathways as a molecular protective mechanism, accelerating the epigenetic landscape to adopt a more mature state. The later increase in and lasting expression of H4K12ac could aberrantly reopen developmental pathways and increase sensitivity to environmental stimuli, modulating susceptibility to future stress exposure. It remains an open question to discover the mechanism behind this shift in expression.

The modifications to residues on histone H3 have been shown to be interesting key players in modulating lasting increased sensitivity to future stress by ELS. In both the mPFC and the NAc, increased di-methylation of H3, notably at lysine K27, is observed in adulthood after ELS in a long-lasting way[6,8,19]. H3K4 methylation is also impacted by ELS. The gene *Mll3* is stably upregulated in mPFC ~1 month after initial ELS experience[15]. *Mll3* encodes KMT2C, a histone methyltransferase that deposits mono-, di-, and tri-methyl groups onto lysine 4 of histone 3 (H3K4meX) to regulate transcription via changes in chromatin structure[15].

Data in the NAc mirrors this finding[6]. Initially downregulated after ELS at P21, H3K4 methylation (mono-, di-, and tri-) increases in expression from P35 on[6]. Interestingly for female NAc, however, a continuation of the initial downregulation for H3K4 methylation throughout the lifespan is found[6]. To round out the impact of this modification in the reward pathway, stable H3K4 methylation, namely H3K4me1, is also found in the VTA after ELS[7]. H3K4 is monomethylated by the enzyme SETD7, a mark of poised enhancers [20]. Balouk et al found that overexpression of SETD7 early in life impacts susceptibility to future stress experienced in

adulthood. This showcases the lasting effect of histone modifications as a functional aspect in epigenetic memory of ELS[16,19].

While this section highlights specific histone residue modifications implicated in ELS epigenetic response, reactivation of the epigenetic memories facilitated by them most likely requires their ELS-driven alterations to be present and work in tandem. Future research should uncover how these concerted modifications maintain a poised epigenetic state in reward regions of the brain.

1.3 Transcription factors affected by ELS

Transcription factors (TFs) are a key aspect of the poised epigenetic state. The transcription factor can act as an anchoring signal to coactivators or corepressors so that upon further stimulation, gene transcription or inhibition can take place. Additionally, the expression of upstream TFs that work early in a response pathway may be modulated by various initial stimuli to signal downstream gene expression, preparing the cell for further stimulus response.

Peña et al reports that in the reward pathway (VTA, NAc, and medial PFC (mPFC)) ELS-affected gene promoters house motifs for TFs in pathways relating to development, immune response, and response to extracellular stimuli. As described and further discussed in Chapter 2, I also observe changes in TFs in these pathways (SMAD1, WT1, REST, and IRG3, among various others)[9,19]. In the case of stimulus and immune response pathways, these TFs may act as epigenetic memory initiators by binding to genes to either recruit initiation or repressor complexes. With a vulnerable, ELS-primed epigenetic landscape at regions implicit in these pathways, a second hit of stress could be activated to induce a transcriptional profile that disrupts an organism's ability to properly respond both transcriptionally (in the case of developmental and

immune pathways) and behaviorally (in the case of external stimulus response). Further insight into ELS-driven changes in overall chromatin architecture could aid in deepening our understanding of how TFs function in ELS response.

1.4 Chromatin remodeling after ELS could reflect epigenetic memory signature

Recent studies, along with those showing changes in TFs and histone modifications, show unique transcriptional activity profiles emerge after adult stress based on ELS history[9,21]. A requirement for this transcriptional activity is a unique primed chromatin architecture profile after ELS (but before adult stress). Chapter 2 of this thesis describes exactly this chromatin architecture change. We show that ELS can induce an epigenetic memory signature within the NAc in the face of dynamic changes within individual cells composing that region.

Using leading experimental and bioinformatic techniques, we posit that the nature of ELS-primed chromatin is a form of epigenetic memory. We also speculate on potential mechanisms behind the interesting finding that even with changes in individual cells over time, a number of the same genes and pathways in the NAc are affected to increase epigenetic vulnerability.

Overall, the epigenetic landscape maintained by ELS memory within cells of the reward pathway may provide the environment for heightened mood disorder risk later in life. Continued research may uncover the building blocks of this formation and activation process to lay the foundation for clinical therapeutics for alleviating or preventing negative outcomes for those who have experienced ELS.

CHAPTER 2: Early-life stress alters postnatal chromatin development in nucleus accumbens

This work has been adapted from the research article: *Early-life stress alters postnatal chromatin development in the nucleus accumbens* by Rebekah L. Rashford, Dr. Michael DeBerardine, Dr. Hye Ji J. Kim, Dr. Nibal Arzouni, Laura W. Hirschfield, Oluwamayowa M. Oke, Shannon N. Bennett, and Dr. Catherine Jensen Peña. All authors have consented to their work being presented in this dissertation. This article is currently under review at Nature, and is posted as a preprint on the bioRxiv server: https://www.biorxiv.org/content/10.1101/2024.04.12.589272v1

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Components of Chapter 2 have been presented in the following formats:

- Rebekah L. Rashford (PNI - talk (2024 + 2022))
- Rebekah L. Rashford (Park City 2024 - talk)
- Rebekah L. Rashford (Dev Bio Meeting 2023 - talk)
- Rebekah L. Rashford (SfN 2023 - talk, 2022 - poster)
- Rebekah L. Rashford (SBN 2021 - poster)

2.1 Abstract

Early-life stress sensitizes individuals to subsequent stressors to increase lifetime risk for psychiatric disorders. Within the nucleus accumbens (NAc) — a key limbic brain region — early-life stress sensitizes both cellular and transcriptional response to later stress. However, the molecular mechanisms linking initial activation of neurons by early-life stress with continued stress sensitivity across the lifespan are poorly understood. Using a combination of activity-dependent cellular tagging and ATAC-sequencing across postnatal development, we find that early-life stress initially opens chromatin in stress-activated cells and that chromatin opening predicts gene expression response to adult stress, suggesting epigenetic priming as a mechanism of stress sensitization. Moreover, early-life stress accelerates both chromatin development within these activated cells, and H3K4me1 deposition broadly in NAc — a post-translational histone modification associated with open chromatin and epigenetic priming. By adulthood, we observe chromatin remodeling throughout the NAc, indicating that the effects of stress are long-lasting and propagate across development into the broader cell population. Lastly, through viral-mediated epigenome editing and behavioral quantification, we find that deposition of H3K4me1 in NAc during early postnatal development is sufficient to mimic early-life stress and prime hypersensitivity to subsequent stress. Together, our results show that a molecular memory of early-life stress is encoded at an epigenetic level through changes in chromatin architecture. This constitutes a novel biological mechanism by which early-life stress programs lifelong stress sensitivity.

2.2 Introduction

Millions of children worldwide experience early-life stress (ELS), which increases lifetime risk for developing mood, anxiety, and substance use disorders [22–25]. ELS broadly encompasses many adverse experiences including maltreatment (neglect, and/or physical, sexual, and/or emotional abuse), death or loss of a caregiver, poverty, systemic racism, exposure to community violence, or experience of a natural disaster [26–30]. Studies in humans and preclinical animal models have shown that ELS sensitizes individuals to future stressors, which may be at the root of increased psychiatric disease risk [1,11,31–33].

ELS alters function and regulation of the nucleus accumbens (NAc), a ventral striatal brain region extensively implicated in the pathophysiology of psychiatric disease [3,34]. In children, ELS alters development and connectivity of the ventral striatum, and changes in NAc activity are associated with blunted reward processing, anhedonia, and heightened response to stressors [35–38]. At a cellular level, ensembles of NAc neurons initially activated by experience of ELS are hyper-reactive to stress across the lifespan, contributing to stress sensitivity in mice [7]. Interestingly, ELS-activated neurons are not hyperactive in the absence of additional stress. This indicates a latent hypersensitivity to stress which may be encoded at a molecular level within this ELS-responsive cell population [39].

At a transcriptional level, ELS induces both long-lasting changes to baseline gene expression, and latent gene expression changes revealed by a second hit of stress in adulthood [9], pointing to epigenetic regulatory mechanisms. While experience of ELS can alter histone variants and post-translational histone modifications in brain reward circuitry [6,16,17,40], it is unknown whether ELS indeed primes chromatin architecture in the NAc to facilitate or sensitize

transcriptional response to subsequent stressors. Here, we sought to determine whether ELS alters the chromatin landscape in ELS-activated cells during postnatal development, investigating the degree, timing, and endurance of such architectural changes, and whether such chromatin changes were functionally relevant for response to future stress. Our results provide novel insights into how the epigenome encodes long-lasting stress sensitivity.

2.3 Results

2.3.1 Tagging and capturing stress-responsive and non-responsive cells of the NAc

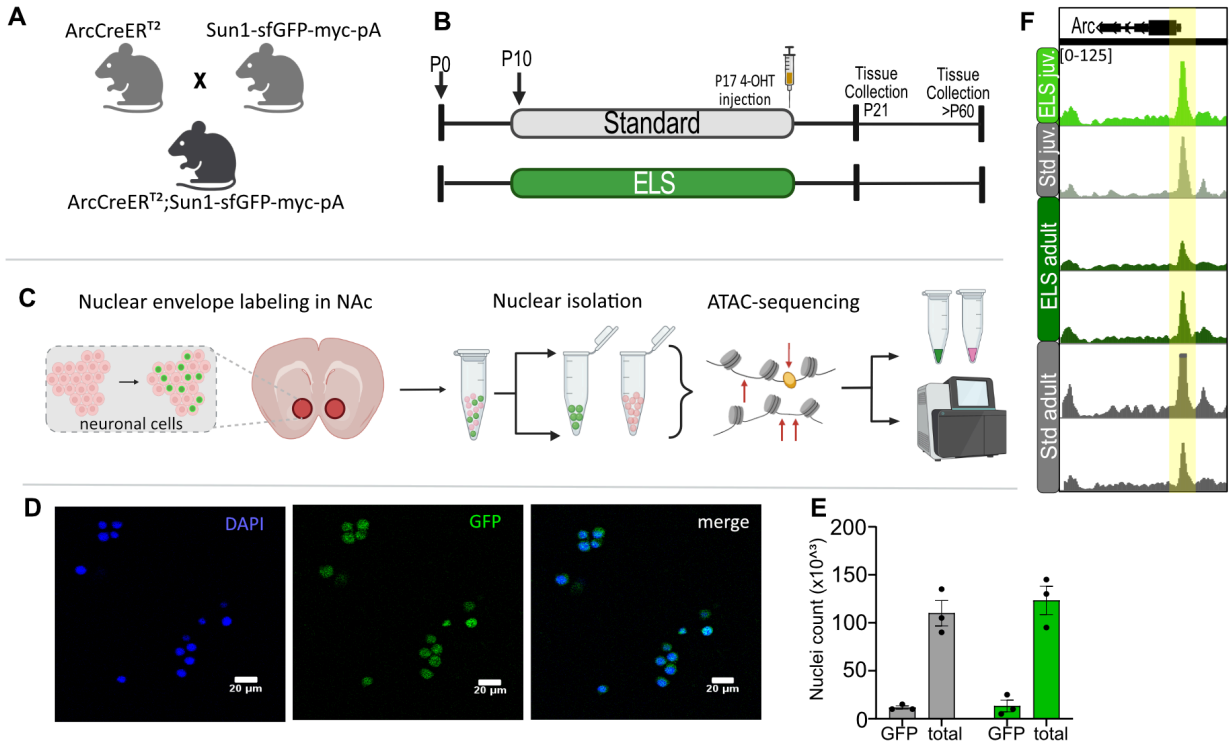


Figure 1 | Tagging and capturing stress-responsive and non-responsive cells of the NAc. (A) Breeding scheme of ArcCreERT2 x Sun1-sfGFP-Myc mice. (B) Experimental timeline for ELS from P10-17, 4-OHT -induced recombination at P17 in standard-reared (Std) and ELS pups, and tissue collection at either P21 or >P60 in adulthood. (C) Schematic of 4-OHT- and activity-dependent recombination and tagging of NAc neurons, followed by nuclear isolation, affinity-purification of tagged cells, and ATAC-sequencing and analysis in both tagged and untagged cells. (D) Example images of purified nuclei expressing SUN1-GFP in the nuclear membrane (scale bar 20 µm). (E) Average counts of total and affinity-purified (GFP) nuclei from standard-reared (gray) and ELS (green) samples. Error bars are mean±SEM. (F) Example browser tracks of ATAC peaks at the Arc promotor from experience-activated samples.

Given previous findings that ELS-activated cells in the NAc are preferentially reactivated by stress [7], we examined chromatin profiles in ELS-activated and non-activated cell populations separately. To permanently tag and isolate these separate cell populations, we utilized double transgenic ArcCreERT2 x Sun1-sfGFP-Myc mice (Figure 1A) in which transgene expression is both neuronal activity- and ligand-dependent [7,41]. ELS occurred in a stress sensitive window from postnatal day P10-17 as previously described [2,11]. Genetic recombination was induced on P17 by administration of 4-OHT either at the beginning of a period of maternal separation (ELS) or exploration of an enrichment object (standard-reared pups, Std) (Figure 1B). Permanent expression of nuclear membrane-localized SUN1-sfGFP-Myc (Figure 1C and D) enabled us to affinity-purify tagged nuclei within four days after recombination (P21) or later in adulthood (>P60; Figure 1B) by the INTACT method [42,43]. Both male and female samples were collected from each group at each age. For both Std and ELS groups, approximately 10% of nuclei in the NAc were tagged and captured (Figure 1E), consistent with previous count estimates of experience-activated cells by immunohistochemistry [7]. We then performed ATAC-sequencing (assay for transposase-accessible chromatin; [44,45]) in both tagged and untagged cell populations to characterize and compare open chromatin regions across treatment conditions and cell populations (Supplemental Table 1). We further validated that experience-activated cells have open chromatin peaks at the Arc gene promoter used to drive transgene expression, in both Std and ELS samples, thus demonstrating the potential for these cells to have been activated (Figure 1F).

2.3.2 ELS opens chromatin in experience-activated cells of the NAc following stress

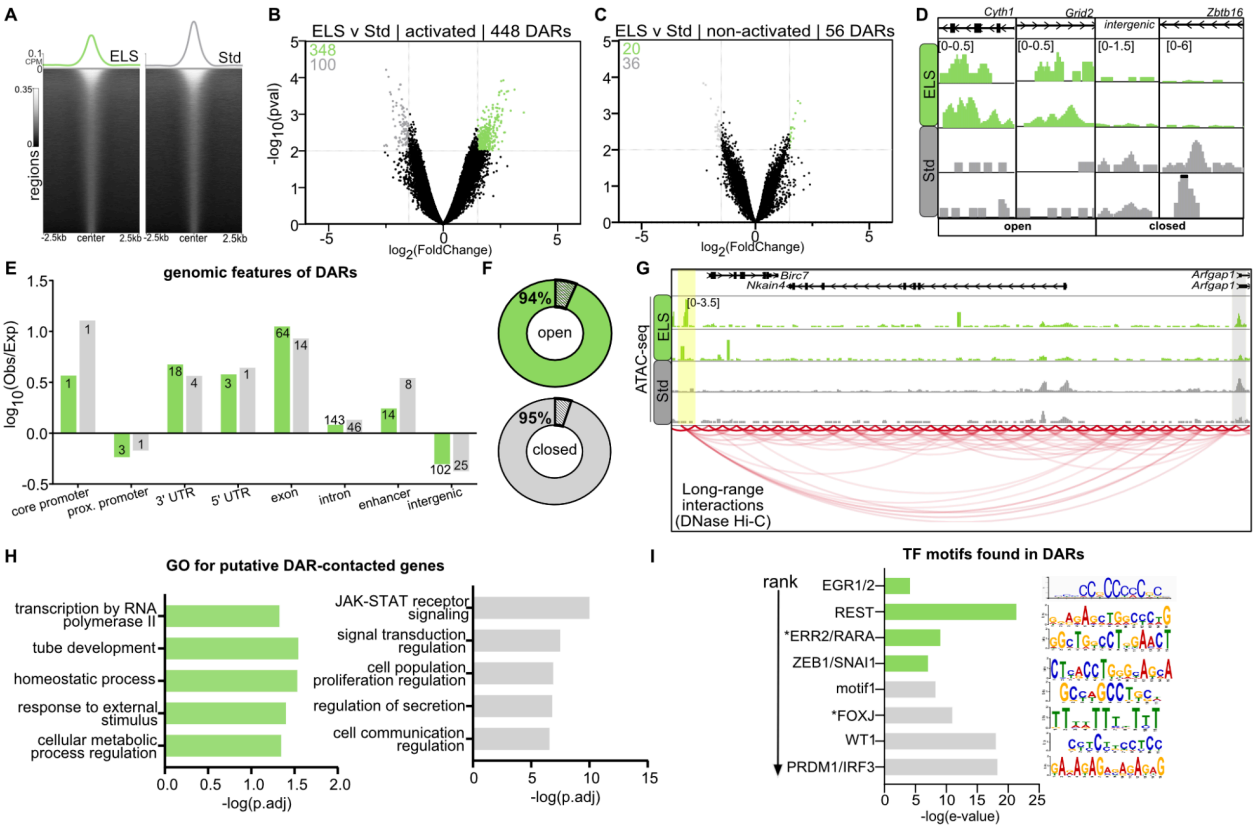


Figure 2 | ELS opens chromatin in experience-activated cells of the NAc following stress. (A) Line plots (top) and heatmaps (bottom) of mean chromatin accessibility CPM ±2.5 kb around center of all ATAC peaks within experience-activated cells. Volcano plot of ELS vs Std differentially accessible regions (DARs) within P21 experience-activated (B) and P21 non-activated (C) cells. Differentially accessible regions (DARs: edgeR, p-value <0.01 and |logFC|>1.5) opened or closed by ELS in are shown in green and gray, respectively. (D) Example browser tracks of ATAC peaks either opened or closed by ELS in P21 experience-activated cells, normalized to CPM. (E) Count and enrichment or depletion (log10 of observed over expected for each genomic feature in the mm10 genome) of DARs mapped to each genomic feature for regions opened (green) or closed (gray) by ELS within experience-activated samples. (F) Proportion of DARs in putative cis-regulatory elements (>1kb from the nearest TSS) for regions opened (green) or closed (gray) by ELS within experience-activated samples. (G) Example browser tracks and long-range interactions between an open DAR (yellow highlight) and a putative target gene promoter (gray highlight). (H) Gene Ontology (GO) biological process terms for genes putatively contacted by ELS-opened (green) or closed DARs. (I) Top transcription factor motifs found in open (green) and closed (gray) DARs ; * denotes motifs found in <60% of input regions.

Among experience-activated NAc cells, we identified 150,895 total accessible regions of chromatin (Figure 2A). In the non-activated cells, we found 108,286 total ATAC peaks. To determine how chromatin is remodeled in the aftermath of ELS, we calculated differences in

chromatin accessibility (differentially accessible regions (DARs); p-value<0.01 and |logFC|>1.5; Figure 2 B-D) at P21 between ELS and Std samples in both experience-activated and non-activated cells. Within experience-activated cells, we identified 348 genomic regions opened by ELS and 100 genomic regions closed by ELS (Figure 2B, D), such that the predominant response to ELS was to open chromatin. In contrast, within non-activated cells, we found minimal effect of ELS wherein ELS impacted a total of 56 regions (Figure 2C), demonstrating that the immediate impact of ELS is within ELS-activated cells.

We next sought to characterize these activated-cell DARs to better understand how ELS alters gene regulatory functions and biological processes. Both open and closed DARs were enriched for 3'UTRs, 5'UTRs, exons, introns, and enhancer genomic features compared to expected proportions of the mouse genome (Figure 2E). However, in contrast to putative enhancer regions, proximal promoter and intergenic regions were underrepresented among DARs. Overall, 94-95% of opened and closed DARs from the activated population were in putative cis-regulatory elements, defined as >1kb from the nearest transcription start site, as enhancers may also be found within intronic, exonic, and intergenic regions (Figure 2F; [46]). The relatively small fraction of DARs within promoter regions suggests that changes to gene transcription following ELS are generally not associated with changes to promoter accessibility, but rather functional changes at long-range cis-regulatory elements that in turn modulate the frequency of transcription at their target genes. Given an enrichment of DARs within putative cis-regulatory regions of activated cells, we next sought to identify what genes and functions these loci regulate. While many enhancers regulate their nearest gene neighbor, enhancers can also regulate genes hundreds of kilobases away [47]. We therefore mapped putative long-range

interactions between DARs and predicted target genes using publicly available Hi-C data from mouse brains [48–50] and performed gene ontology enrichment analysis for biological processes associated with the putatively contacted genes (Figure 2G-H). Genes in, or putatively contacted by, open DARs were enriched for functions related to transcription, response to stimulus, development, and cellular metabolism (Figure 2H, left). Genes in or putatively contacted by closed DARs were enriched for functions related to JAK-STAT signaling, signal transduction, proliferation, and regulation of cell communication (Figure 2H, right).

To determine whether DARs were enriched for any functional elements such as transcription factor sites, we performed motif analysis [51]. Genomic loci that gained ATAC peaks after ELS were enriched for binding sites for EGR1/2, REST, ERR2/RARA, and ZEB1/SNAI1, indicating that ELS alters transcriptional regulation and response to stimuli (Figure 2I; [52–57]. Genomic loci that lost ATAC peaks after ELS were enriched for a novel motif and binding sites for FOXJ, WT1, and PRDM1/IRF3, indicating that ELS may close regions that regulate developmental programs and immune response (Figure 2I;[58–61]. Taken together, these results demonstrate that ELS largely opens chromatin in NAc, that chromatin remodeling is specific to ELS-activated cells and persists for at least several days, and that such remodeling may serve to alter transcriptional response to stimulus.

2.3.3 ELS impacts chromatin accessibility across a broad NAc cell population over time

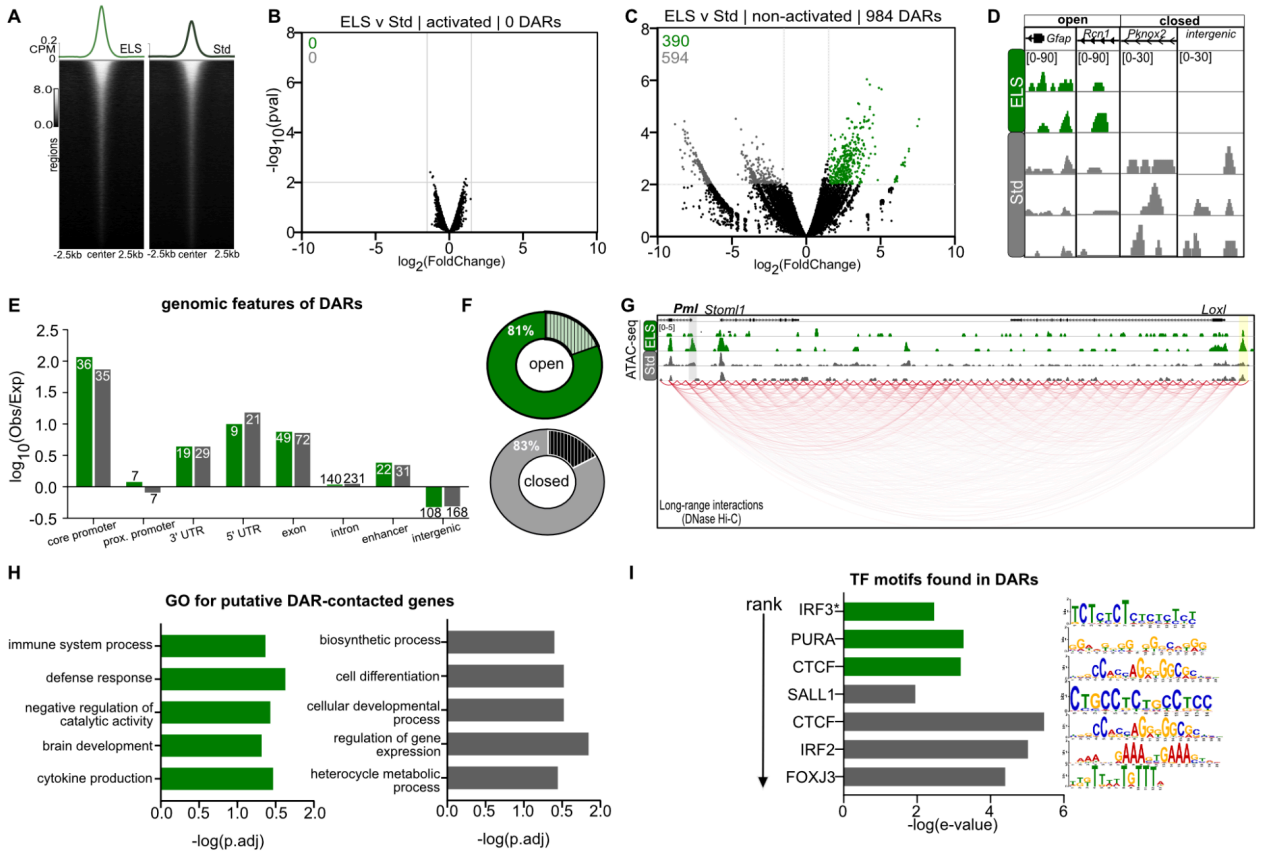


Figure 3 | ELS impacts chromatin accessibility across a broad NAc cell population over time. (A) Line plots (top) and heatmaps (bottom) of mean chromatin accessibility CPM ±2.5 kb around center of all ATAC peaks within non-activated cells. Volcano plot of ELS vs Std differentially accessible regions (DARs) within adult experience-activated (B) and adult non-activated (C) cells. Differentially accessible regions (DARs: edgeR, p-value<0.01 and |logFC|>1.5) opened or closed by ELS are shown in green and gray, respectively. (D) Example browser tracks of ATAC peaks either opened or closed by ELS in experience-activated cells normalized to CPM. (E) Count and enrichment or depletion (log10 of observed over expected for each genomic feature in the mm10 genome) of DARs mapped to each genomic feature for regions opened (green) or closed (gray) by ELS within experience-activated samples. (F) Proportion of DARs in putative cis-regulatory elements (>1kb from the nearest TSS) for regions opened (green) or closed (gray) by ELS within experience-activated samples. (G) Example browser tracks and long-range interactions between an open DAR (yellow highlight) and a putative target gene promoter (gray highlight). (H) Gene Ontology (GO) biological process terms for genes putatively contacted by ELS-opened (green) or closed DARs. (I) Top transcription factor motifs found in open (green) and closed (gray) DARs ; * denotes motifs found in <60% of input regions.

In order to determine whether these near-term changes in chromatin persist into adulthood, we compared the differential accessibility profiles between the ELS and Std-reared

conditions in adulthood using the same total ATAC peak sets from the experience-activated and non-activated cells. As described above, we identified a total of 108,286 ATAC peaks within the non-activated cells (Figure 3A). In contrast to the chromatin opening observed at P21 in ELS-activated cells, there were no DARs in adult experience-activated cells (Figure 3B). Instead, there were 390 opened and 594 closed regions in cells that were not initially activated in response to ELS (Figure 3C-D), indicating that the effect of ELS propagated across development into the broader NAc cell population.

We therefore characterized these opened and closed regions within initially-non-activated cells from adult NAc. Adult DARs both opened and closed by ELS were enriched for nearly all genomic features except intergenic regions, which were depleted (Figure 3E), and were largely in putative cis-regulatory elements (81-83% of DARs; Figure 3F). As with juvenile peaks, we mapped putative long-range interactions and performed gene ontology analysis in order to identify what genes and functions these loci may regulate (Figure 3G-H). Genes within — or putatively contacted by — open DARs were enriched for functions related to immune system processes and brain development, while closed-DAR-contacted genes regulated biosynthetic processes, cellular differentiation and development, and gene expression (Figure 3H).

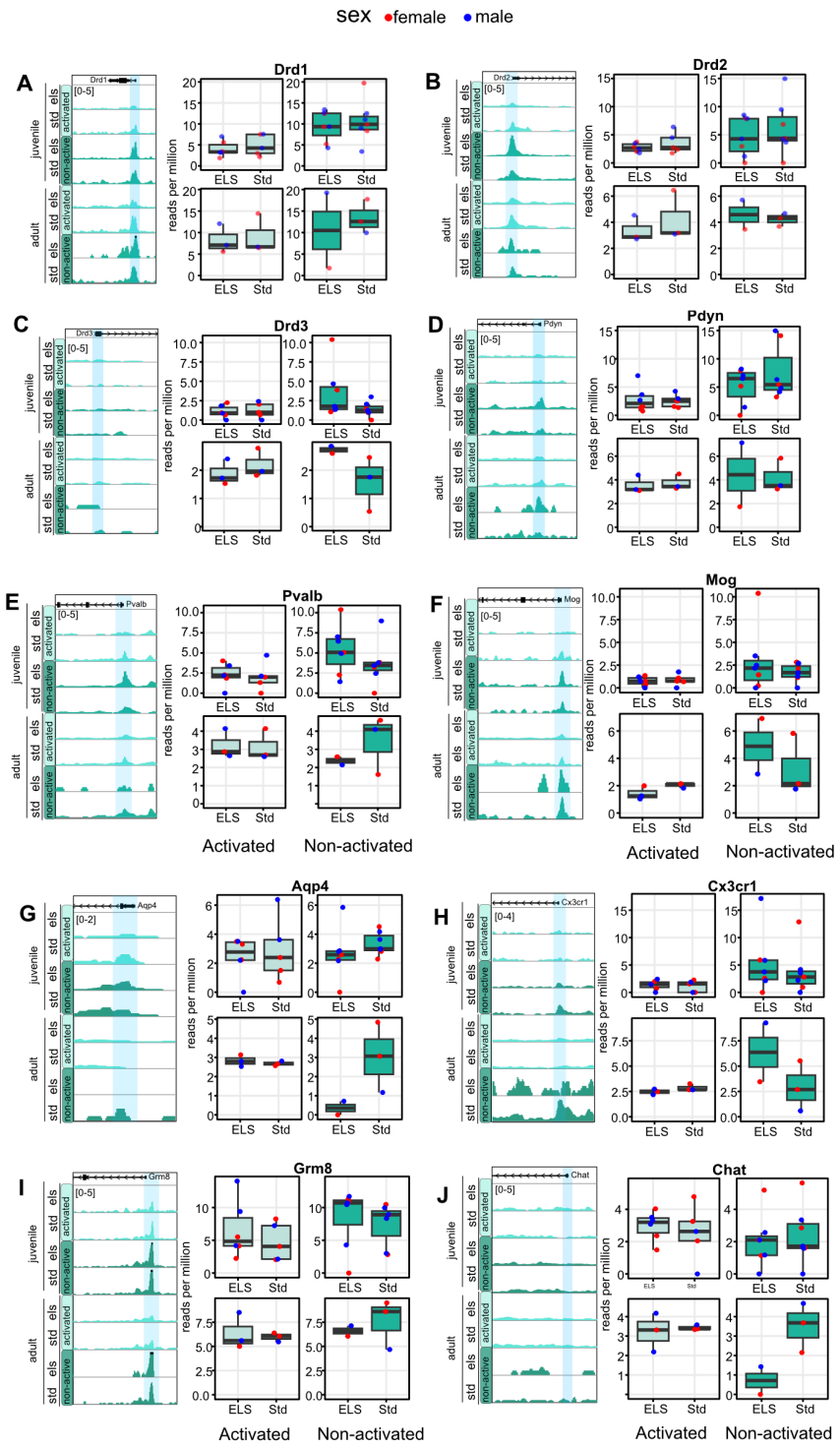
Genomic loci that decreased in accessibility by adulthood after ELS were enriched for binding sites for transcription factors in the FOXJ and IRF families (Figure 3I), demonstrating that some changes in these transcription factor programs persist across development. Genomic loci that gained accessibility in adulthood after ELS were enriched for transcription factor binding sites implicated in immune response pathways (IRF3 and PURA) (Figure 3I; [58,62–64]. Interestingly, DARs that both gained and lost accessibility after ELS were enriched for CTCF

motifs. CTCF is an architectural protein which facilitates enhancer-promoter interactions through three-dimensional chromatin looping [65], supporting the hypothesis that ELS may alter genomic architecture to impact long-range transcriptional regulation.

While cell-type specificity is obscured by semi-bulk ATAC-seq, we examined enrichment of ATAC peaks in promoters of genes marking different canonical cell types found within the NAc to estimate relative proportions of previously-identified NAc cell types (Supplemental Figure 1A-J). Experience-activated cells from Std and ELS NAc included open chromatin at both the Drd1 and Drd2 promoters (Supplemental Figure 1B). This is consistent with findings that both classes of medium spiny neurons are altered by stress and engaged by both positive and negative valence stimuli [11,66,67]. Experience-activated cells also included open chromatin at the promoter for Grm8 promoter (Supplemental Figure 1I), which encodes a metabotropic glutamate receptor and marks a distinct population of NAc medium-spiny neurons [68]. Within experience-activated cells, little open chromatin was found within promoters of other neuronal population markers including Drd3, Pdyn, Pvalb, or Chat [69]. Juvenile and adult non-activated cells from both Std and ELS NAc also included open chromatin peaks at Drd1, Drd2, and Grm8 promoters as expected, as well as other neuronal (Pdyn and Pvalb) and non-neuronal (Mog, Aqp4, and Cx3cr1) markers examined, reflective of the fact that 90% of NAc cells were not activated and tagged (Figure 1E).

However, adult ELS cells that were not initially activated had relatively greater ATAC peaks at promoters for Mog (marking oligodendrocytes) and Cx3cr1 (marking microglia) and lower peaks within Aqp4 (marking astrocytes) relative to Std-reared and juvenile samples (Supplemental Figure 1F-H). The finding that ELS alters chromatin in these cell populations that

were not initially activated by ELS is consistent with research demonstrating dynamic cross-talk between neurons and glial cells, and that activity within non-neuronal cells adjacent to experience-activated neurons is required for long-term experience encoding [70,71].



Supplemental Figure 1: Cell-type estimation in experience-activated and non-activated cell populations. Cell-type predictions based on ATAC-seq read pileups at cell-type marker gene promoters. Each cell type marker—(A) Drd1, (B) Drd2, (C) Drd3, (D) Pydn, (E) Pvalb, (F) Mog, (G) Aqp4, (H) Cx3xr1, (I) Grm8, (J) Chat – shows CPM normalized track visualizations generated with the WashU Epigenome Browser (left) and RPM normalized read counts at respective promoters (right).

2.3.4 ELS accelerates normal chromatin development in experience-activated cells

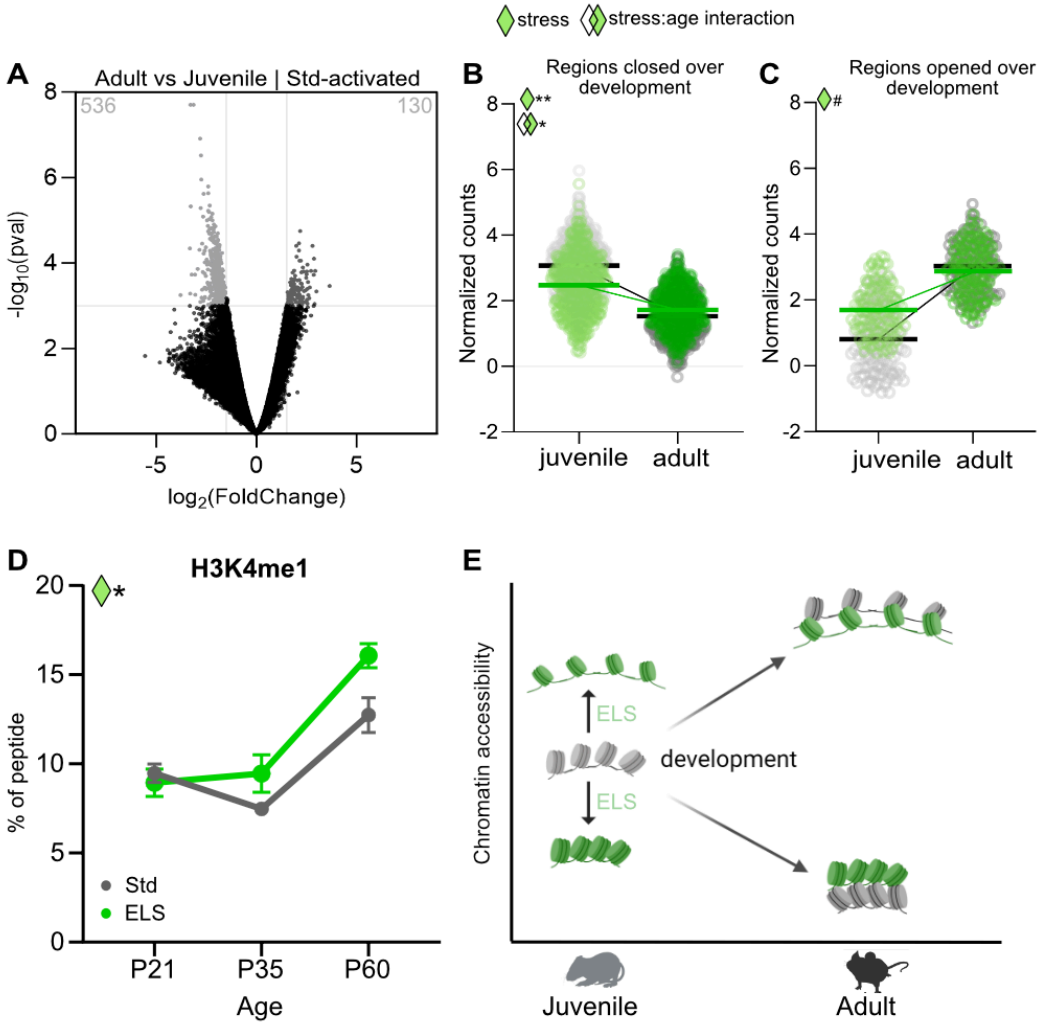
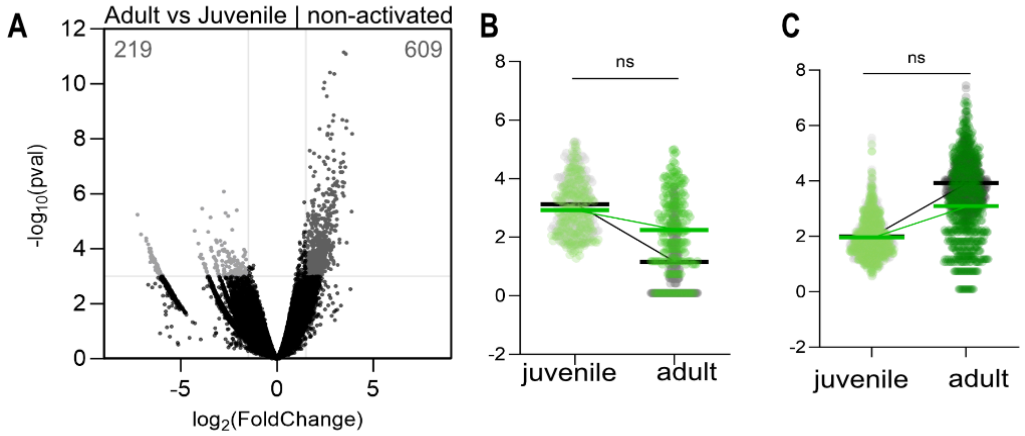


Figure 4 | ELS accelerates normal chromatin development in experience-activated cells. (A) Volcano plot of adult vs juvenile differentially accessible regions (DARs: edgeR, p-value<0.001 and |logFC|>1.5) within standard-reared experience-activated NAc samples. TMM-normalized counts of regions significantly closed (B) or opened (C) from P21 to adulthood among Std samples (gray) overlaid with normalized counts from the same regions among ELS samples (green). Main effects of and interactions between ELS and age are denoted (bars at mean; **p<0.01; *p<0.05; #p<0.1). (D) Proportion of monomethylated histone-3 lysine-4 peptide fragments in male NAc across postnatal development (mean±SEM; reanalyzed mass spectrometry data published in Kronman et al, Nature Neuroscience, 2021). (E) Schematic of the impact of ELS on chromatin architecture across postnatal development.

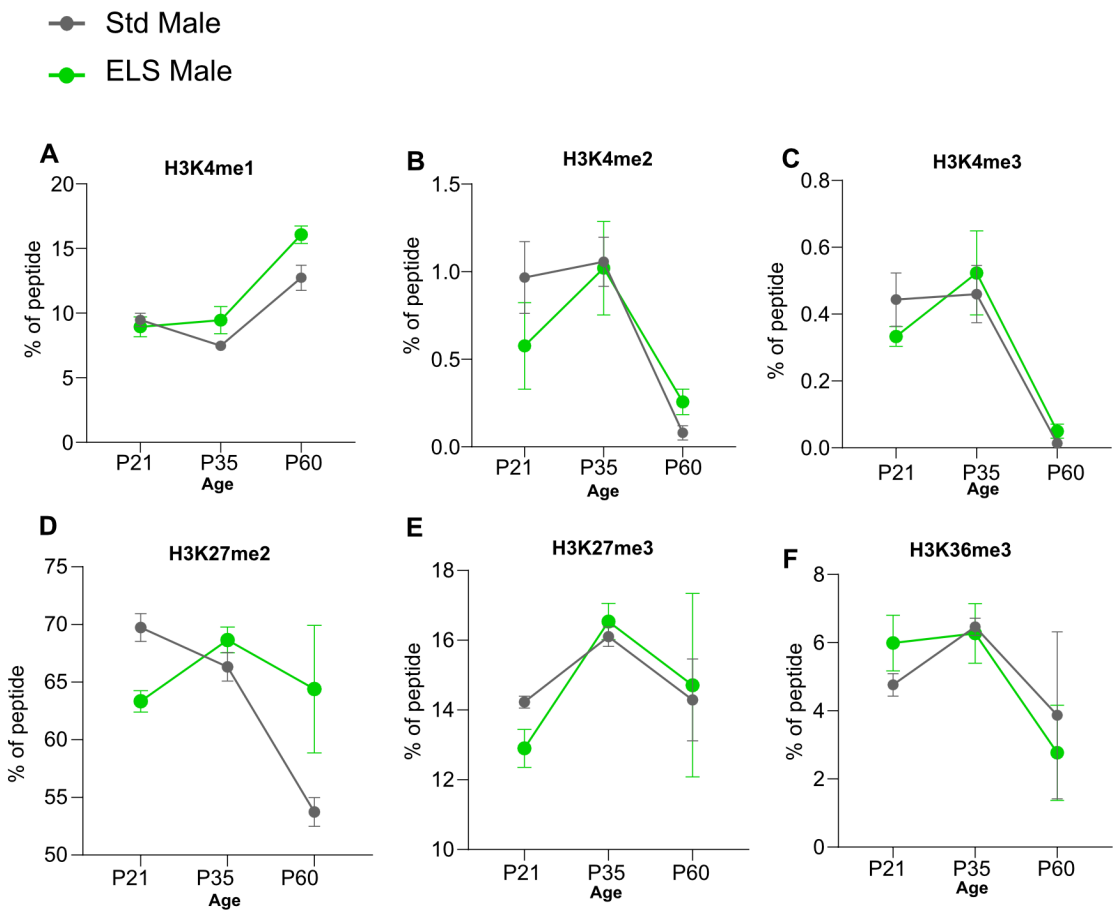
Recent work has shown that enhancer usage continues to change between rodent postnatal weeks 1-3 as neurons mature [72]. To identify how chromatin changes across normal development in NAc, we performed differential accessibility analysis between juvenile and adult samples from standard-reared mice. Within experience-activated cells, chromatin accessibility largely decreased, with 536 regions closing and 130 regions opening from P21 to adulthood (Figure 4A). This is reminiscent of widespread decommissioning of enhancers that drive genes involved in neuronal proliferation and migration during early development [72]. Among non-activated cells, chromatin accessibility largely increased into adulthood, with 219 regions closing and 609 regions opening (Supplemental Figure 2A). This is consistent with earlier work showing that overall more enhancers become activated than repressed across later stages of postnatal development [72,73].

Given that the ELS sensitive period from P10-17 overlaps with a postnatal period of enhancer maturation – and that enhancer maturation is modulated by neuronal activity and experience during this period [72] – we hypothesized that ELS may disrupt postnatal chromatin maturation. To determine whether ELS alters the timing of chromatin maturation, we compared normalized chromatin accessibility counts within these standard opening and closing regions using a linear mixed effects model to account for sex and shared variance within individuals. By comparing the rate of chromatin maturation in ELS versus Std samples across time, we were also able to distinguish between three possibilities for how ELS opens chromatin following stress: 1) that ELS blunts closing of regions that should normally close during development, leaving chromatin in a more open, immature state; 2) that ELS aberrantly opens chromatin de novo, potentially through recruitment of pioneer factors; and 3) that ELS accelerates chromatin

maturation such that genomic loci set to open normally, open earlier. As expected, all analyses had a main effect of age (Supplemental Table 2). Sex was not a significant factor in any analysis. Among chromatin regions that close across standard development within experience-activated cells, there was a main effect of ELS [b= -0.874, SE=0.272, p=0.008] and an interaction between ELS and age [b=0.912, SE=0.338, p=0.021], such that closing regions were already more closed by P21 (Figure 4B). Similarly, among chromatin regions that open across standard development within experience-activated cells, there was a trend for a main effect of ELS [b=0.873, SE=0.469, p=0.089], such that opening regions were already more open by P21 (Figure 4C). These effects were specific to experience-activated cells (Supplemental Figure 2B-C). Together, these results demonstrate that ELS accelerates chromatin maturation in ELS-responsive cells. These findings of accelerated chromatin maturation also resolve the earlier puzzling results regarding the lack of differences in chromatin accessibility in adult experience-activated cells, reflecting a change in timing rather than location of chromatin remodeling.



Supplemental Figure 2: Postnatal development of chromatin in initially non-activated cell populations. (A) Volcano plot of adult vs juvenile differentially accessible regions (DARs: edgeR, p-value <0.001 and |logFC| > 1.5) within standard-reared initially-non-activated NAc samples. TMM-normalized counts of regions significantly closed (B) or opened (C) from P21 to adulthood among Std samples (gray) overlaid with normalized counts from the same regions among ELS samples (green).



Supplemental Figure 3: Post-translational histone modifications across development. Changes in post-translational histone modification proportions in male NAc across postnatal development. Reanalyzed mass-spectrometry data published in Kronman et al, Nature Neuroscience, 2021. Mean±SEM. (A) H3K4me1: main effect of ELS: $F(1,12)=6.971$, $p=0.023$; main effect of age: $F(1,12)=35.53$, $p<0.0001$; trending interaction: $F(1,12)=3.498$, $p=0.067$. (B) H3K4me2: not significant. (C) H3K4me3: not significant. (D) H3K27me2: interaction between ELS and age: $F(1, 12)=5.874$, $p=0.017$. (E) H3K27me3: not significant. (F) H3K36me3: not significant.

We next asked whether ELS accelerates other aspects of chromatin development. We took advantage of published data that used mass-spectrometry to profile more than 200 individual and combinatorial post-translational histone modifications in the NAc at P21, P35, and P70-80 following ELS [74]. Interestingly, we found evidence for accelerated development of histone-3 lysine-4 monomethylation (H3K4me1) in the NAc (Fig. 4D; [main effect of ELS:

F(1,12)=6.971, p=0.023; main effect of age: F(1,12)=35.53, p<0.0001; trending interaction: F(1,12)=3.498, p=0.067]). H3K4me1 is associated with open chromatin at gene promoters and enhancers, and marks primed enhancers in the absence of H3K4me3 and H3K27Ac [75]. However, there were no main effects or interactions with ELS for H3K4me2, H3K4me3, or H3K27Ac, which are associated with active gene expression (Supplemental Figures 3B-C), suggesting that H3K4me1 differences following ELS may be specific to enhancer priming. We also found an interaction between ELS and age for H3K27me2, a histone modification associated with enhancer regulation [76], such that this modification was decreased following ELS but elevated in adulthood [Supplemental Figure 3D; F(1, 12)=5.874, p=0.017]. There were no main effects or interactions with ELS for H3K27me3 or H3K36me3, both associated with inhibition of gene expression (Supplemental Figures 3E-F). Taken together, our findings of accelerated chromatin maturation and accelerated H3K4me1 accumulation in the NAc, without simultaneous changes in markers of active gene expression, are consistent with premature development and priming of chromatin architecture (Figure 4E).

2.3.5 Chromatin opening after ELS predicts gene expression response to adult stress

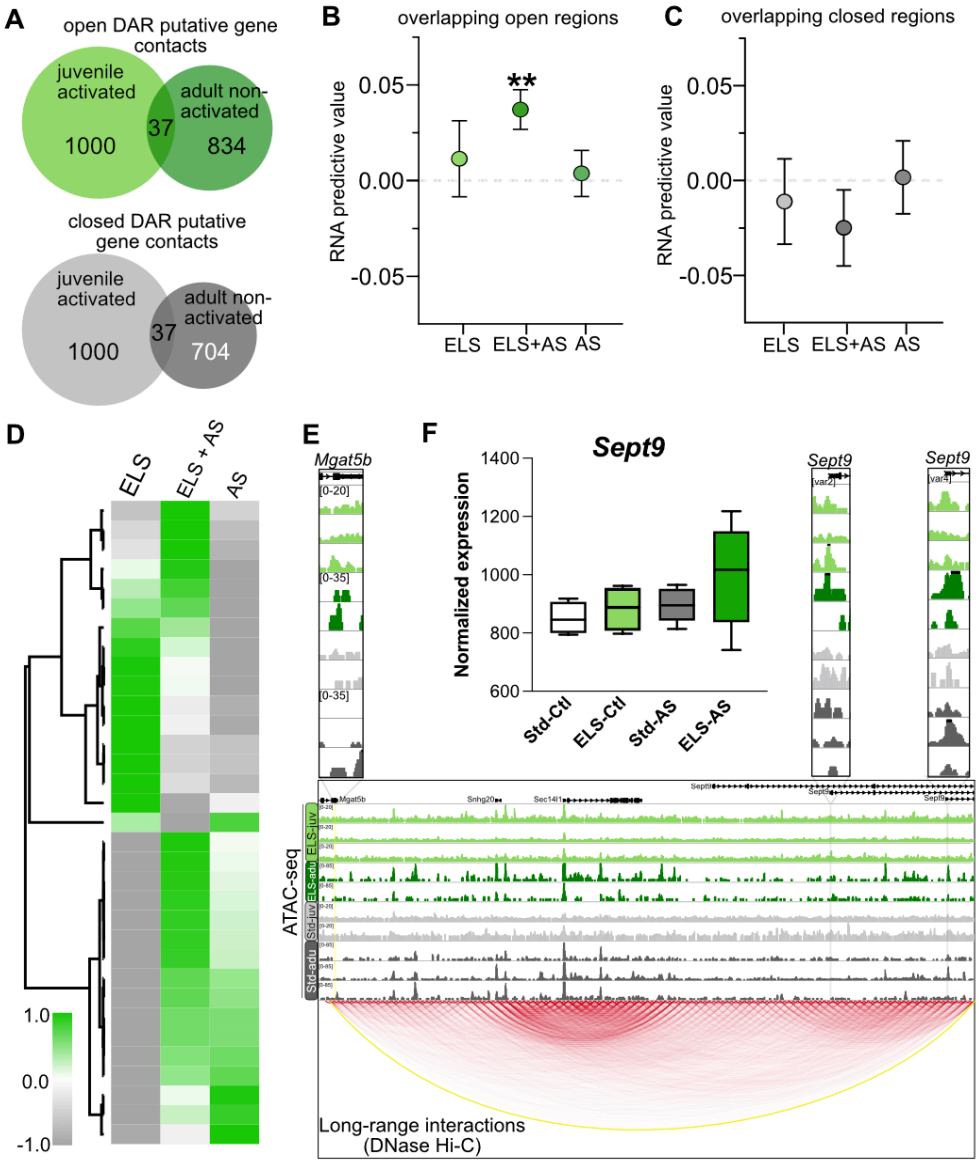


Figure 5 | Chromatin opening after ELS predicts gene expression response to adult stress. (A) Venn diagrams showing overlap of genes putatively contacted by ELS-opened or ELS-closed DARs from P21 and adult samples (maximum predicted gene contacts limited to 1000). Measure of how well (B) open or (C) closed DARs predict NAc gene expression after ELS alone, ELS with a second hit of stress (ELS+AS), or adult stress (AS) alone, with positive values representing greater congruent prediction (mean±SEM, **p<0.01). (D) Heatmap of expression changes of 37 genes contacted by open juvenile and adult DARs (row-normalized DESeq2 log2(FoldChange) values from ELS, ELS+AS, or AS, each vs Std-reared; hierarchically clustered). (E) Example browser tracks and long-range interactions between an opened DAR (yellow highlight) and a putative target gene, Sept9 (gray highlight). (F) Boxplots of Sept9 gene expression (CPM) among Std control, ELS control, Std+AS, or ELS+AS groups (min to max and mean represented) as an example. All gene expression data reanalyzed from adult male NAc RNA-seq published in Peña et al., Nature Communications, 2019; GEO GSE89692.

An important question is whether the observed changes in chromatin accessibility after ELS are relevant to gene expression. Given an enrichment of DARs within putative cis-regulatory regions (Figures 2G and 3G) and histone modifications associated with enhancer priming (Figure 4E), we speculated that chromatin changes may govern transcriptional response to future stress, in addition to the long-lasting changes to gene expression following ELS that we previously observed [9]. We first determined whether any DARs or genes putatively regulated by DARs (based on long-range interaction mapping) were maintained across development in activated and non-activated cell populations. None of the DARs from activated juvenile cells overlapped with DARs from non-activated adult cells. However, 37 genes putatively regulated by open DARs from each condition overlapped, and 37 genes putatively regulated by regions closed by ELS in both conditions overlapped (Figure 5A; Supplemental Table 3). This indicates that even though the impact of ELS on chromatin accessibility is dynamic across time and cellular populations, there is some consistency in the functional effect at the level of gene regulation.

Using previously published RNA-seq data from adult male NAc [9], we mapped the expression changes of the putatively-contacted genes after different stress experiences: ELS, ELS + adult social defeat stress, or adult social defeat stress alone, compared to Std controls (Figure 5D, 5F). We calculated an "RNA predictive value" of chromatin opening or closing to predict increased or decreased expression, respectively, based on direction, magnitude, and significance of differential gene expression for each gene. Chromatin opening by ELS only had predictive value for the two-hit stress (ELS+adult stress) experience [two-tailed one-sample t-test: $t(1,30)=3.583$, $p=0.0012$; Figure 5B], but not to ELS or adult stress alone. Chromatin

closing by ELS was not significantly predictive of RNA expression for any group (Figure 5C). We also found that among a subset of genes (e.g., *Sept9*), there is an increase in expression in response to adult stress only if previously primed by ELS (Figure 5F). These results indicate that long-lasting increases in chromatin accessibility induced by ELS can predict transcriptional responses to adult stress later in life, consistent with the concept of epigenetic priming.

2.3.6 Epigenetic priming via postnatal H3K4me1 enrichment in NAc increases sensitivity to future stress

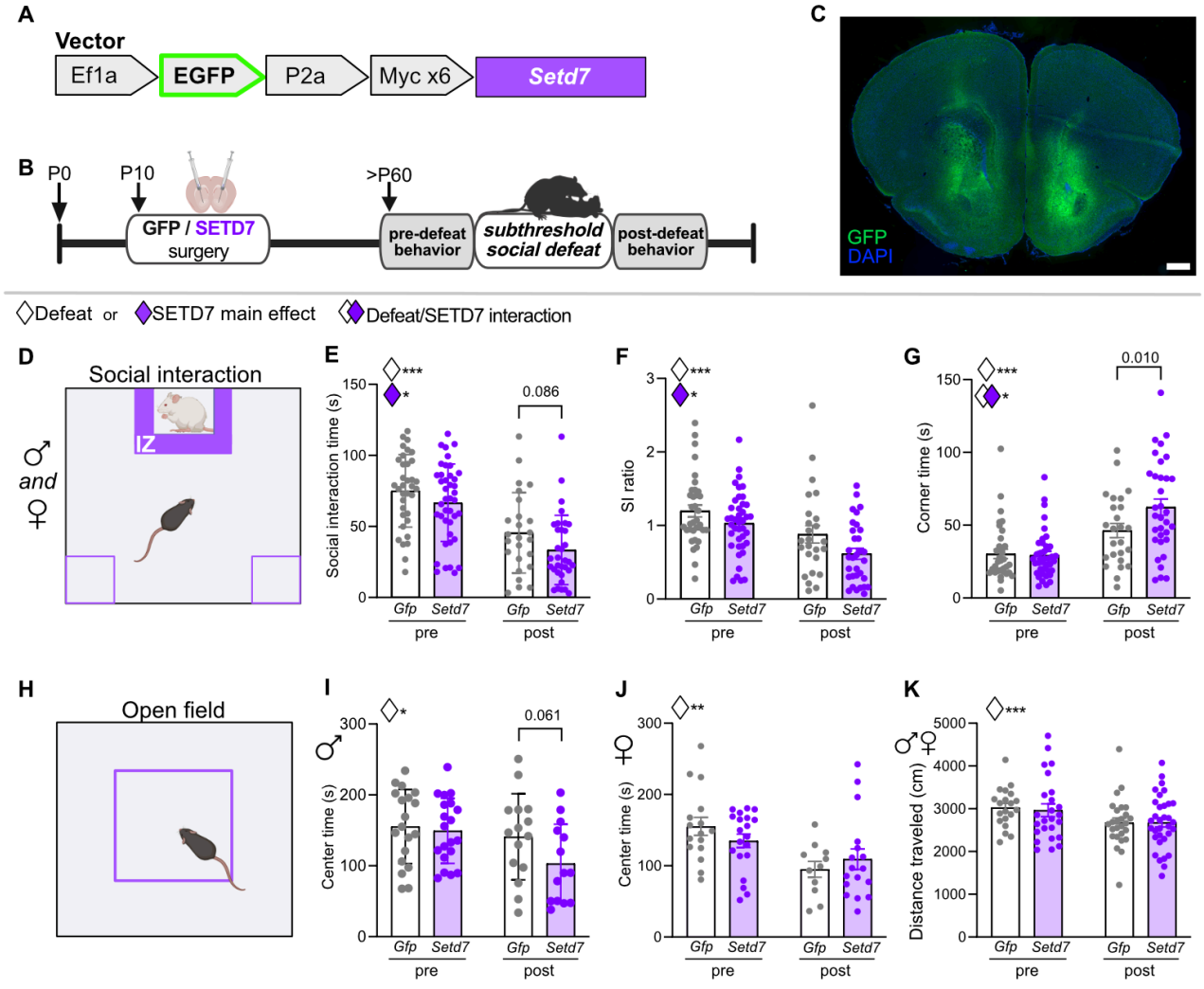


Figure 6 | Postnatal H3K4me1 enrichment in NAc increases sensitivity to future stress. (A) Experimental timeline. (B) Schematic of the Setd7 over-expression vector packaged into AAVs; control vector was identical without P2A-Myc-Setd7. (C) Representative image showing viral targeting of NAc. Scale bar, 1mm. (D) Social interaction testing schematic: time spent in the interaction zone (IZ) or corners was measured. Within the social interaction test, (E) social interaction time, (F) social interaction ratio (lower ratio is associated with greater social avoidance), and (G) time spent in far corners among male and female mice before (pre) and after (post) social defeat. (H) Open field testing schematic. Within the open field test, time spent exploring the center among males (I) and females (J). (K) Total distance traveled among males and females in the open field arena. Main effects of and interactions between Setd7 and social defeat are denoted in the key (mean±SEM; ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05; p-values of post-hoc tests are shown).

To test whether aberrant chromatin opening primes future stress response, we manipulated chromatin during postnatal development and tested behavioral sensitivity to adult stress. Because we found that ELS predominately opened chromatin within activated neurons after stress, which ultimately led to aberrant chromatin changes within the broader NAc cellular population in adulthood, we sought to widely deposit a histone modification associated with open chromatin, agnostic to neuronal activation state. To do this, we took advantage of an epigenome editing tool recently validated by our group to over-express the histone mono-methyltransferase SETD7 to enrich for H3K4me1 (Figure 6A[16]). AAV-Setd7 broadly enriches for H3K4me1 without inducing further H3K4 methylation or other histone modifications associated with active gene expression[16]. Thus, we predicted that global enrichment of H3K4me1 would induce or maintain open chromatin states similar to the epigenetic priming and H3K4me1 enrichment we observed after ELS (Figure 3; Figure 4).

Because ELS accelerated chromatin development, we over-expressed AAV-Setd7 or AAV-Gfp in NAc at P10 to match the start of ELS (Figure 6B). Immunohistochemical staining showed successful targeting of the virus to the NAc even at this young age (Figure 6C). We used a within-subject design to test all male and female mice on behavioral tests both before and after sub-threshold adult social defeat stress (Figure 6D-K[7,77–79]). We powered this study to detect sex

differences. Notably, there were no main effects of sex on measures of social avoidance behavior, and thus males and females were analyzed together using a repeated-measures mixed effects model.

Both social defeat stress and epigenetic priming decreased social interaction time, a measure of threat avoidance [Figure 6E, Supplemental Figure 4, Supplemental Table 4; main effect of defeat: $F(1,56)=62.97$, $p<0.0001$; main effect of AAV-Setd7: $F(1,74)=4.63$, $p=0.034$; post-hoc comparison between GFP-Defeat and SETD7-Defeat with Benjamini FDR correction: $p=0.086$]. Similarly, both stress and AAV-Setd7 decreased social interaction ratio [Figure 6F; main effect of defeat: $F(1,55)=23.86$, $p<0.0001$; main effect of Setd7: $F(1,74)=5.69$, $p=0.019$]. Time spent huddling in corners, an additional measure of heightened vigilance and stress sensitivity [80] was also increased in an interaction between stress and Setd7 [Figure 6G; main effect of defeat: $F(1,56)=45.9$, $p<0.0001$; main effect of Setd7: $F(1,73)=3.05$, $p=0.085$; interaction between stress and Setd7: $F(1,56)=5.94$, $p=0.018$; post-hoc comparison between GFP-Defeat and SETD7-Defeat: $p=0.010$].

Time spent exploring the center of an open field, a more generalized measure of threat avoidance, was sensitive to stress, AAV-Setd7, and sex, and thus male and female mice were analyzed separately [trending three-way interaction between sex, stress, and Setd7: $F(1,10=3.37)$, $p=0.096$]. Among males, both defeat and, to a lesser extent, Setd7 decreased open field center time [Figure 6I; main effect of defeat: $F(1,27)=6.26$, $p=0.019$; main effect of Setd7: $F(1,37)=2.49$, $p=0.123$; post-hoc comparison between GFP-Defeat and SETD7-Defeat: $p=0.061$]. Among females, defeat but not Setd7 decreased center time [Figure 6J; main effect of defeat: $F(1,26)=14.67$, $p<0.001$]. There was also a main effect of defeat on distance traveled

among both males and females, but no effects of Setd7, indicating Setd7 in the NAc did not impair mobility [Figure 6K; main effect of defeat: $F(1,34)=15.20$, $p<0.001$].

Taken together, this functional manipulation and behavioral results show that broad, developmental deposition of a histone modification associated with open chromatin mimics the effects of ELS to prime sensitivity to stress across the lifespan.

2.4 Discussion

ELS sensitizes, or primes, individuals to subsequent stressors to increase lifetime risk of psychiatric disorders. At a cellular level, our recent work demonstrated that ensembles of neurons in the NAc that are active during ELS are preferentially reactivated by adult stress, which contributes to stress hypersensitivity and altered behavior [7]. At a molecular level, ELS programs both long-lasting baseline gene expression changes in the NAc and other afferent brain regions, and also primes gene expression response to a second hit of stress [9,21]. Here, we tested the hypothesis that ELS encodes these persistent changes in transcriptional reactivity within the NAc at an epigenetic level by priming cis-regulatory regions of the genome. Using a combination of activity-dependent cellular tagging, ATAC-sequencing across developmental time, bioinformatic analyses, viral-mediated epigenome editing, and behavioral quantification, we show that ELS initially opens chromatin in ELS-responsive cells — ultimately leading to chromatin remodeling throughout the NAc — and that deposition of a post-translational histone modification associated with open chromatin is sufficient to mimic ELS and prime hypersensitivity to future stress.

Enhancer priming — a form of molecular memory in which developmental or environmental cues open chromatin at enhancers and which is associated with presence of the

post-translational histone modification H3K4me1 — facilitates a faster, stronger, and/or sensitized transcriptional response to recurring stimuli [12,75,81,82]. This process is known to occur in botany, immunology, embryological development, and cancer [81,83–86]. Elegant work also demonstrated that experience can prime enhancers in the adult hippocampus through changes in 3D enhancer architecture, that priming is stable, and that memory recall activates enhancers [10]. Our results reveal that ELS also leads to stable epigenetic priming. In the aftermath of ELS, we find that ELS largely opens chromatin (Figure 2B), that differentially accessible chromatin is enriched for enhancers and other cis-regulatory elements (Figure 2E, Figure 3E), and that ELS elevates H3K4me1 (Figure 4D). Through long-range interaction mapping to putatively contacted genes, we also show that ELS-opened regions, but not closed regions, predict the transcriptional response to a second hit of stress (Figure 5B-C). Furthermore, early developmental (P10) deposition of H3K4me1 in the NAc increases sensitivity to adult stress and alters behavior (Figure 6), demonstrating that epigenetic priming is functionally relevant and sufficient to recapitulate the effect of ELS at a behavioral level. Together, these data support an emerging role for enhancer priming as a biological mechanism for ELS-induced lifelong stress sensitivity.

While much effort has been made to characterize embryonic chromatin development [87], chromatin development in the brain between birth and adulthood has remained understudied. Recent research has demonstrated that the epigenetic landscape within cortical interneurons continues to mature postnatally and only crystalizes between mouse postnatal weeks 1 and 3, after which it is stable into adulthood [72]. This window of postnatal epigenetic maturation overlaps with the postnatal sensitive period for stress exposure in mice [2,9,88–90], suggesting that ELS may interfere with chromatin development. Indeed, we find that ELS accelerates maturation

of chromatin in stress-responsive cells for both regions that typically open or close across development (Figure 4B-C). We also find that ELS accelerates and augments bulk levels of H3K4me1 (Figure 4D). ELS has similarly been found to accelerate other aspects of brain development, such as connectivity between amygdala and prefrontal cortical regions [91]and markers of hippocampal development including parvalbumin interneuron maturation, myelination, and synaptic plasticity [92]. Accelerated development within some cell types or brain regions decouples otherwise tightly-controlled neurodevelopmental processes, which has been hypothesized to prematurely reduce plasticity and the ability to appropriately learn from expected environmental experiences [93,94]. Our results show that these effects may arise from accelerated epigenetic development.

The finding that ELS initially opens chromatin in experience-responsive cells but ultimately leads to a more generalized chromatin response in the broader cell population — and not within experience-responsive cells — was unexpected. Profiling only tagged cells (as in Marco et al., 2020) or a single time point would have precluded these findings. The lack of differences in chromatin accessibility in adult experience-responsive cells is reconciled by the finding that ELS accelerates standard maturation of chromatin within these cells, reflecting a change in timing of rather than overall chromatin opening (Figure 4B-C). However, several possibilities remain for our findings of delayed chromatin remodeling within initially non-activated cells. One possibility is that this epigenetic response is due to neurons initially activated by ELS remaining active and recruiting activation of surrounding neurons. However, we rejected this possibility given our earlier work showing that ELS leads neither to spontaneous neuronal reactivation of initially-responsive cells nor broader neuronal hyperactivity in the adult

NAc in the absence of additional stress [7]. A second possibility is that this delayed epigenetic response reflects changes over time within the non-neuronal cell population. This hypothesis is supported by an enrichment of open chromatin in marker genes for oligodendrocytes, astrocytes, and microglia within non-activated cells after ELS (Supplemental Figure 1). Indeed, activation of neurons stimulates surrounding "peri-engram" astrocytes and other glia, which likely induces chromatin remodeling within these cells [71]. Additionally, microglia adjacent to stress-responsive cells in the hypothalamus are disrupted by ELS and fail to prune excitatory synapses [95]. Neuronal activity also recruits surrounding microglia to aid in negative feedback of neuronal responses [96], which may involve chromatin alterations to support changes in gene expression. This is an intriguing possibility because a lack of microglia-driven negative feedback also contributes to hyper-responsiveness of neurons to stimuli [96], which could contribute to ELS-induced hyperresponsivity to stress. Further studies using spatially-resolved ATAC-seq are needed to determine whether adult chromatin alterations are greater among glial cells surrounding ELS-activated neurons.

In sum, these data provide novel evidence that ELS primes sensitivity to future stress though developmental changes in chromatin, akin to an epigenetic memory of stress within the NAc. Our research provides an important foundation for additional research to develop therapeutic strategies targeting chromatin to ameliorate the impact of ELS on brain development and psychiatric disease risk.

2.5 Methods

Further Supplemental Materials and Data Availability: Supplementary Tables further supporting this data can be found in the preprint on bioRxiv. All sequencing data will be deposited in NCBI's Gene Expression Omnibus (GEO). All other data will be made available upon reasonable request.

Mice

All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at Princeton University. Mice were housed in a temperature- and humidity-controlled vivarium and maintained on a 12-h light/dark cycle (lights on at 4a.m.) and were group-housed in sets of three to five with *ad libitum* access to food and water. Mice were considered to be "male" or "female" based on external genitalia (adult) and/ or anogenital distance (pups). For all experiments, mice were bred in-house in trios, males were removed after 5-7 days, and females were separated into individual cages 1-3 days prior to parturition. All offspring regardless of early life experience were weaned at postnatal day P21, with males and females weaned into separate cages, keeping littermates together and only combining pups from different litters of the same experimental condition in order to maintain at least three mice/cage. Males and females were used in all experiments.

In order to tag and isolate experience-responsive cells for ATAC-seq experiments, we generated Arc-CreERT2 X Sun1-GFP double-transgenic mice on a C57BL/6J background as previously described [7]. Briefly, heterozygous Arc-CreERT2/+ mice (stock #022357, The Jackson Laboratory; [41]were bred in-house with R26-CAG-LSL-Sun1-sfGFP-Myc knock-in mice

("Sun1-GFP"; stock #030952, The Jackson Laboratory; (Mo et al., 2015). Genotyping of double-transgenic mice was performed as previously described [7].

Wild-type C57BL/6J mice were bred in-house and used for viral-mediated gene manipulation and behavior experiments. We powered viral manipulation and behavioral experiments to potentially detect sex differences based on power calculations of behavioral effect sizes in the lab [(n=15-19 Gfp males, 11-16 Gfp females, 16-20 Setd7 males, 17-21 Setd7 females)]. Retired Swiss-Webster male breeder mice (Taconic) were housed individually and used as aggressor mice in social defeat stress.

Administration of 4-hydroxytamoxifen

To induce experience-dependent recombination, 4-hydroxytamoxifen (4-OHT) was administered as described [7]. 4-OHT (catalog #H7904, Sigma-Aldrich) was first prepared as 25mg/ml stock solutions in ethanol and frozen at -20°C. For injections, 4-OHT was prepared fresh daily from stock to 5mg/ml, dissolved in ethanol and corn oil (1:4). Double-transgenic Arc-CreERT2 X Sun1-GFP pups were first habituated to injections of saline (60 µL i.p.) from P14-16. To drive recombination on P17, (the last day of the stress for ELS pups), transgenic pups were injected with 4-OHT (50 mg/kg body weight, approximately 60 µL/pup, i.p.) at the beginning of maternal separation. Standard-reared pups were returned to their home cage and given a novel enrichment object (running wheel: pups were observed to explore the novel object but not necessarily run on it) in order to tag a specific positive-valence experience. The mice were then left undisturbed for at least three days (P17-P21) to allow time for expression of the transgenes to develop before collecting tissue and isolating nuclei. We previously validated the

use of these mice for experience-specific tagging, and showed that we were able to tag similar numbers of neurons in ELS and Std NAc so as not to artificially bias downstream analyses.

Stress paradigms

Early-life stress (ELS): Litters for ATAC-seq were randomly assigned at birth to standard (Std)-rearing or ELS conditions. The ELS paradigm used was a combination of maternal separation and limited nesting resources during a stress sensitive period from postnatal day P10-17, based on established protocols[11]. Briefly, pups in ELS-assigned litters were separated from their dams together into a clean cage containing a tablespoon of bedding from an adult male aggressor mouse for 3-4 h/day from P10-17. During the week of daily separations, home cage nesting material was also reduced to 1/2 of a nesting puck, which was replaced with a full nesting puck after the last day of stress.

Adult sub-threshold social defeat stress: In order to determine whether chromatin manipulation increased sensitivity to later stress, male and female mice were subject to sub-threshold adult social defeat stress for 5 consecutive days. Previous studies have found that among control males, susceptibility to chronic social defeat stress develops across 10 days, but is not apparent after only 5[78,97,98]. Here, both male and female mice were exposed to social defeat but were run in separate cohorts. Males were defeated as described in standard protocols [99]. Females were defeated in a "non-discriminatory" version of social defeat as previously described (Yohn et al. 2019; Bennett et al. 2024; Geiger and Balouek 2024). Briefly, on each day of social defeat, experimental mice were placed into the cage of a novel Swiss-Webster retired breeder ("aggressor"; Taconic) mouse. Male lures were introduced first for ~1-2 minutes, then an experimental female mouse was added into the cage for an additional 5 minutes. After defeat,

mice were moved across a perforated plexiglass barrier for the remainder of the day for continued sensory contact. Experimental mice were introduced to a new aggressor each day. Control mice were housed in a standard mouse cage in pairs of the same sex, separated by a perforated plexiglass barrier. Behavioral testing began one day after the last bout of social defeat.

Stereotaxic surgery

Standard-reared male and female C57BL/6J pups were randomly assigned to either experimental Setd7 over-expression group or a control Gfp group and surgeries were performed as previously described (Geiger and Balouek 2024) with the exception of NAc-targeting surgical coordinates. P9-12 pups were anesthetized with isoflurane (2-3% induction; 1-3% maintenance) for surgeries and fitted into dual-arm stereotax with the nose fitted in the nose cone above the bite bar and the head stabilized using blunt ends of ear bars between the jaw and ear. Pups were bilaterally administered either AAV-GFP (AAV9-EF1a-EGFP) or AAV-SETD7 (AAV9-EF1a-EGFP-P2A-MYC-Setd7) into the nucleus accumbens. The surgical coordinates were +/-1.4 (x = medial/lateral plane), +1.0(y = anterior/posterior plane), -3.15(z = dorsal/ventral plane), with both Hamilton syringes angled at 7˚. 250nL of virus was administered into each hemisphere at a rate of 0.1μL/minute. Both vet glue (Covetrus, cat#: 001505) and two-four sterile sutures (Fisher, cat#: 501180710) were used to ensure complete healing of incisions. Mice were given a perioperative topical dose of bupivacaine (0.25%, 2 mg/kg) and an additional dose 24h following surgery. Mice recovered in a partially-warmed cage before being returned to their home cage. To prevent cannibalization of pups by dams following surgery, pups were only returned to the home cage with the dam once completely ambulatory. Mice were given an

additional topical dose of bupivacaine 24h following surgery, monitored for five days, weaned at P21, and aged to adulthood in standard group housing conditions.

Behavioral paradigms and analysis

Mice were tested on a set of two behavioral tests twice for within-subject controls: once prior to ("pre") and once after social defeat stress ("post"). Groups are presented as GFP-control (AAV-Gfp, no social defeat stress), GFP-defeat (AAV-Gfp, social defeat stress), SETD7-control (AAV-Setd7 virus, no social defeat stress), SETD7-defeat (AAV-Setd7, social defeat stress). Social interaction and open field exploration testing were performed as previously described [11,16,100]. All behavior was recorded and initially analyzed with Ethovision software.

Social interaction: To assess social avoidance behavior, experimental mice were tested in a two-stage social interaction test. In the first 2.5-minute stage, experimental mice were placed into a novel empty arena (44 x 44 x 20 cm) with an empty enclosure along one wall. Mice were then removed, a novel aggressor mouse was placed into the enclosure in the "social interaction zone," and experimental mice were reintroduced into the arena for an additional 2.5 minutes. Time spent in the interaction zones and in the corners was measured. Social interaction ratio was calculated as time spent in the interaction zone with the aggressor present divided by time spent in the interaction zone without the aggressor present.

Open field: Each mouse was allowed to explore an empty arena (44 x 44 x 20 cm) for 10 minutes and time spent in the center of the arena and overall distance traveled were measured.

Statistical analysis: Behavioral data were graphed and analyzed using Graphpad PRISM software (v. 10.0.3). Outliers were considered to be >2 standard-deviations away from group mean and were removed to avoid type-II error. Main effects and interactions between viral

manipulation and social defeat were determined by mixed effects model to account for within-subject repeated testing, with Sidak's multiple comparisons testing for post hoc pairwise analysis. All significance thresholds were set at $p<0.05$; trends were reported for $p<0.1$.

Nuclear isolation and ATAC-sequencing

Double-transgenic Arc-CreERT2 X Sun1-GFP mice allowed for affinity purification of nuclei expressing the SUN1-GFP-MYC fusion protein in the nuclear membrane by the INTACT approach [42,43]. Brains were harvested from mice after cervical dislocation and rapid decapitation into ice-cold homogenization buffer at either P21 (juvenile time point, three days after recombination) or in adulthood (P60-90). Tissue was used immediately or flash-frozen until use. To isolate nuclei, 14-gauge bilateral punches of the NAc were made on 1mm slices of brain tissue and dounce homogenized in 1.5mL of 1X Unstable homogenization buffer [45]. Homogenate was transferred to 2mL lo-bind tubes then spun down at 500 x g for 5 min (at 4C). Nuclei were collected by iodixanol gradient and counts were collected using a hemocytometer. Nuclei from tagged cells were separated from non-tagged nuclei by incubation with anti-GFP (Sigma; cat#SAB4301138-100UL) and anti-myc (Sigma; cat#: SAB4300318-100UG) antibodies that bound to magnetic Protein G Dynabeads (ThermoFisher; cat#10004D). Total yield of nuclei for all samples was ~600k nuclei, and activated nuclei made up ~10% of the total yield. Both populations of cells were kept and immediately used for ATAC-sequencing. All reactions were performed on ice.

Nuclei from activated and non-activated cells were transposed using Illumina's transposition kit (Illumina; cat#20034198) for 35 minutes for non-activated and 40 minutes for activated nuclei at 37°C at 1000RPM. Fragmented DNA was recovered with Qiagen MinElute

kits (Qiagen; cat# 28604). Samples were amplified and purified according to Corces et al. (2017) using Illumina UD indexes (Illumina; cat#20027214 A or B) and AMPure XP beads (ThermoFisher; cat#NC9959336). Resulting yield of DNA fragment sizes was determined by Bioanalyzer dsDNA High Sensitivity protocol and normalized by concentration for sequencing.

Experience-activated samples: Juvenile samples included 5 Std and 6 ELS samples (ELS: 3 male and 3 female samples, Std-activated: 2 male and 3 female samples) harvested from two different cohorts. Samples from the first cohort (two per group) included pooled bilateral NAc tissue punches from two mice per sample, while samples from the second cohort (3 Std and 4 ELS) were from individual mice. Adult samples included 3 Std and 3 ELS samples (ELS: 2 male and 1 female samples, Std: 1 male and 2 female samples) and were from individual mice.

Non-activated samples: Juvenile samples included 7 Std and 7 ELS samples (ELS: 4 male and 3 female samples, Std-activated: 4 male and 3 female samples). Adult samples included 3 Std and 2 ELS samples (ELS: 1 male and 1 female samples, Std: 1 male and 2 female samples). All samples were from individual mice.

Sequencing and statistical analysis

All samples were sequenced within the Genomics Core Facility of the Lewis-Sigler Institute at Princeton University on a NovaSeq using S1 100nt chemistry to yield ~1.3-1.6 B read depth (adult time point and juvenile cohort 2). NovaSeq using SP 100nt chemistry to yield ~325-400M read depth was used for juvenile cohort 1. FASTQ quality controls were used to show quality of reads on average (>30 Phred) for all samples, and only samples with a Phred score >30 were used. Alignment was performed to mm10 (/grcm38) genome by Bowtie2 and low quality or duplicate reads were filtered out. Libraries were normalized and adjusted for

differences in read depth. Broad peaks were defined using MACS2, with a q-value cutoff of 0.05. Consensus peaksets were created with bedtools. featurecounts was used to create single count matrices for use in differential analysis. Differential analysis was performed using edgeR with TMM normalization to allow for comparisons between samples, and low count regions (less than 2 CPM in at least 8 samples in each neuronal activation class) were removed.

Cell-type analysis

To estimate cell type composition, we quantified rpm/cpm-normalized ATAC-seq reads within 500 bp of any annotated TSS (GENCODE VM23) for each marker gene (Drd1, Drd2, Drd3, Pdyn, Grm8, Chat, Pvalb, Mog, Aqp4, Cxcr1). Normalization and quantification was performed using the R/Bioconductor package BRGenomics (v1.13.0; DeBerardine, 2024[101]; 10.18129/B9.bioc.BRGenomics; [102] and boxplots were generated using ggplot2 (v3.4.4). Visualizations of tracks for these genes were generated using the WashU epigenome browser (https://epigenomegateway.wustl.edu/browser/) using rpm/cpm-normalized bigWig files.

Bioinformatic analysis: genomic features, motif analysis, contacted gene prediction, looping visualizations, and gene ontology analysis

To determine which genomic features differentially accessible regions (DARs) belonged to, the Bioconductor package GenomicDistributions(1.8.0) was used. The mm10 package was loaded and enhancers were added to the list of features (partitionList from November 2020 ENSEMBL build [https://nov2020.archive.ensembl.org/]).

Motif Analysis was carried out using XTREME, of the MEME suite (https://meme-suite.org/meme/tools/xstreme)[51]. Significantly upregulated and downregulated regions were supplied to the motif discovery pipeline, using the HOCOMOCO Mouse v11 FULL

parameter for known motifs. All other parameters were kept at default; an E value of ≤ 0.05 was used as a significance threshold, and motifs were only reported if they appeared in >60% of input regions*. (*Except for ERR2/RARA and FOXJ in the activated juvenile condition, which were each present in 55% of input regions, and IRF3 in the adult non-activated population, in which 40% and 20% of open DAR regions had respectively 1 or 2 IRF3 motifs.)

In order to identify genes putatively contacted and regulated by DARs, we used the T-Gene tool within MEME-suite [49]. We first lifted-over our ATAC-seq peaks from mm10 to mm9 using a chain file downloaded from the UCSC genome browser (http://hgdownload.cse.ucsc.edu/goldenpath/mm10/liftOver/mm10ToMm9.over.chain.gz) and the re-implementation of UCSC liftOver found in the R/Bioconductor package rtracklayer (v1.58.0; [103]). During liftOver, peaks that failed to lift over, or lifted over into multiple non-contiguous peaks, were dropped from this analysis (n=0 for adultBB_open_annotations.bed; n=1 for adultBB_closed_annotations.bed and juvBB_closed_annotations.bed; and n=3 for juvBB_open_annotations.bed). Regions that survived the lift over were then used in T-Gene. Output of T-Gene is limited to 1000 putative gene contacts. We applied a cutoff of $p<0.1$ to identify the strongest contact predictions. We visualized contacts between regions of open chromatin and potential-contacted genes using the WashU Epigenome Browser (http://epigenomegateway.wustl.edu/; [104–107]) with mm10 Brain Tracks from the 4D Nucleome Network, in Arc mode [50]. Potential chromatin contacts are shown with ATAC-seq tracks as indicated. Publicly available and pre-loaded brain Hi-C data was used for looping visualizations between gene promoters and putative regulatory elements. Hi-C data was collected from the dataset (https://data.4dnucleome.org/experiment-set-replicates/4DNESRWDFFF8/), from [48].

Gene Ontology (GO) biological process terms were generated using the DOSE R package for genes most likely contacted by open and closed regions and plotted by –log10(adjusted p-value).

Gene expression predictions

In order to predict the impact of changes in chromatin accessibility on gene expression response to adult stress, we filtered lists of genes putatively-contacted by DARs to only those consistently associated with open or consistently associated with closed chromatin at both juvenile and adult time points. We then used previously published RNA-seq from adult NAc to determine how the expression of these genes changed from Std-Control conditions with ELS, Social Defeat, or ELS+Defeat [9]; GEO GSE89692; differential expression calculated by DEseq2). Genes with expression <5 CPM were excluded. We visualized the log2Fold-Change of these expression changes using Morpheus (https://software.broadinstitute.org/morpheus) with rows transformed by subtracting row mean and dividing by row standard deviation. Hierarchical clustering was then performed on the gene rows. We calculated a "RNA predictive factor" as [L2FC * (1-p-value)] such that stronger statistical significance and greater fold-change would be more heavily weighted, and that positive L2FC values (corresponding to increases in gene expression as would be predicted from open chromatin at gene regulatory regions) would produce positive predictive factors, and negative L2FC (corresponding to decreases in gene expression, opposite to open chromatin predictions) would produce negative predictive factors. For RNA predictive factors for closed regions, [-L2FC * (1-p-value)] was used so that more positive values always indicated higher predictive value, assuming closed chromatin would predict decreased gene expression. Predictive factors were then averaged across all genes for

each group. Analysis of group differences was performed by two-tailed one-sample t-test with a null of 0 (no predictive value) and alpha=0.05.

Linear mixed effects analysis for chromatin development

A linear mixed effects model was used to allow both fixed and random effects given multiple levels of variability in the data (i.e., ELS, sex, and age) and non-independence among the chromatin regions (i.e. each individual sample contributed multiple open chromatin regions), using the LME function in R. Normalized counts represent the relative accessibility of chromatin. Individual samples were taken as random effects in this hierarchical model to examine the significance and interaction effects of stress and age, stress and sex, and the individual fixed effects. Beta estimates, standard-error, and p-values are reported.

Histone modification analysis

Analysis of post-translational histone modifications in NAc across three developmental time points (P21, P35, P70-90, n=3 males/group/age) was done by re-analyzing previously published histone post-translational modification mass spectrometry (data from[6]). This data represents percentage of each measured histone modification per fragmented histone peptide (for example, percentage of unmodified histone-3 lysine-4, H3K4me1, H3K4me2, and H3K4me3). Data for H3K27me2 and H3K27me3 are summations of the modification of interest alone or in combination with any H3K36 modification measured on the same peptide fragment. For each histone modification of interest, two-way ANOVA was done to assess main effects and interactions of ELS with age, followed by post-hoc testing with Sidak's multiple comparisons correction to assess impact of ELS at each age. Alpha=0.05.

Immunohistochemistry and imaging

To confirm viral targeting of the NAc, following behavior testing, a subset of mice was anesthetized by i.p. injection of 100 mg/kg ketamine and 10 mg/kg xylazine, then euthanized and transcardially perfused with ice-cold saline followed by 4% paraformaldehyde (PFA). Brains were dissected and submerged in fresh 4% PFA at 4°C overnight, then stored in 30% sucrose solution for an additional 2 days (until the tissue became isotonic). Dry ice was used to flash-freeze the brains, after which they were kept at -80°C until slicing. For slicing, the frozen brains were embedded in Tissue-PlusTM O.C.T. Compound (Fisher Scientific) to maintain structural integrity. A cryostat held at -20°C was used to slice the brains at 20µm. Brain slices were mounted directly onto SuperfrostTM Plus microscope slides (Fisher Scientific) and stored at -20°C ahead immunohistochemical staining.

Immunohistochemistry entailed the following steps (at room temperatures unless noted): (1) Rinsing the slices in 1 X phosphate-buffered saline (PBS) 3 times for 5 min each. (2) Incubating the slices in 1.5% normal donkey serum (NSD) in 1 X PBS-Triton X-100 solution for 1 h. (3) Incubating the slices in primary antibody [1:500 Anti-Green Fluorescent Protein (Chicken) Antibody (Aves Labs); 1:150 SETD7 Mouse Monoclonal Antibody (OriGene); and 1.5% NSD in 1 X PBS-Triton X-100 solution] overnight at 4°C. (4) Rinsing the slices in 1 X PBS 3 times for 5 min each. (5) Incubating the slices in a secondary antibody solution [1:500 Alexa Fluor® 488 AffiniPure Donkey Anti-Chicken IgY (IgG) (H+L) (Jackson ImmunoReseach Labs); 1:500 Cy™3 AffiniPure Donkey Anti-Mouse IgG (H+L) (Jackson ImmunoReseach Labs); and 1.5% NSD in 1 X PBS-Triton X-100] for 1 h. (6) Rinsing the slices in 1 X PBS 3 times for 5 min each. (7) Incubating the slices in 0.0025% DAPI solution for 5 min. (8) Rinsing

the slices in 1 X PBS 3 times for 5 min each. Following the last rinse, Fluoromount-G® mounting media (SouthernBiotech) was used to coverslip the immune-stained slices. Samples were kept at 4°C until imaging. Fluorescent imaging was performed with a Nanozoomer S60 slide scanner (Hamamatsu). All images were processed using the ImageJ (version 1.2.0) software containing the FIJI plugin (NIH, Bethesda, MD, USA).

CHAPTER 3: A brief investigation into a method of specific chromatin manipulation, *in vitro*

***Note:** The dSaCas9-SETD7 [pVC 319: EFS-dSaCas9-Setd7] plasmic was designed and generated by Dr. Julie-Anne Balouek and Dr. Catherine Pena in collaboration with the Viral Core at the Princeton Neuroscience Institute. The experiments described here with this plasmid were run in collaboration with Dr. Hye Ji J. Kim and Laura Hirschfield ('24).* All authors have consented to their work being presented in this dissertation.

Components of Chapter 3 have been presented in the following formats:

- Rebekah L. Rashford (Dev Bio Meeting 2023 - talk)

3.1 Abstract

Early-life stress (ELS) has been shown to be a high predictor for development of mood disorders and their behavioral analogs in rodent models. Chapter 2 shows that changes in chromatin accessibility reflect the impact of ELS on epigenetic response mechanisms. Whether direct manipulation of chromatin architecture can modulate behavioral susceptibility has yet to be established. Here, I aimed to explore a method of CRISPR/dCas9 manipulation at specific genomic sites in Neuro-2a cells (N2a) to directly change chromatin accessibility. The promoter and two enhancers for the gene *Fos* were targeted to pilot this approach. Ultimately, while the different types of manipulation complexes tested (Cre-dependent dSaCas9-KRAB, dSaCas9-KRAB, and dSaCas9-SETD7) could be expressed in N2a cells, no functionality of the vectors could be elucidated. The shortcomings and potential improvements in this method are discussed.

3.2 Introduction

Early life stress (ELS) is known to increase the risk for development of psychological disorders upon additional stress experience. These effects of ELS on altered behavioral phenotype development are reflected at the transcriptional level in the NAc and VTA[9]. Previous experiments show that the effects of ELS can also be seen at the level of chromatin accessibility, and may give insight into the mechanisms modulating observed transcriptional changes[19]. Chromatin adopts more of an open conformation closely following the ELS experience, with a spread of the ELS effect to the wider cell population over time. Further, I've shown that overexpression of the methyltransferase SETD7, responsible for H3K4me1 to induce open but not active chromatin, is sufficient for promoting behavioral susceptibility to future mild stress exposure. To further explore the establishment of a causal relationship between chromatin conformation and transcriptional profile and behavioral outcomes, we developed a potential approach to manipulate chromatin state at regulatory loci to test sufficiency and necessity in behavioral susceptibility or resilience to future stress experience. I hypothesize that closing regions of chromatin found to be significantly differentially opened after ELS will prevent the development of altered transcriptional and behavioral profiles. I further posit that the reverse is true: opening regions of chromatin found to be significantly differentially closed after ELS will increase the likelihood of altered transcriptional and behavioral susceptibility.

To do this, we employed the widely used CRISPR/dSaCas9 approach, in which a catalytically dead Cas9 enzyme fused to a functional effector enzyme is guided to a specific genomic region by guide RNAs (gRNAs)[108–111]. We used a smaller Cas9 variant, isolated from *Staphylococcus aureus*, SaCas9[108]. The effectors used here are the functional domain of the

Krüppel-associated box protein (KRAB) to decrease accessibility (dSaCas9-KRAB), and the whole SETD7 enzyme to increase accessibility (dSaCas9-SETD7). We first piloted these manipulation complexes on their functionality at the promoter and two enhancer regions of the *Fos* gene in Neuro-2a (N2a) cells (male neural progenitor cells, an *in vitro* model for neuronal cells in the brain). As *Fos* is an immediate early gene (IEG), its modulated expression measured by quantitative PCR (qPCR) due to the CRISPR manipulation will be easily detected after activation of N2a cells, which models the stimulation provided by the ELS experience[112]. I hypothesize that dSaCas9-KRAB will drive a blunted *Fos* expression at the promoter and enhancer regions after stimulation of the cells. For dSaCas9-SETD7, augmented expression of *Fos* at experimental target regions after cellular stimulation should be observed.

We tested the efficacy of this experimental approach using confocal imaging and qPCR after plasmid transfection of the manipulation components. Each manipulation complex and gRNA was packaged into a separate DNA plasmid with an AAV backbone due to the size constraints of AAVs (~5kb). The plasmids were concurrently transfected into the N2a cells. *Npas4*, a neuron-specific IEG was used as a control to detect proper activation of the N2a cells.

3.3 Results

3.3.1 dSaCas9-KRAB

To close chromatin by promoting heterochromatin formation, we decided to use a form of the catalytically dead SaCas9 protein fused to the enzymatic domain of KRAB. This approach has been widely used in the literature[112]. We adopted it to be utilized in two different ways: (1) in a Cre-dependent manner in which dSaCas9-KRAB would only be expressed in cells also housing

the Cre-recombinase enzyme, and (2) in a widespread fashion in which dSaCas9-KRAB would be administered throughout the cellular population.

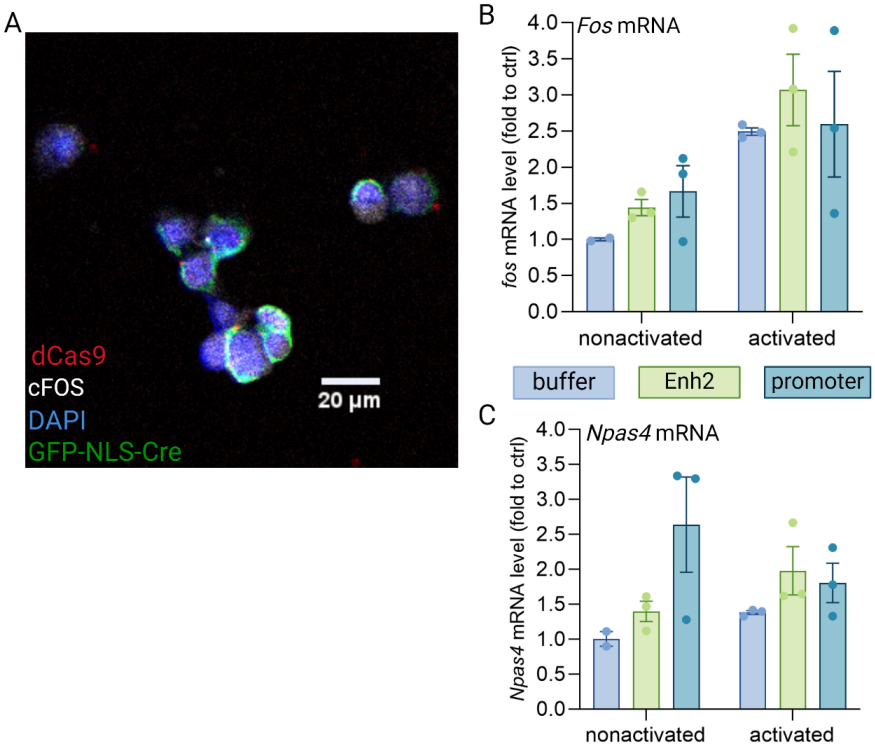


Figure 1: Cre-dependent KRAB -- dSaCas9-KRAB can be integrated and expressed in N2a cells. (A) Confocal image of activated N2a cells after transfection and activation. 40X-oil, scale bar = 20um. (B) mRNA levels for *Fos* and (C) mRNA levels for *Npas4* after transfection and activation. Coral blue: transfection with transfection kit Buffer EC (no plasmid - negative control). Light Green: gRNA for *Fos* Enhancer 2. Sea Blue: gRNA for *Fos* gene promoter. Error bars represent S.E.M.

We first attempted manipulation of the *Fos* gene and one of its enhancers using the Cre-dependent approach. The motivation for Cre-dependence emerged from the desire to determine whether specific manipulation only in an activated subset of cells would be sufficient to influence behavioral resilience. Our double-transgenic mouse setup with ArcCreER^{T2} mice drove the expression of the Cre recombinase in activated cells in response to stress or the standard-reared stimulation. I concurrently transfected the 3 plasmids housing the Cre-recombinase, manipulation Cas9 complex, and gRNAs into N2a cells.

We found, as expected, that N2a cells expressed Cre-recombinase, dSaCas9-KRAB, and FOS in activated cells (Figure 1A). While qualitatively it may seem as though blunting may have occurred in manipulation at the *Fos* promoter, no statistical difference was detected (Figure 1B). The varied activation of *Npas4* in the activated condition may be due to incomplete development of the N2a neural progenitor cells, and thus underdeveloped signaling NPAS4 signaling (Figure 1C). Therefore, conclusions from *Npas4* data here and throughout are drawn with caution. This data suggested some off-target or unintended effects of the Cas9 complex. Because of this, we sequenced the gRNA plasmids and confirmed they were faulty guides.

After this experiment, we updated two aspects of the approach: (1) we updated the *Fos* gRNAs and confirmed by sequencing they represented the genomic regions we intended to target, and (2) we decided to manipulate chromatin in a widespread manner with a non-Cre-dependent plasmid.

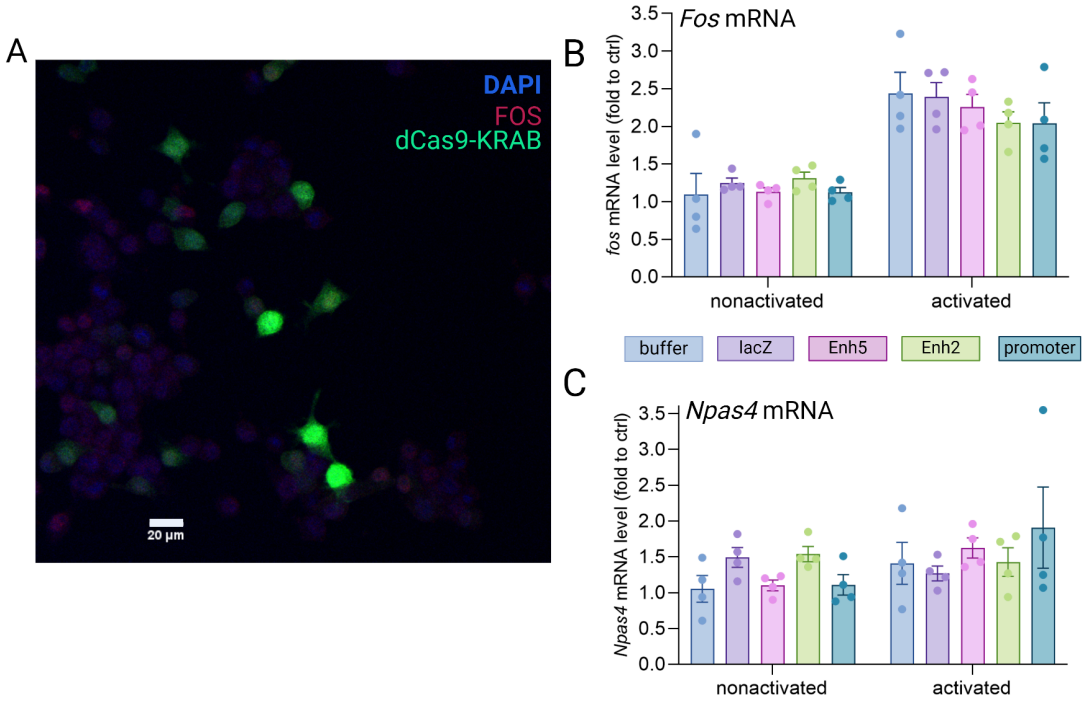


Figure 2: Non-Cre-dependent KRAB -- dSaCas9-KRAB can be integrated and expressed in N2a cells. (A) Confocal image of activated N2a cells after transfection and activation. 40X-dry, scale bar = 20um. (B) mRNA levels for *Fos* and (C) *Npas4* after transfection and activation. Coral blue: transfection with transfection kit Buffer EC (no plasmid - negative control). Purple: gRNA for *lacZ*. Pink: gRNA for *Fos* Enhancer 5. Light Green: gRNA for *Fos* Enhancer 2. Sea Blue: gRNA for *Fos* gene promoter. Error bars represent S.E.M.

In this case, we used an identical setup as the Cre-dependent experiment, save for the updated guides, adding another *Fos* enhancer target and a loci target of *lacZ* as a positive control (*lacZ* gene is not present in mouse genome and so should not result in detectable manipulation of *Fos*). After concurrent transfection with the dSaCas9-KRAB and gRNA plasmids, again we confirmed the N2a cells were able to express the manipulation complexes (Figure 2A). *Fos* mRNA levels increased with activation as expected, but the level of blunting that activation by the manipulation complex, if any, was minimal and not significant for complexes targeting the promoter or any of the enhancers of *Fos* (Figure 2B). The level of *Npas4* mRNA again was variable or not affected by activation (Figure 2C). Further experiments with more samples could be used to better determine the efficiency of manipulation of *Fos* through targeting its promoters or enhancers.

Overall, both the specific and widespread manipulation complexes using dSaCas9-KRAB seemed to get the complexes into the cells to express, but the efficiency at *Fos* was uninterpretable and further experiments need to be done to flesh out the potential of this manipulation tool in the context of promoting resilience to stress.

3.3.2 dSaCas9-SETD7

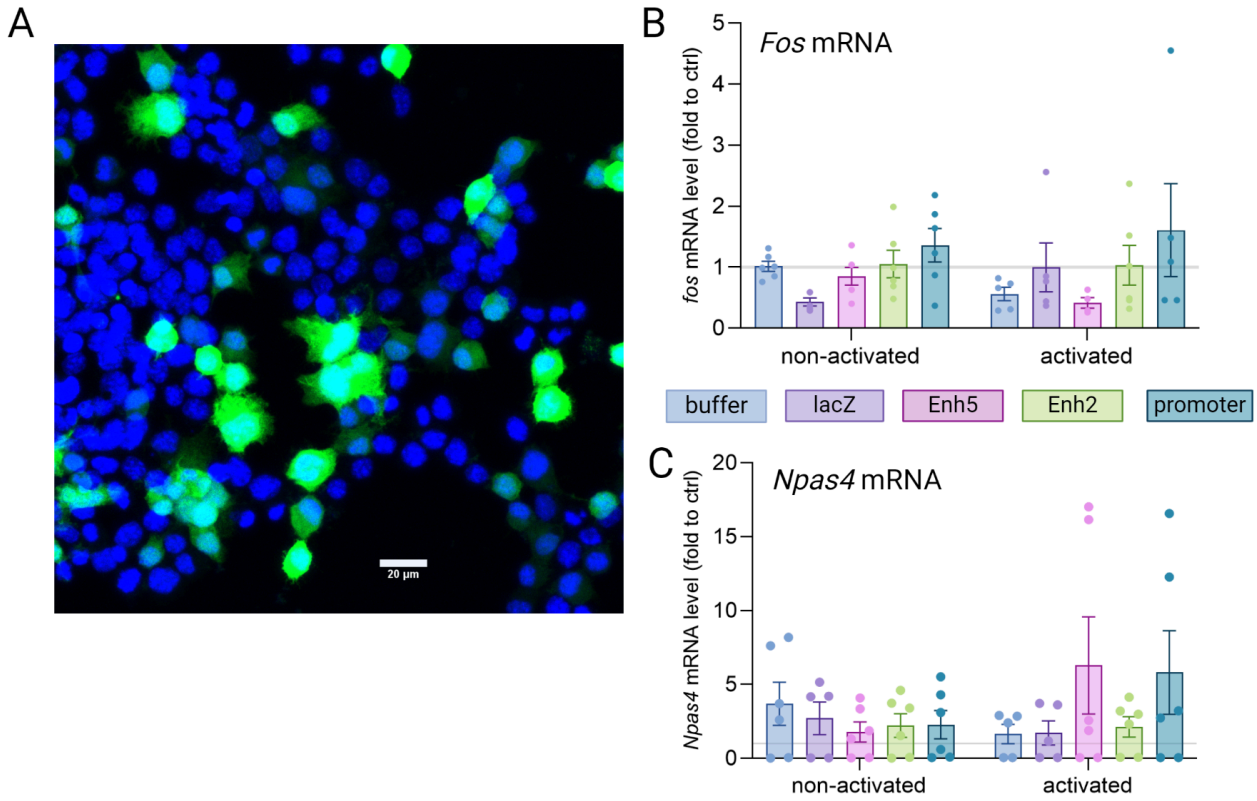


Figure 3 – dSaCas9-SETD7 can transfect the nucleus of N2a cells *in vitro*. (A) Confocal images generated by Dr. Hye Ji J. Kim showing two areas of cells transfected with dSaCas9-SETD7 plasmids. 40X-oil, scale bar = 20um. (B) mRNA levels for *Fos* and (C) *Npas4* after transfection and activation. Coral blue: transfection with transfection kit Buffer EC (no plasmid - negative control). Purple: gRNA for *lacZ*. Pink: gRNA for *Fos* Enhancer 5. Light Green: gRNA for *Fos* Enhancer 2. Sea Blue: gRNA for *Fos* gene promoter. Error bars represent S.E.M.

To open chromatin, we decided to again use a form of the catalytically dead SaCas9 protein, but this time fused to the SETD7 enzyme. All aspects of this approach were new, as a dSaCas9-SETD7 complex has not, to our knowledge, been constructed yet. SETD7 is a mono-methyltransferase that marks open enhancers by H3K4me1 deposition.

We excitingly found that dSaCas9-SETD7 could be integrated into and expressed by N2a cells, which is a promising first step in this tool development process (Figure 3A). However, much like the experiments with dSaCas9-KRAB, we did not observe evidence of reliable

functionality at targeted regions for either *Fos* or *Npas4*. Yet the issues that could be begetting the results with this vector could be more complex than those plaguing the KRAB manipulation. As this is a new construct, we do not know what mechanism of action occurs at the sites where the complex is brought by the gRNAs. Two aspects of the mechanism of action need to be determined before further optimization is conducted. First, whether the complex has a transient effect. This is improbable because (1) the effect of SETD7 in general lasts long enough for previous experiments in the lab to be conducted[7,19] and (2) the H3K4me1 mark itself is stable, so the potential transience of the complex itself does not need to be considered.

Secondly, the complex could be drawn to *Fos* sites that are already open. In this case, depositing a methyl group here would not functionally impact that site. Further experiments potentially targeting other genes and regions will continue to be conducted in collaboration with Dr. J. Kim to explore this possibility.

3.4 Discussion

The CRISPR-dSaCas9-effector system has been a go-to for experimenters since its boom of popularity in the early 2010s[113]. Utilizing this approach to target specific gene regulatory elements *in vivo* would be a cool way to rank necessity of certain regions as more important for driving epigenetic susceptibility to future stress stimulus, or if a concerted approach of many regions is necessary to produce the observed ELS-induced hyper-response to future stress.

In the case of dSaCas9-KRAB, we recapitulated from the literature that the complexes, both Cre-dependent and nonspecific, could be expressed well in N2a cells. Yet the functionality of these complexes concerning targeted chromatin manipulation need more optimization for conclusions to be drawn in this context, either through using different versions of the designed

gRNAs or picking a different pilot gene altogether. The original paper using this plasmid reported low efficiency with their Cre-dependent approach, so another avenue for improvement in this method development could be transfecting at a higher concentration and/or have more samples manipulated to elicit an effect[111]. Additionally, the use of *Npas4* was not informative in these experiments, and so a potential improvement for this would be choosing a different IEG to measure activation within the cells.

An exciting aspect of the experiments using the novel dSaCas9-SETD7 was that this custom complex could be expressed in cells. This is promising in continuing to determine the functionality of the construct, although issues determining its mechanism of action have yet to be elucidated.

The approaches described here manipulating cells in a generalized way (non Cre-dependent dSaCas9-KRAB and dSaCas9-SETD7) could impact both neuronal and non-neuronal cell types. This brings up interesting questions of the role that non-neuronal cells may play in ELS response originally raised in Chapter 2 (See Chapter 2.4). The Cre-dependent dSaCas9-KRAB system would be an exciting tool to utilize in determining whether manipulation of chromatin architecture within only the activated subset of the neuronal population is sufficient to promote resilience to future stress.

Overall, these experiments were interesting avenues that we were excited to explore. These methods could become useful tools in future studies aiming to use changes in chromatin architecture to prime specific genomic sites *in vivo* to be reactive or resilient to future stress.

3.5 Methods

N2a cells

Cells were ordered from ATCC (#CCL-131) and kept frozen in a liquid nitrogen chamber until use. Purchased cells were seeded and split to create stock of 16 vials of passage 2 or 3 (P2, P3) cells for further experimental use.

Transfection, Imaging, and qPCR

Plasmid transfection into N2a cells: Transfection by Effectene© was performed according to Qiagen protocol, optimized for 12-well and 24-well plates (Qiagen: cat# 301247).

Confocal Imaging: To prepare cells for confocal imaging, a subset of freshly thawed passage 3 cells were seeded onto a poly-L-lysine (0.1%) treated coverslip in a 12-well plate for ~65% confluency. 24 hours post seeding, transfection was performed. Complexes remained on the cells in the media for 6-12, then was changed to avoid cell toxicity due to transfection complexes. 48 hours after transfection, when cells reached ~85% confluency, cells were activated with 70uL of 1M potassium chloride. One hour after activation, cells were fixed with 500uL of 4% paraformaldehyde for 20 minutes at room temperature. Cells were then washed, prepared with 1.5% normal donkey serum for antibody blocking for 1 hour at room temp, and stained with anti-Cas9 (isolated from mouse; Sigma: cat# SAB4200701-100UL) and anti-cFos isolated from rabbit (Cell Signaling: cat # 2250), each at a 1:500 dilution overnight at 4C. Cells were washed and treated with secondary antibodies donkey anti-Mouse (fluor 488; Thermo Fisher: cat# NC0192065) and donkey anti-rabbit (fluor 555; Thermo Fisher: cat# NC9805921), incubating for 1 hour at room temperature protected from light. Cells were washed, stained with DAPI and mounted onto coverslips and imaged on a Leica confocal microscope.

qPCR: To determine the effect of the manipulation complexes on *Fos* expression, a subset of cells were harvested and the RNA was extracted according with the Qiagen RNeasy Micro Kit, using the standard protocol (Qiagen: cat# 74004). cDNA was then generated from RNA molecules by reverse transcription (Thermo Fisher: cat# 4368814). qPCR was performed with in-house primers for *Fos*, along with housekeeping gene *Hprt* and immediate early gene *Npas4* as controls. Standard SYBR and deltaCt qPCR methods were used.

Plasmid and Primer Design

Primer and gRNAs ordered as DNA fragments (gBlocks) were transformed into AAV plasmid backbones using a New England Biolabs Transformation kit (NEB: cat# C3040H) according to kit protocol, then amplified through bacterial culture. Plasmids from bacterial cultures were recovered with Qiagen's Mini- or Maxi-prep kit according to kit protocol (Qiagen: cat# 12123-mini, 12163-maxi).

Guide RNA (gRNA) gBlocks were designed according to Church et al 2013: "the following 457bp fragments bear all components necessary for gRNA expression for *Sa*Cas9, namely: U6 promoter (318bp) + target sequence (22bp) + guide RNA scaffold (77bp) + termination signal (6bp). gBlocks were synthesized and ordered with IDT." All guide RNA plasmids were validated through sequencing by Plasmidsaurus.

Primers for gRNAs, *Fos*, and *Npas4*, were designed with Benchling, according to adapted protocol by Dr. Liza Brusman from Zoe Donaldson's lab.

Plasmid	Product
AAV backbone (gRNAs and dSaCas9-SETD7)	https://www.addgene.org/89060/
GFP-Cre plasmid	https://www.addgene.org/49056/
Cre-dependent dSaCas9-KRAB	https://www.addgene.org/113686/
dSaCas9-KRAB (non-Cre dependent)	https://www.addgene.org/113677/
dSaCas9-SETD7 [pVC 319: EFS-dSaCas9-Setd7, 1.69 ug/ul - 10ul aliquot]	This plasmid was a custom construct in collaboration with our lab and the Princeton Viral Core in the Princeton Neuroscience Institute.

Primer	Sequence
forward_FosgRNA1.p1	CCATATTAGGACATCTGCGTCA
forward_FosgRNA1.p2	GACCATCTCCGAAATCCTACAC
forward_FosgRNA2.p1	CCATATTAGGACATCTGCGTCA
forward_FosgRNA2.p2	CCATATTAGGACATCTGCGTCA
reverse_FosgRNA1.p1	GAATGGATGGACTTCCTACGTC
reverse_FosgRNA1.p2	GAATGGATGGACTTCCTACGTC
reverse_FosgRNA2.p1	GAATGGATGGACTTCCTACGTC
reverse_FosgRNA2.p2	CGTCTTGGCATACATCTTTCAC
Fos-mRNA_West-F1	TTTATCCCCACGGTGACAGC
Fos-mRNA_West-R1	CTGCTCTACTTTGCCCCTTCT
Npas4-mRNA_West-F1	GCTATACTCAGAAGGTCCAGAAGGC
Npas4-mRNA_West-R1	TCAGAGAATGAGGGTAGCACAGC
Fos_ChIP_Promoter_West-F1	CAAGACGGGGGTTGAAAGCC
Fos_ChIP_Promoter_West-r1	TCACTGCTCGTTCGCGGAAC
Fos_ChIP_3UTR_West-F1	TGACACCTGAGAGCTGGTAGTTAG
Fos_ChIP_3UTR_West-r1	ATCAGCTGCACTAGATACAATCCA
Fos_ChIP_Enh1_West-F1	TAAAGCCTATTGCCGTGACCTG

Fos_ChIP_Enh1_West-r1	TCTTTCCCTTACAATGCCCTTACC
Fos_ChIP_Enh2_West-F1	GTCTACTGTCTGAGGAGAAGTGGTTAG
Fos_ChIP_Enh2_West-r1	AGAACAGATTCTGGAACAGTGTCTAC
Fos_ChIP_Enh5_West-F1	GGATTCAGTGCTACCCCCTG
Fos_ChIP_Enh5_West-R1	CAGGCTGCTACCAGACAGAG
Fos_ChIP_Enh5_West-F2	AGAAAACGTGCTCAACGTGC
Fos_ChIP_Enh5_West-R2	GCCGACGTCCTGACACTAAA
Fos_ChIP_Enh5_West-F3	CCCCACAGATGACATCGCTC
Fos_ChIP_Enh5_West-R3	GGTATGAACGGCTCCGACTG

CHAPTER 4: Summary and Discussion

4.1 Summary

Early-life stress is a high predictor for psychiatric disorders in adulthood. The impact it has on the formation of epigenetic memories, namely at the level of chromatin accessibility through changes in TF and histone modification expression, could underlie this increased risk in behavioral susceptibility. The global epigenetic profile generated by these molecular memory states can then be activated, particularly in cells of the brain's reward pathway, to robustly respond to additional stress experienced during the lifetime to drive negative behavioral outcomes. How these epigenetic memories can be formed by ELS is an interesting question and was explored by examining the process of remodeling chromatin accessibility into a vulnerable state.

Looking into the effect of ELS on postnatal chromatin development (in the nucleus accumbens (NAc) specifically) provided foundational understanding of ELS and how it affects lifelong psychiatric risk. Investigation of the chromatin accessibility profiles at both the juvenile and adult time points showed initial chromatin opening in cells activated by the stress experience, followed by a diffusion of the ELS impact over time to the wider NAc cell population. Interestingly, regulatory elements were most impacted, and TF motif and GO analysis of differentially accessible regions and their potential gene contacts showed development, stimulus response and immune response pathways to be most affected by ELS. An experiment overexpressing the enzyme SETD7 to increase widespread H3K4me1 deposition throughout the NAc showed sufficiency of increasing behavioral susceptibility to future stress experiences.

The findings shown in our empirical study led us to question whether or not manipulation at specific genomic sites would also lead to modulation of susceptibility to future stress. This prompted a brief investigation into piloting a CRISPR/dSaCas9 system to open or close chromatin at the promoter and enhancers of the *Fos* gene. Ultimately this path ended up down a convoluted avenue. We found that while the complexes could be integrated into the genome as expected (a replication of knowledge in the case of dSaCas9-KRAB, but excitingly novel in the case of dSaCas9-SETD7), the functionality of the manipulation of the *Fos* gene was unclear. Improvements and optimizations to this approach through potentially updating gRNAs or further elucidating the complexes' mechanisms of the action could remediate this method design.

4.2 Remaining Questions and Future Directions

The research presented here shows exciting strides forward in discovering how changes at the epigenetic level due to ELS can predict susceptibility to future stress. Yet there remain many interesting questions and potential avenues for future research. Brief discussion on some of these topics are described here.

An interesting future direction of research would deepen exploration into the steps in the mechanism stabilizing the epigenetic landscape after ELS to prime future transcriptional reactivity. What specific steps or pathways are being utilized to set up this primed chromatin state? Is the global molecular response after ELS driving specific changes at particular TF and histone modification loci that are then stabilized in the epigenetic memory, or the other way around? Many experiments have tested whether specific manipulations are sufficient or necessary for mediating future stress susceptibility after ELS, but it remains to be speculated how potential interactions between these individual factors affect the concerted vulnerability of

the epigenetic landscape. Understanding the individual serial events impacting the epigenetic mechanisms after ELS may reveal further understanding. This could shed light on whether and how possible interactions between specific factors could be contributing to the epigenetic memory of ELS to increase vulnerability.

Relatedly, a necessary question to complete this mechanism of the stability of ELS-induced epigenetic memory would be exploring the nature of TF binding to differentially accessible regions. Are all transcription factors binding with stable interactions, forming complexes to prime chromatin architecture and await further stress stimuli, or are most transient, acting at the level of signaling other epigenetic modifiers and transcriptional machinery to prepare for a repeat of stimuli? Both scenarios could be occurring, but in what order and to what extent? Biochemical and protein domain experiments such as chromatin immunoprecipitation followed by sequencing (ChIP-seq) could uncover how TFs bind to DNA to maintain primed chromatin architecture.

Another question concerns the switch in expression (downregulation after ELS to upregulation in adulthood, or vice versa) for some specific genes, TFs, or histone modifying enzymes over time. Many of the changes to TFs and histone modifications and their associated enzymes go through some type of modulation during adolescence that persists into adulthood[5,6,15]. One thought is that the developmental timescale and process could be altered by the effect of ELS. Indeed, there is evidence of altered developmental timing because of ELS[19]. But could the arrival at the normal appointed end of a critical developmental period drive an overcompensation or rebalancing attempt for these genes and regions whose developmental trajectory was perturbed by ELS? Analyzing genome-wide association studies from both ELS

and typical developmental trajectories over a time course could provide insight on these interesting changes in expression levels over the lifespan to maintain susceptibility to future stress.

Considering that epigenetics ultimately impacts cellular activity, future research should continue to elucidate how ELS impacts the brain's overall activity network to increase susceptibility. For example, in the case of the NAc which is primarily composed of inhibitory medium spiny neurons (MSNs): How does epigenetically increasing sensitivity to further stress stimuli in a primarily inhibitory region work to signal to other regions to be in a state of behavioral vulnerability? We see that there's an effect of histone modifications in MSNs in contexts that affect repression/inhibition[6,19]. Indeed, it has been shown that a majority of genes impacted by ELS are downregulated in NAc[9]. But does widespread downregulation necessarily signal the potential increase in activation of surrounding neurons or projecting excitatory neurons from other regions? Electrophysiological techniques could start to uncover the downstream, network wide impact of the epigenetic effects on cells affected by ELS.

4.3 Final Conclusions

In conclusion, the work presented in this thesis adds to the body of knowledge concerning the impact that ELS has on heightened risk for future psychiatric disorders by investigating changes in chromatin architecture and development. It is my hope that the findings and discussion herein will support and spur future research aimed at developing novel techniques to alleviate or prevent psychiatric and behavioral disorders in adulthood caused by early-life stress.

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Additional Quotes:

"Intelligence is one of the greatest human gifts.

But [do not let] a search for knowledge [drive] out the search for love."

-*Flowers for Algernon* (adapted) By Daniel Keyes

"He who testifies to these things says, 'Surely I am coming soon.'

Amen. Come, Lord Jesus! The grace of the Lord Jesus be with all. Amen."

Revelation 22:20-21, Holy Bible, ESV