

# Temporally specific gene expression and chromatin remodeling programs regulate a conserved *Pdyn* enhancer

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## Abstract

## Summary

Neuronal and behavioral adaptations to novel stimuli are regulated by temporally dynamic waves of transcriptional activity, which shape neuronal function and guide enduring plasticity. Neuronal activation promotes expression of an immediate early gene (IEG) program comprised primarily of activity-dependent transcription factors, which are thought to regulate a second set of late response genes (LRGs). However, while the mechanisms governing IEG activation have been well studied, the molecular interplay between IEGs and LRGs remain poorly characterized. Here, we used transcriptomic and chromatin accessibility profiling to define activity-driven responses in rat striatal neurons. As expected, neuronal depolarization generated robust changes in gene expression, with early changes (1 h) enriched for inducible transcription factors and later changes (4 h) enriched for neuropeptides, synaptic proteins, and ion channels. Remarkably, while depolarization did not induce chromatin remodeling after 1 h, we found broad increases in chromatin accessibility at thousands of sites in the genome at 4 h after neuronal stimulation. These putative regulatory elements were found almost exclusively at non-coding regions of the genome, and harbored consensus motifs for numerous activity-dependent transcription factors such as AP-1. Furthermore, blocking protein synthesis prevented activity-dependent chromatin remodeling, suggesting that IEG proteins are required for this process. Targeted analysis of LRG loci identified a putative enhancer upstream of *Pdyn* (prodynorphin), a gene encoding an opioid neuropeptide implicated in motivated behavior and neuro-psychiatric disease states. CRISPR-based functional assays demonstrated that this enhancer is both necessary and sufficient for *Pdyn* transcription. This regulatory element is also conserved at the human *PDYN* locus, where its activation is sufficient to drive *PDYN* transcription in human cells. These results suggest that IEGs participate in chromatin remodeling at enhancers and identify a conserved enhancer that may act as a therapeutic target for brain disorders involving dysregulation of *Pdyn*.

## eLife assessment

This is an **important** study that uses chromatin accessibility as a measure to determine the impact of neuronal activity on the state of chromatin regulatory elements in striatal neurons. The authors provide **convincing** evidence of how Pdyn gene expression is highly dependent on a distal regulatory genomic region both at basal and upon neuronal activation in this particular system, a mechanism conserved as well in human neuronal cells. Although the basic idea of accessibility changes have been studied before, this paper ties previous findings all together in one place and uses the analysis to identify a functionally relevant and conserved enhancer for the prodynorphin gene with potential relevance for neuropsychiatric disorders beyond basic cellular neuroscience. The study will be of interest to neuroscientists studying the striatum, neuronal plasticity, or related neuropsychiatric disorders.

## Introduction

Experience-dependent cellular adaptations within the brain circuits that control motivated behaviors are critical for learning, memory, and longterm behavioral change. In psychiatric disorders such as drug addiction, these cellular changes are hijacked to drive maladaptive behavioral changes that promote drug seeking<sup>1,2</sup>. Furthermore, mutations that alter the function of activity-dependent transcription factors have been implicated in a host of neurodevelopmental and autism spectrum disorders<sup>3,4</sup>. Adaptations to novel stimuli are regulated by temporally and functionally distinct activity-dependent transcriptional programs. For example, various forms of neuronal activation result in the rapid induction of immediate early genes (IEGs), including transcription factors such as *Fos* (aka c-Fos), *Npas4*, and *Nr4a2*. These genes follow a temporally dynamic profile, with elevated expression within 1 h of a stimulus and rapid return to baseline levels. In contrast, the same stimuli promote expression of a more delayed gene expression program, termed late response genes (LRGs)<sup>5,6</sup>. This set of genes includes kinases, neurotrophic factors, and neurotransmitter receptors. Current models of activity-dependent gene expression suggest that these distinct transcriptional waves work together to promote enduring cellular and behavioral adaptations.

While genes within the IEG expression program are required for cellular and behavioral changes following stimulation, the exact mechanisms by which they contribute to LRG expression and the functional consequences of this process remain poorly characterized. Recent evidence suggests that chromatin remodeling at genomic enhancers is a key event linking LRGs to IEGs<sup>7,8</sup>. In non-neuronal systems, AP-1, an activity-dependent transcription factor consisting of *Fos* and *Jun* family members, directly contributes to chromatin remodeling at LRG enhancers<sup>7,9</sup>. However, despite ample evidence for activity-dependent transcription of AP-1 members in multiple brain regions and cell types, understanding the nature and functional consequences of LRG induction remains a challenge for several reasons. First, emerging evidence has revealed that different classes of neurons induce distinct LRG programs in response to the same neuronal activation<sup>9–14</sup>. Second, different types of stimuli may give rise to non-overlapping constellations of IEG transcription factors, which could promote activation of distinct LRGs to tune neuronal responses<sup>6,15</sup>. Finally, even where chromatin remodeling has been identified at candidate enhancers near LRGs<sup>7,16</sup>, the consequences of this remodeling have not been concretely linked to transcriptional activation of candidate LRGs.

Here, we used next generation sequencing approaches to characterize temporally distinct, activity-dependent transcriptomic and epigenomic reorganization in cultured rat embryonic striatal neurons, an *in vitro* model containing many cell types implicated in learning, motivation, reward, and substance use disorders<sup>17</sup>. These experiments comprehensively characterized activity-responsive genes in both the IEG and LRG expression programs in striatal neurons, and revealed a temporal decoupling between IEG activation and activity-dependent chromatin remodeling. Further functional studies suggest that translation of IEGs is necessary for activity-dependent chromatin remodeling, and that sites of chromatin opening are enriched for AP-1 transcription factor motifs. Combining transcriptional and epigenomic profiling allowed us to identify a putative enhancer upstream of the prodynorphin (*Pdyn*) gene locus that is conserved in the human genome. CRISPR-based activation and repression experiments provided functional validation that this region serves as a *Pdyn* enhancer in both rat neurons and dividing human cell lines. Collectively, these results highlight the mechanisms through which neuronal activity promotes gene expression changes to modify neuronal function, and have relevance for brain disease states characterized by alterations to this process.

## Results

### Characterization of temporally and functionally distinct transcriptional programs following neuronal depolarization

Activity-dependent transcriptomic and epigenomic reorganization has been heavily implicated in neuropsychiatric diseases, such as drug addiction. Previously, our lab established cultured rat embryonic striatal neurons as an *in vitro* model for studying activity-dependent processes as these cultures contain the same cell types affected by drugs of abuse in the rat ventral striatum<sup>17</sup>. To characterize IEG and LRG expression programs in this model system, we performed RNA-seq following neuronal depolarization with 10mM KCl for 1 or 4 h (**Fig. 1a**). Following 1 h of depolarization, we identified 207 differentially expressed genes (DEGs; defined as genes with an adjusted p-value < 0.05 and |log2FoldChange| > 0.5). Notably, ~75% of these genes were upregulated by KCl, including activity-dependent transcription factors such as *Npas4*, *Fos*, *Fosb*, *Fosl2*, and *Nr4a1* (**Fig. 1b**, **Table S1**). In contrast, 4 h of depolarization resulted in significant transcriptomic reorganization, with 1,680 genes identified as DEGs (**Fig. 1c**, **Table S1**). DEGs upregulated by KCl at this timepoint included the opioid propeptide *Pdyn*, as well as the voltage-gated potassium channel *Kcnf1* (**Fig. 1c**). Interestingly, overlap between 1 h DEGs (putative IEGs) and 4 h DEGs (putative LRGs) primarily occurred when IEGs such as *Fos* were upregulated at both 1 and 4 h (**Fig. 1d,e**; **Fig. S1a**). In contrast, most 4 h DEGs such as *Pdyn* demonstrated temporally specific upregulation and were only activated following 4 h of depolarization (**Fig. S1b**).

To determine whether 1 h and 4 h specific DEGs were functionally distinct, we used gProfiler<sup>18</sup> to identify enriched cellular component and molecular function gene ontology (GO) terms in each gene list. 1 h DEGs, or IEGs, were enriched for cellular component terms such as “Chromatin”, “Chromosome”, “Nucleus”, and “Transcription regulator complex” (**Fig. 1f**), suggesting that most DEGs were transcription factors or genes encoding proteins involved in transcriptional regulation. While not in the top 10 cellular component GO terms, “Nucleus”, “Nucleoplasm”, and “Transcription regulatory complex” are also significantly enriched in the 4 h DEGs. However, 4 h DEGs, or LRGs, were also enriched for cellular component terms such as “Synapse” and “Neuron projection” (**Fig. 1f**), suggesting that these DEGs encode proteins required for cellular adaptations to stimulation. Molecular function GO term analysis found overlap between IEGs and LRGs (**Fig. S1c**), with both IEG-specific and overlapping molecular function GO terms including transcription factor activity (**Table S2**). However, LRG molecular function GO terms were distinct

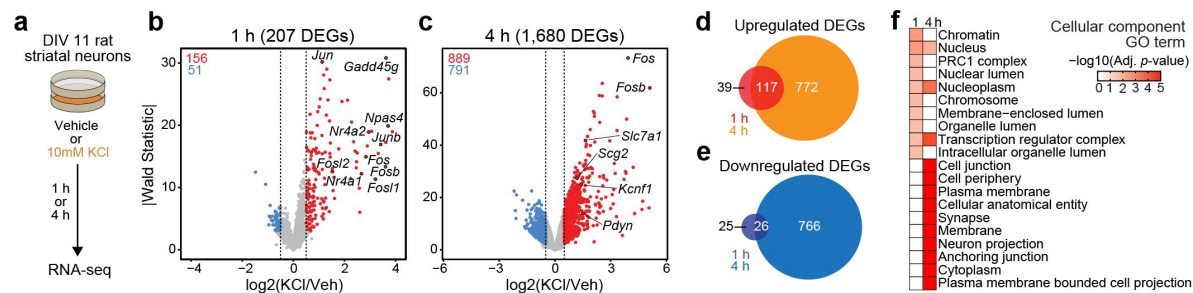


**Figure 1.**

Characterization of temporally and functionally distinct activity-dependent gene expression programs in cultured striatal neurons. **a**, Experimental design. DIV11 cultures were depolarized for 1 or 4 h with 10mM KCl. Following treatment, RNA-seq libraries were constructed. **b-c**, Volcano plots displaying gene expression changes after 1 and 4 h of neuronal depolarization. **d-e**, Venn diagrams comparing 1 and 4 h upregulated and downregulated DEGs. **f**, Top 10 cellular component GO terms for 1 and 4 h upregulated DEGs.

and included “voltage gated channel activity” and “G protein-coupled receptor binding” (Table S2). Together, these results demonstrate that striatal neuron depolarization induces temporally and functionally distinct gene expression programs that can be classified as IEGs and LRGs.

Given that most LRGs emerged only after 4 h of KCl depolarization, we next sought to determine whether this was due to the length of the depolarization stimulus or the time since stimulus onset. Striatal neurons were again treated with 10mM KCl for 1 h, followed by either a media wash off (replacement with conditioned media) or no wash off for 3 h (Fig. S1d). RT-qPCR for a representative IEG (*Fos*) and a representative LRG (*Pdyn*) revealed that while *Fos* mRNA began to return to baseline in the wash off (1 h stimulation) condition, *Pdyn* mRNA was equally elevated in response to 1 h KCl (followed by 3 h wash off) and 4 h KCl stimuli (Fig. S1e-f). Additionally, we found that both *Fos* and *Pdyn* expression were significantly elevated by 4 h treatment with a variety of other stimuli (Fig. S1g-h), including brain derived neurotrophic factor (BDNF) and forskolin (FSK, an adenylyl cyclase activator). Moreover, the overall level of *Pdyn* mRNA was correlated with the level of *Fos* mRNA across individual replicates (Fig. S1i), suggesting a direct link between IEGs and LRGs.

## Activity-dependent chromatin remodeling primarily occurs in non-coding genomic regions

In non-neuronal systems, LRG induction is dependent on activity-dependent chromatin remodeling at genomic enhancers.<sup>7</sup> To identify the potential mechanisms governing transcriptomic reorganization following 4 h of depolarization, we first sought to identify whether activity-dependent chromatin remodeling occurs in striatal neurons. Cultured rat embryonic striatal neurons were treated with 10mM KCl for 1 or 4 h and assay for transposase accessible chromatin followed by next generation sequencing (ATAC-seq) libraries were prepared (Fig. 2a). Strikingly, 1 h of depolarization did not induce any chromatin remodeling that passed genome-wide cutoffs for statistical significance (Fig. 2b). However, 4 h of depolarization induced genome-wide chromatin remodeling with 5,312 differentially accessible regions (DARs, defined as regions with adjusted *p*-value  $\leq 0.05$ ), all of which become more open with depolarization (Fig. 2c; Table S3). Previous studies have suggested that activity-dependent chromatin remodeling and other epigenetic processes occur in non-coding regions associated with genomic enhancers.<sup>7,19,20</sup> To understand whether activity-dependent chromatin remodeling preferentially occurred in non-coding genomic regions, we calculated the odds ratio of enrichment for all 4 h DARs in comparison to baseline peaks identified in vehicle treated samples that did not overlap 4 h DARs (termed “vehicle” peaks; Fig. 2d). Activity-regulated DARs were depleted in coding regions and promoters, but were enriched in intergenic and intronic regions of the genome (Fig. 2e). This distribution is consistent with a function for these DARs as distal cis-regulatory enhancer elements.

Enhancers are marked by distinctive histone modifications, including histone acetylation (at H3K27) and increased ratios of histone monomethylation to trimethylation at H3K4 (H3K4me1:H3K4me3). In contrast, while promoters are also marked by H3K27ac, they tend to exhibit a lower H3K4me1:H3K4me3 ratio.<sup>21,22</sup> To investigate whether 4 h DARs were enriched for enhancer-associated histone modifications, we next leveraged a recent study<sup>23</sup> that profiled histone modification enrichment throughout the mouse striatum. While DARs and vehicle peaks were enriched for H3K27ac and H3K4me1 (Fig. S2a,b), DARs had a significantly higher H3K4me1:H3K4me3 ratio than vehicle peaks (Fig. S2c,d). The preferential enrichment of DARs in non-coding regions coupled with the presence of H3K27ac and increased ratios of H3K4me1:H3K4me3 suggests that activity-dependent chromatin remodeling occurs at genomic enhancers in striatal neurons.

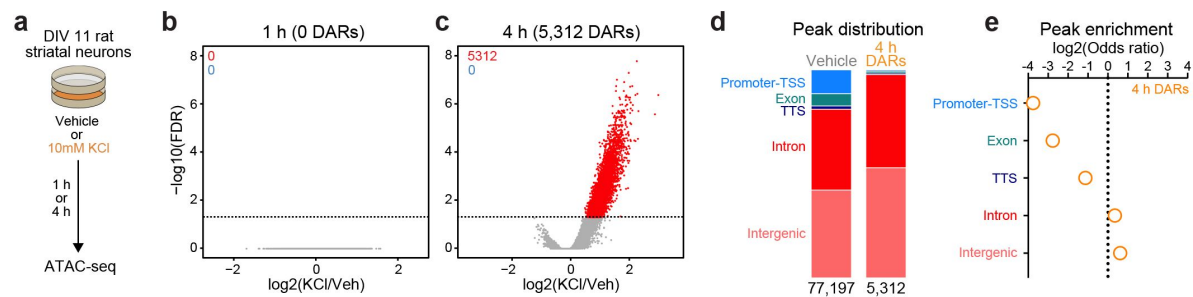


**Figure 2.**

Activity-dependent chromatin remodeling in cultured primary rat striatal neurons. **a**, DIV11 primary rat striatal neurons were treated with 10mM KCl for 1 or 4 h. Following treatment, ATAC-seq libraries were prepared. **b-c**, Volcano plots displaying differentially accessible regions (DARs) after 1 and 4 h of neuronal depolarization. **d**, Genomic location of vehicle and 4 h DAR ATAC peaks. **e**, Odds ratio for genomic annotations of 4 h DARs.

## Transcription factor motifs associated with IEGs are significantly enriched in 4 h DARs

The combination of RNA-seq and ATAC-seq results suggests a temporal decoupling between induction of IEG transcription factors and activity-dependent chromatin remodeling. IEG transcription factors were activated following 1 h of depolarization (**Fig. 1b**), but activity-dependent chromatin remodeling only occurred following 4 h of depolarization (**Fig. 2c**).

Furthermore, IEG transcription factors are critical mediators of activity-dependent chromatin remodeling in neuronal<sup>8</sup> and non-neuronal cells<sup>7</sup>. Thus, we predicted that IEG transcription factor motifs would be enriched in 4 h DARs.

To test this possibility, we searched for enriched motifs using a database of experimentally validated transcription factor binding motifs with HOMER<sup>24</sup>. Additionally, because millions of IEG motifs are found throughout the genome, we compared the enrichment between 4 h DARs and peaks found in vehicle treated samples. HOMER uses randomly selected background regions to compare the enrichment of motifs within a user identified peak set. This allowed us to calculate a percent enrichment, or the percent of background sequences with the motif subtracted from percent of target sequences with the motif. While no transcription factor motifs exhibited enrichment greater than 50% in the vehicle peak dataset (**Fig. 3a**), 8 motifs exceed this threshold within 4 h DARs (**Fig. 3b**). Interestingly, all motifs correspond to versions of the consensus motif for the AP-1 family of activity-dependent transcription factors, with 93% of 4 h DARs containing an AP-1 motif (**Fig. 3c**). In addition to calculation of a percent enrichment, we calculated the enrichment of specific motifs across DARs and vehicle peaks. AP-1, as well as its subunits FOS, FOSL2, and JUNB, were significantly enriched at the center of DARs and not in vehicle peaks (**Fig. 3d**). Recently, binding sites for the AP-1 family member  $\Delta$ FosB were assayed in the adult mouse nucleus accumbens using CUT&RUN<sup>23</sup>. Bindings sites identified in this study were then mapped to the rat genome.  $\Delta$ FosB is significantly enriched in DARs, but not in vehicle peaks or a set of 5,312 random regions that are the same size as DARs (**Fig. S2e**). Additional analyses identified that MEF2C motifs were enriched at the center of DARs (**Fig. 3c-d**). MEF2C is a member of the MEF2 family of proteins that are integral regulators of synaptic plasticity in the developing brain and interact with histone deacetylases to alter chromatin accessibility<sup>4,25,26</sup>. ISL1, is an integral regulator of MSN development<sup>27</sup>, was also enriched at the center of DARs, with 95% of DARs containing an ISL1 motif (**Fig. 3c-d**).

The HOMER database allowed us to investigate the enrichment of over 400 transcription factor motifs in over 5,300 DARs. To understand if DARs could be separated based on the transcription factor motifs present within these regions, we used uniform manifold approximation and projection (UMAP; a dimensionality reduction technique)<sup>28</sup> and density-based clustering<sup>29</sup> to separate DARs based on the presence, absence, or count of annotated transcription factor motifs. This analysis identified 3 major clusters with some DARs labeled as outliers (cluster 0) (**Fig. 3e**). Unsurprisingly, clusters were not defined by the presence of AP-1, FOSL2, MEF2C, or ISL1, transcription factors that are significantly enriched at the center of DARs (**Fig. 3f**). However, cluster 2 was marked by the presence of the KLF10 motif and cluster 1 was marked by the presence of a motif corresponding to OCT6 (**Fig. 3f**). Taken together, these results demonstrate a significant enrichment of IEG motifs within 4 h DARs and suggests that IEGs may participate in activity-dependent chromatin remodeling in striatal neurons.

## De novo protein translation is required for activity-dependent chromatin remodeling

The significant enrichment of IEG transcription factor motifs within 4 h DARs suggests that these inducible transcription factors may be required for subsequent activity-dependent chromatin remodeling. Furthermore, the temporal decoupling between IEG expression and activity-

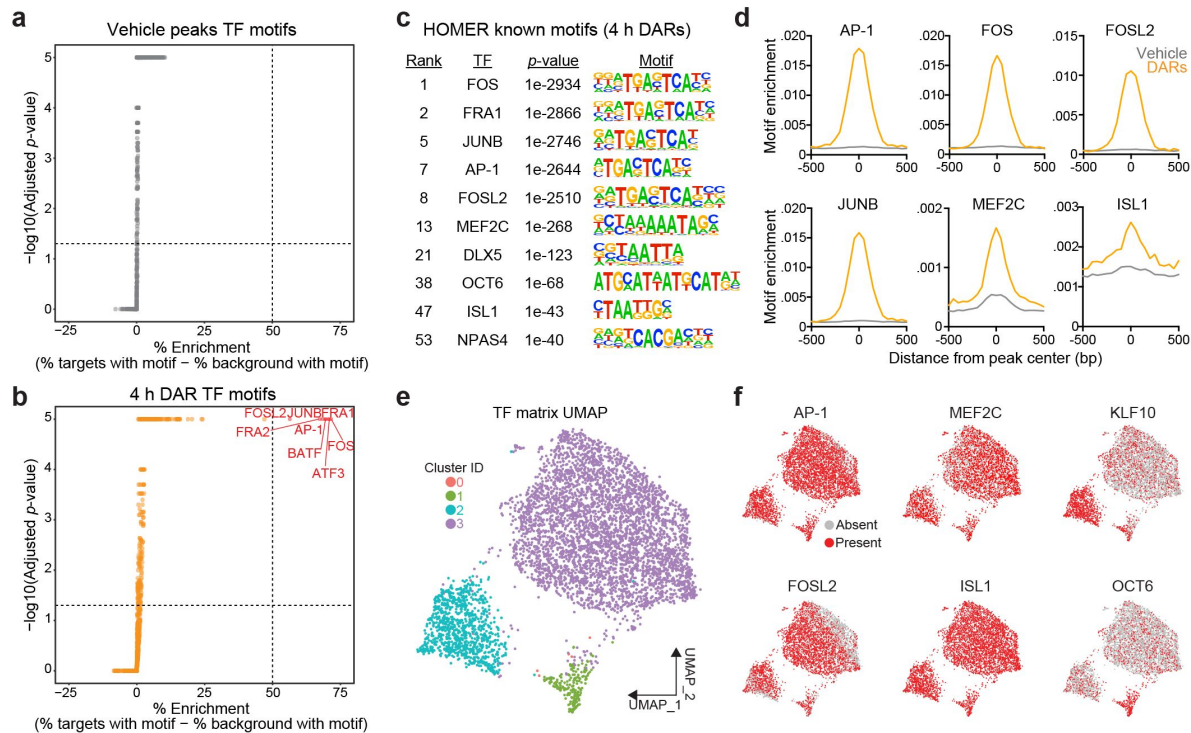


**Figure 3.**

Motifs for activity-dependent transcription factors are significantly enriched in 4 h DARs. **a-b**, Plots showing enrichment of specific transcription factor motifs in vehicle peaks and 4 h DARs. Motifs with significant adjusted p-values and a percent enrichment greater than 50% are shown in red and labeled with the corresponding transcription factor. **c**, Representative results from HOMER known motif enrichment analysis conducted using 4 h DAR peak set. **d**, Motifs for activity-dependent TFs (AP1, FOS, FOSL2, JUNB), MEF2C, and ISL1 are enriched at the center of 4 h DARs but not baseline (vehicle) peaks. Motif histogram distribution is represented as motifs/bp/peak. **e**, UMAP generated using transcription factor motif enrichment within the 4 h DARs. **f**, UMAPs colored by the presence of absence of specific transcription factor motifs. KLF10 and OCT6 specifically mark clusters 2 and 1, respectively.

dependent chromatin remodeling suggests that IEGs must be translated before acting on genomic regions to induce an open chromatin state. To test the hypothesis that *de novo* protein translation is required for activity-dependent chromatin remodeling, we repeated ATAC-seq after 4 h of depolarization in combination with protein synthesis inhibition. Cultured rat embryonic striatal neurons were pretreated with 40μM anisomycin, a translation inhibitor, for 30 minutes followed by 10mM KCl for 4 h (**Fig. 4a**). As expected, 40μM anisomycin was sufficient to block depolarization-induced translation of FOS protein, as determined by immunoblotting (**Fig. 4b**). Targeted analysis of the previously identified 5,312 DARs demonstrated replication of activity-dependent chromatin remodeling at these regions (**Fig. 4c**). Pretreatment with anisomycin completely blocked activity-dependent chromatin remodeling across the genome (**Fig. 4c,d**), demonstrating that *de novo* protein translation is required for activity-dependent changes in chromatin accessibility. Further, this result suggests that chromatin remodeling induced by neuronal depolarization is regulated by IEG transcription factors.

## Activity-dependent *Pdyn* transcription is regulated by IEGs and protein translation

Given that activity-dependent chromatin remodeling across the genome required protein translation, we next sought to identify discrete regions near LRGs that may serve as activity-dependent enhancers. We predicted that regions serving as activity-dependent enhancers for LRGs would be: 1) located in non-coding regions of the genome, 2) inaccessible at baseline and accessible following depolarization, and 3) inaccessible when depolarization was paired with protein synthesis inhibition. These regions would be fundamentally different from enhancers regulating IEGs (such as the enhancers within the *Fos* locus), which are accessible at baseline and do not undergo activity-dependent chromatin remodeling (**Fig. S3**). We identified a putative enhancer ~45 kilobases upstream of the *Pdyn* TSS that met all of these criteria (**Fig. 5a**). While *Pdyn* is an LRG that plays important roles in striatal function, the *Pdyn* promoter does not undergo activity-dependent chromatin remodeling (**Fig. 5a**). Next, we predicted that if this differently accessible chromatin region in the *Pdyn* locus serves as enhancer for *Pdyn*, then blocking protein translation should also attenuate activity-dependent *Pdyn* transcription. In support of this prediction, anisomycin pretreatment completely blocked activity-dependent *Pdyn* transcription, as detected with RT-qPCR (**Fig. 5b**). Additionally, anisomycin pretreatment attenuated baseline *Pdyn* mRNA levels in the absence of stimulation, suggesting that basal *Pdyn* expression is comprised of both constitutive and activity-dependent transcriptional events (**Fig. 5b**).

To determine whether *Pdyn* is directly regulated by IEG transcription factors, we leveraged a previously published RNA-seq dataset that used CRISPR activation tools to overexpress 16 IEGs that are upregulated in striatal neurons following dopamine stimulation<sup>17</sup> (**Fig. 5c**). CRISPR-based activation of 16 IEGs (including *Fos*, *Fosb*, and *Junb*) resulted in significant upregulation of *Pdyn* mRNA (**Fig. 5d**), demonstrating that activation of IEGs is sufficient to upregulate *Pdyn* expression. To further investigate the transcriptional regulation of *Pdyn*, we leveraged a publicly available single nucleus RNA-sequencing dataset from rats treated with a single dose of cocaine (or saline as a control; **Fig. 5e**)<sup>17</sup>. Because these rats were sacrificed one hour after injection, we reasoned that LRGs have not yet been fully activated by cocaine experience at this timepoint. However, using pseudotime analysis to reconstruct gene regulatory networks allowed us to identify predicted upstream regulators of *Pdyn* transcription. In prior work, we demonstrated that distinct populations of Drd1-MSNs have differential responses to cocaine<sup>30</sup>. Therefore, pseudotime trajectory graphs were constructed such that the highest pseudotime (cells colored bright red) marked cells that were transcriptionally activated by cocaine (i.e., expressed high levels of IEGs) (**Fig. 5f-g**). *Pdyn* mRNA levels were also higher in cells with high *Fos* and *Fosb* expression (**Fig. 5g**). Finally, a gene regulatory network was constructed for cells with high IEG levels. This network predicted that the IEG transcription factors *Fos*, *Fosb*, *FosL2*, *Nr4a1*, and *Nr4a2* all regulate *Pdyn* (**Fig. 5h**). As a positive control, we also generated a gene regulatory network

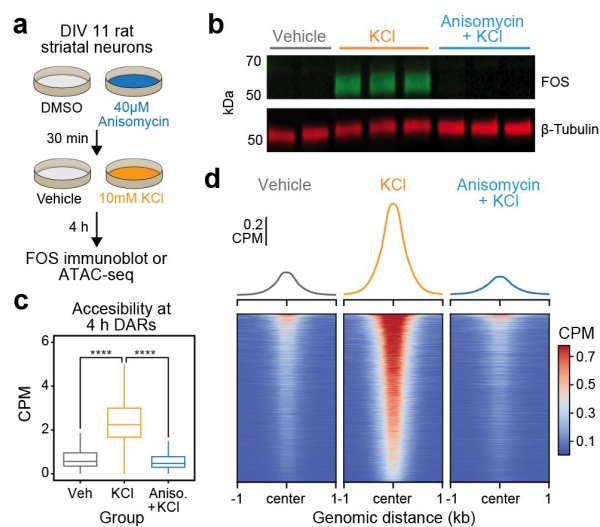


**Figure 4.**

Activity-dependent chromatin remodeling requires protein translation. **a**, Experimental design. DIV11 primary rat striatal neurons were treated with DMSO or anisomycin for 30 minutes followed by 4 h of depolarization with 10 mM KCl. **b**, Western blot for FOS and β-tubulin from striatal neurons treated with vehicle, 10 mM KCl, or 10 mM KCl + 40 μM Anisomycin. **c**, Boxplots demonstrating the effects of anisomycin on activity-dependent chromatin remodeling. One way ANOVA with Tukey's multiple comparisons test, \*\*\*\*p < 0.0001. **d**, Heatmaps and mean accessibility plots from 4 h DARs. For heatmaps, each row represents a single DAR. CPM = counts per million.



for *Scg2*, an LRG that was previously demonstrated to be regulated by *Fos*<sup>31</sup>. This network also predicted *Fos* as a regulator of *Scg2* (Fig. S4), demonstrating that this computational technique can replicate experimental findings.

## CRISPR-based functional validation of a novel *Pdyn* enhancer

The observation that *Pdyn* DAR accessibility and *Pdyn* mRNA were both activity- and translation-dependent suggests that this site acts as a stimulus-regulated enhancer for *Pdyn*. To test this hypothesis, we used a catalytically dead version of Cas9 (dCas9) fused to the transcriptional activator VPR (CRISPRa) or the transcriptional repressor KRAB-MeCP2 (CRISPRi)<sup>32,33</sup> to activate or silence this region on demand (Fig. 6a,b). To test the necessity of the DAR in mediating activity dependent *Pdyn* transcription, we used single guide RNAs (sgRNAs) to target dCas9-KRAB-MeCP2 to the DAR in the presence of depolarization. As a negative control, we transduced cultured striatal neurons with a sgRNA for *lacZ*, a bacterial gene not found in the mammalian genome. As expected, activity-dependent *Pdyn* upregulation was observed in neurons transduced with *lacZ* sgRNAs and dCas9-KRAB-MeCP2 (Fig. 6c). However, KCl-induced *Pdyn* upregulation was blocked in neurons transduced with DAR-targeting sgRNAs and dCas9-KRAB-MeCP2 (Fig. 6c). Additionally, baseline *Pdyn* mRNA levels were attenuated by CRISPRi of the *Pdyn* DAR (Fig. 6c), suggesting that this enhancer may be accessible at baseline in some cells or as a consequence of spontaneous neuronal activity. These results demonstrate that the DAR upstream of the *Pdyn* TSS is necessary for activity-dependent *Pdyn* transcription.

Next, we transduced neurons with the same sgRNAs targeting *lacZ* or the *Pdyn* DAR and dCas9-VPR (CRISPRa) to test if activation of this DAR is sufficient to promote *Pdyn* transcription. *Pdyn* mRNA levels were significantly increased in neurons transduced with DAR-targeting sgRNAs (Fig. 6d), demonstrating that transcriptional activation of the DAR is sufficient for upregulation of *Pdyn* mRNA levels. To test the specificity of the DAR for regulation of *Pdyn*, we also measured mRNA levels of the two closest genes within the *Pdyn* locus, *Sirpa* and *Stk35*. CRISPRa based transcriptional activation of the DAR did not result in significant upregulation of *Sirpa* or *Stk35*. Taken together, these CRISPR-based functional assays demonstrate that the DAR upstream of the *Pdyn* TSS is a genomic enhancer that is necessary, sufficient, and specific for *Pdyn* transcription.

## PDYN is a cell type-specific LRG in the human genome regulated by a conserved genomic enhancer

Because genomic enhancers are critical regulators of genes important for shared biological functions, many of these elements are conserved across species. Furthermore, regions undergoing activity-dependent chromatin remodeling in human neurons harbor genetic variants associated with the development of neuropsychiatric disorders<sup>34</sup>. Thus, we next set out to understand if *PDYN* is an LRG in the human genome, and whether the identified rat enhancer is conserved. To test whether *PDYN* is an LRG in the human genome, we leveraged publicly available RNA-seq datasets from cultured human GABAergic neurons<sup>34</sup>. These neurons were depolarized for 0.75 or 4 h, timepoints that allow for the investigation of IEGs and LRGs, respectively. Like cultured rat embryonic striatal neurons, *PDYN* is only upregulated following 4 h of depolarization (Fig. 7a-c). This study also performed the same experiments in cultured human glutamatergic neurons, allowing us to investigate whether *PDYN* is a cell type-specific LRG. Analysis of *PDYN* at several timepoints in both glutamatergic and GABAergic neurons demonstrated that *PDYN* is only upregulated in GABAergic neurons following 4 h of depolarization (Fig. 7d).

This published study also performed ATAC-seq on human glutamatergic and GABAergic neurons following 0 and 90 minutes of depolarization. While 90 minutes does not provide the same temporal resolution for chromatin remodeling at genomic enhancers as our experiments in rat neurons, it is a timepoint in which chromatin remodeling may initially occur following stimulation. Interestingly, the rat *Pdyn* DAR is conserved in a region that is also upstream of the human *PDYN* TSS (Fig. 7e), and this region undergoes time-dependent increases in chromatin

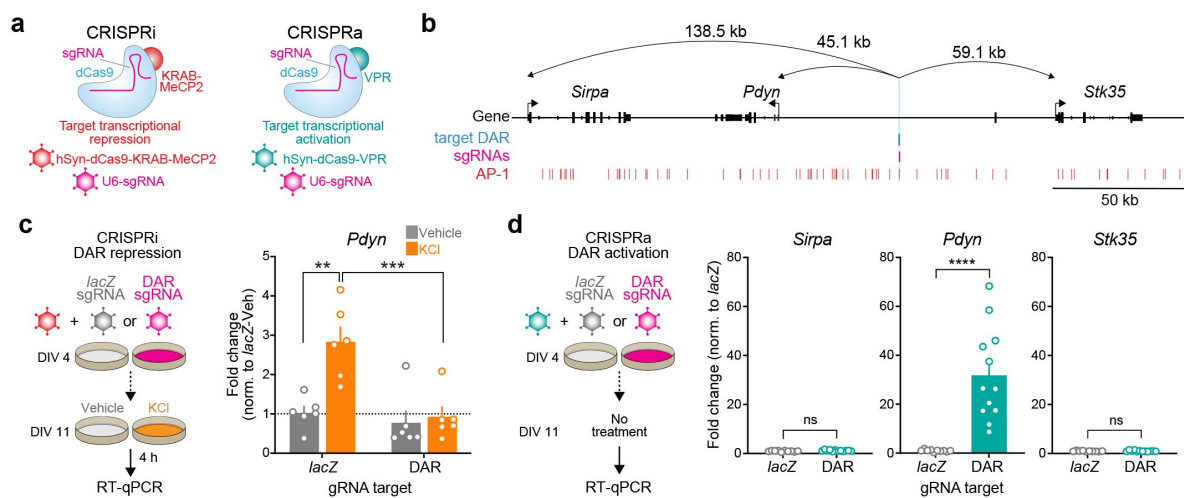


**Figure 6.**

CRISPR-based functional validation of a novel *Pdyn* enhancer. **a**, Viral strategy for functional validation of putative enhancers using CRISPR interference with dCas9-KRAB-MeCP2 and CRISPR activation with dCas9-VPR. **b**, CRISPR sgRNAs (4x) were designed to target the activity-regulated DAR 45.1 kb upstream of *Pdyn* in the rat genome. **c**, CRISPRi at the *Pdyn* DAR blocks activity-dependent induction of *Pdyn* mRNA. Cultured embryonic striatal neurons were transduced with dCas9-KRAB-MeCP2 and sgRNAs targeting *lacZ* (non-targeting control) or the *Pdyn* DAR. Neurons were then treated with vehicle or 10mM KCl for 4 h prior to RT-qPCR. One-way ANOVA with Tukey's multiple comparisons test  $**p < 0.01$ ,  $***p < 0.001$ . **d**, CRISPRa at the *Pdyn* DAR selectively upregulates *Pdyn* mRNA without altering expression of the nearest upstream and downstream genes. Striatal neurons were transduced with dCas9-VPR and sgRNAs targeting *lacZ* or the *Pdyn* DAR. Following RNA extraction at DIV 11, RT-qPCR was used to measure expression of *Pdyn*, *Sirpa*, and *Stk35*. Mann-Whitney test  $****p < 0.0001$ .

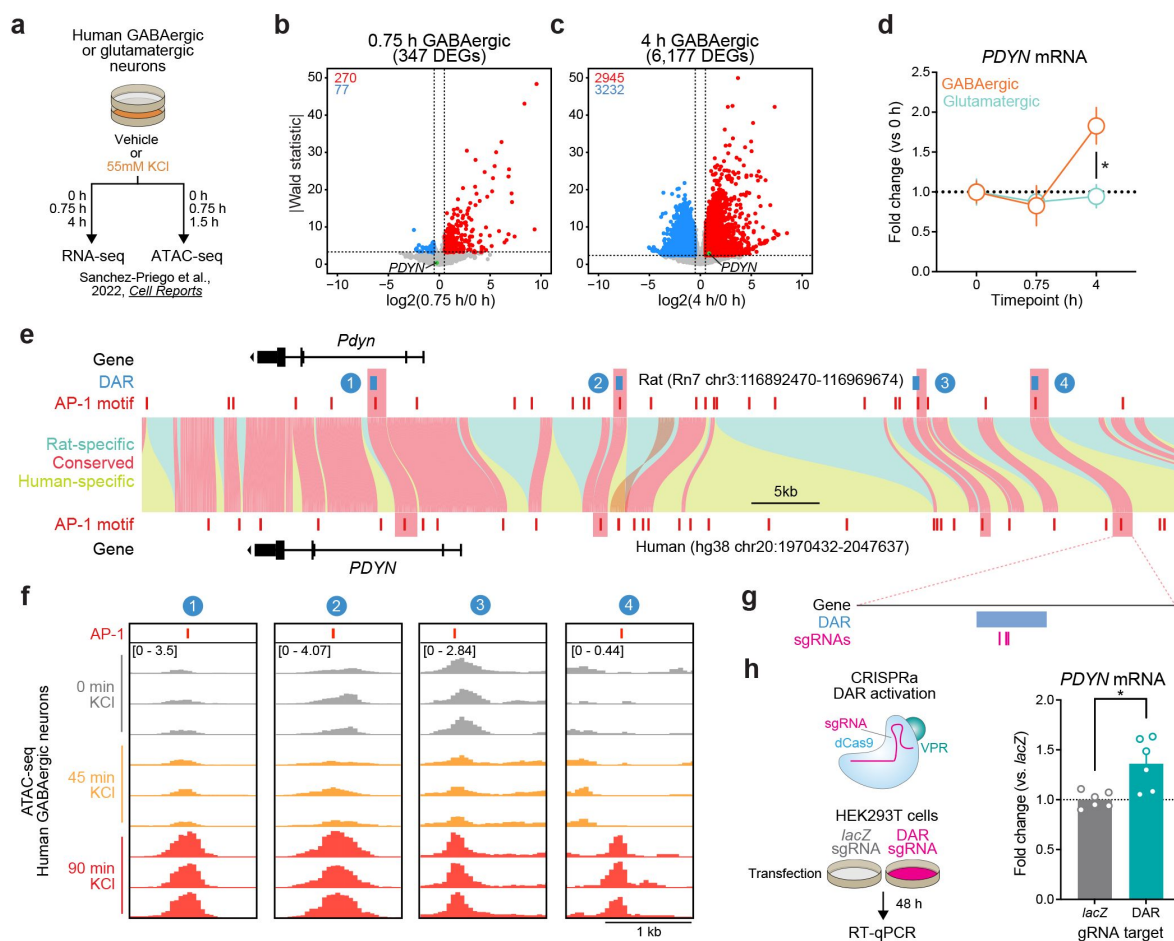


**Figure 7.**

Identification and validation of a conserved *PDYN* enhancer in the human genome. **a**, Experimental design for published RNA-seq and ATAC-seq datasets from human GABAergic and glutamatergic neurons treated with 55mM KCl. **b-c**, Volcano plots for human GABAergic neurons treated with 55mM KCl for 0.75 or 4 h. *PDYN* is a significant DEG at 4 h, but not 0.75 h. **d**, *PDYN* is significantly upregulated 4 h after a KCl stimulus, but only in GABAergic neurons. Two-way ANOVA with Tukey's multiple comparison's test. \* $p < 0.05$ . **e**, Linear synteny view of the rat and human *Pdyn*/*PDYN* locus reveals shared conservation of 4 distinct activity-dependent DARs identified in rat striatal neurons. **f**, ATAC-seq tracks of GABAergic neurons treated with 55mM KCl for 0, 0.75, or 1.5 h at the human *PDYN* locus. Regions conserved between rats and humans undergo dynamic remodeling in human GABAergic cells at 1.5 h after stimulation. A region homologous to the rat *Pdyn* enhancer characterized in [Figure 6](#) is 63.7 kb upstream of the human *PDYN* gene. **g**, Location of CRISPR sgRNAs in the human genome for CRISPR-based activation of the *PDYN* DAR in human cells. **h**, CRISPRa at the human *PDYN* DAR is sufficient to upregulate *PDYN* mRNA in HEK293T cells. Mann-Whitney test. \* $p < 0.05$ .

accessibility following KCl stimulation along with other identified DARs in this locus (**Fig. 7f**). Furthermore, activity-induced chromatin remodeling at the conserved and experimentally validated DAR only occurs in human GABAergic neurons (**Fig. S5**). To test if this enhancer is sufficient for upregulation of *PDYN* transcription in human cells, we transfected HEK-293T cells with plasmids to express dCas9-VPR machinery and sgRNAs targeting the conserved region (**Fig. 7g**). Transcriptional activation of the conserved DAR was sufficient to upregulate human *PDYN* transcription (**Fig. 7h**). Together, these results suggest that *PDYN* is a cell type-specific LRG within the human genome that is regulated by a conserved enhancer element.

## Pdyn enhancer is accessible in the adult rat striatum in a cell type-specific manner

To determine whether the activity-dependent *Pdyn* enhancer characterized *in vitro* is also functional in the adult brain, we performed snATAC-seq using nuclei from male and female adult Sprague-Dawley rats that received once daily intraperitoneal cocaine injections for 7 consecutive days (repeated cocaine; **Fig. 8a**). 10,085 snATAC-seq nuclei were sequenced and integrated with a previously published snRNA-seq dataset<sup>30</sup> that contained NAc nuclei from rats treated with repeated cocaine injections to identify cell types. Nuclei were distributed across 14 distinct cell types (**Fig. 8b**) that include previously identified neuronal and non-neuronal cell populations<sup>17,30</sup>. Visualization and accessibility peak-calling identified that the validated *Pdyn* enhancer locus was accessible in both *Drd1*- and *Grm8*-MSNs (**Fig. 8b**), populations that also express high levels of *Pdyn* at the mRNA level. Furthermore, co-accessibility analysis identified that accessibility of the *Pdyn* enhancer was correlated with accessibility of the *Pdyn* promoter across individual cells (**Fig. 8c**). This result demonstrates that the region is accessible, and coaccessible with the *Pdyn* promoter, *in vivo*. Furthermore, this dataset suggests that the *Pdyn* enhancer is functional in the adult rat brain in selected cell populations that also express *Pdyn* mRNA.

## Discussion

Here, we used transcriptomic and epigenomic profiling to characterize activity-dependent transcriptional and epigenomic waves in cultured embryonic rat striatal neurons, an *in vitro* model relevant for studying striatal function and neuropsychiatric disease. These experiments characterized a well-studied IEG expression program, which consisted primarily of activity-dependent transcription factors, as well as a delayed LRG expression program (**Fig. 1a-c**). While IEGs are required for cellular and behavioral adaptations, they control this process through the transcriptional regulation of LRGs. LRGs are both functionally and temporally distinct from IEGs. IEGs primarily encode transcription factors and co-activators, while LRGs encode opioid peptides, transporters, and other proteins involved in synaptic plasticity<sup>5,6</sup>. For example, *FOS* regulates transcriptional activation of the LRG *Scg2*<sup>31</sup>, a gene that encodes several neuropeptides that regulate inhibitory plasticity<sup>35</sup>. IEGs, such as *Fos*, may regulate LRGs by their involvement in activity-dependent chromatin remodeling, as *Fos* mRNA induction is required for activity-dependent chromatin remodeling in dentate gyrus following neuronal stimulation<sup>8</sup>. Our results highlight how this process modulates the expression of prodynorphin, a neuropeptide with critical functions in the striatum. Further, our work defines the dynamic nature of activity-dependent chromatin accessibility changes in striatal neurons using comprehensive approaches.

Surprisingly, analysis of both transcriptomic and epigenomic data revealed a temporal decoupling between transcriptional activation of IEGs and chromatin remodeling. While 1 h of depolarization was sufficient to induce hundreds of transcriptional changes, genome-wide chromatin remodeling was only observed following 4 h of depolarization. This result contrasts with previously published studies that observed genome-wide chromatin remodeling in the mouse dentate gyrus following 1 h of electroconvulsive stimulation<sup>8</sup>, or in hippocampal excitatory neurons following seizure

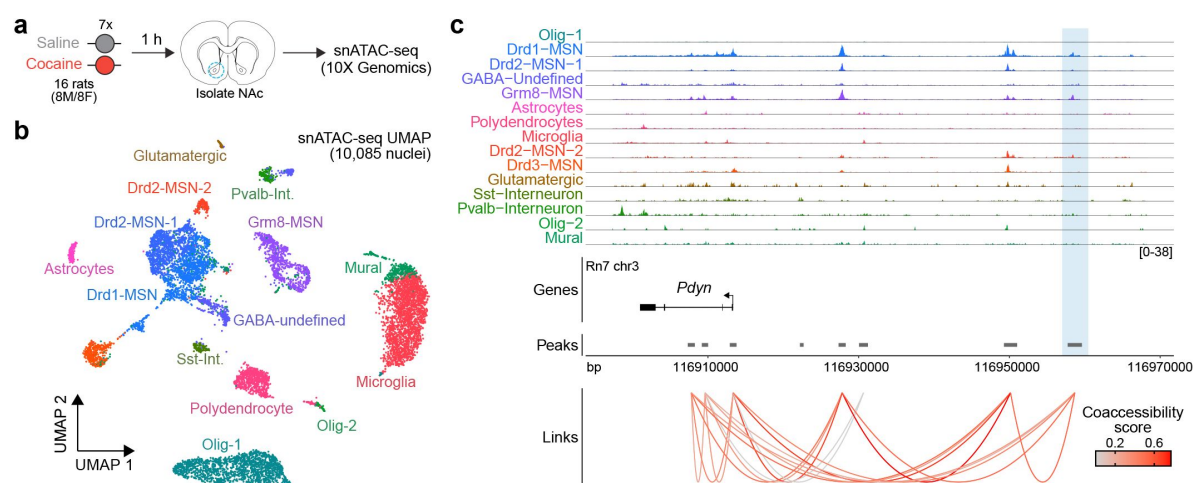


**Figure 8.**

Characterization of the rat *Pdyn* DAR in the adult nucleus accumbens at single cell resolution. **a**, Experimental design. **b**, UMAP of 10,085 nuclei from adult rat nucleus accumbens (NAC). **c**, Genome tracks displaying snATAC-seq data at the *Pdyn* locus. The experimentally validated *Pdyn* DAR (highlighted in blue) exhibits high co-accessibility with the proximal promoter for *Pdyn* as well as other nearby accessible regions.

induction with kainic acid<sup>36</sup>. Differences in the temporal dynamics of activity dependent chromatin remodeling could be due to differences in both the cell type of interest (hippocampal vs. striatal neurons), as well as the type of stimulation (e.g., electroconvulsive stimulation vs. depolarization). Nevertheless, our studies agree in that a significant proportion of regions undergoing remodeling become more accessible following stimulation (**Fig. 2c**). While it is known that IEGs engage in activity-dependent chromatin remodeling at LRGs in other brain regions and cell types, this process has seldom been studied in an addiction-relevant cell type such as striatal neurons. Additionally, drugs of abuse engage IEGs in the striatum<sup>17,30,37–42</sup>, and interference with IEG induction prevents subsequent cellular and behavioral adaptations caused by psychoactive drugs<sup>40,43,44</sup>. Thus, investigation of IEG-dependent chromatin remodeling at enhancers regulating addiction-relevant LRGs is important for understanding how rapid gene expression changes might result in persistent cellular and behavioral adaptations.

The accepted model of activity-dependent transcription posits that LRG activation is dependent on IEGs. To this point, blocking *Fos* mRNA induction via shRNA in the dentate gyrus is sufficient to significantly attenuate activity-dependent chromatin remodeling<sup>8</sup>. This data demonstrates that *Fos* is required for some level of chromatin remodeling in the brain but does not provide evidence regarding the mechanisms through which it is involved. In non-neuronal cells, AP-1 acts as a pioneer factor at genomic enhancers by guiding the SWI/SNF chromatin remodeling complex to target regions<sup>7,45</sup>. Our data suggests a similar mechanism may regulate activity-dependent chromatin remodeling at genomic enhancers in neurons. First, AP-1 motifs and  $\Delta$ FosB binding sites are significantly enriched within 4 h DARs (**Fig. 3b-d**, **S2e**). Second, blocking translation of IEGs, which include a significant number of AP-1 family members, completely blocks activity-dependent chromatin remodeling (**Fig. 4c,d**). Thus, we speculatively propose a model in which neuronal stimulation results in the transcription and translation of AP-1 family members. These AP-1 family members then interact with the SWI/SNF complex to induce chromatin remodeling at genomic enhancers. However, while AP-1 is thought to aid in enhancer selection, it is expressed in a non-cell type specific manner and must choose from over 1 million potential AP-1 motifs in the genome. Thus, additional factors must be present to ensure precise cell type-specific responses to activity. The use of the HOMER database allowed us to explore the enrichment of over 400 additional transcription factor motifs, including ISL1, an MSN-selective transcription factor. ISL1 is significantly enriched in DARs (**Fig. 3c-d**). The combined enrichment of AP-1 and ISL1 at 4 h DARs suggests that cell type-specific transcription factors may be working in conjunction with activity-dependent transcription factors to induce chromatin remodeling. We envision three possible mechanisms through which cell type-specific transcription factors may be involved in this process. First, population-specific transcription factors may be directly interacting with AP-1 or chromatin remodeling complexes to induce remodeling at these sites. Second, ISL1 may stochastically bind these regions, resulting in specific temporal windows in which AP-1 might bind in a cooperative fashion. Finally, because cell type specific transcription factors are often activated during development, they may epigenetically alter potential binding sites proximal to AP-1 motifs, resulting in a preferential targeting of these AP-1 motifs at later timepoints. Any of these mechanisms would allow distinct cell types to induce specific LRG programs, even with homogenous IEG activation, and ultimately enable the same stimulus to produce different cellular adaptations in different cell types.

Multiple studies have demonstrated that most regions undergoing activity-dependent chromatin remodeling are genomic enhancers<sup>7,16</sup>. Analysis of 4 h DARs from this study identified a significant enrichment for DARs in non-coding regions and a depletion in coding regions (**Fig. 2d-e**), suggesting that activity-dependent chromatin remodeling in striatal neurons is also occurring at genomic enhancers. This data also demonstrates a distinction between the chromatin profiles of IEG and LRG enhancers. IEG enhancers are “poised” at baseline, while LRG enhancers are largely inaccessible in without stimulation. For example, enhancers within the *Fos* locus are accessible in both vehicle and KCl treated samples (**Fig. S3**), while the *Pdyn* enhancer is only accessible with neuronal depolarization (**Fig. 5a**). In addition, LRG enhancers contain motifs for

activity-dependent transcription factors (Fig. 3a-e) and are dependent on *de novo* protein translation (Fig. 4c,d). While LRG enhancers become accessible only with activity, both IEG and LRG enhancers are in noncoding regions of the genome and are marked by specific histone modifications like H3K27ac and H3K4me1<sup>16,21,46,47</sup> (Fig. S2).

While the enrichment of DARs in putative enhancer elements is intriguing, we also wanted to understand whether enhancers regulate nearby LRGs. To do this, we identified a DAR upstream of the *Pdyn* TSS that may serve as a functional enhancer. This region is also accessible in the adult rat NAc (Fig. 8c). Furthermore, this region is co-accessible with the *Pdyn* promoter and has the highest level of accessibility in Drd1- and Grm8-MSNs, suggesting that the enhancer is functional *in vivo* and may exhibit some level of cell type specificity (Fig. 8c). However, a larger dataset containing more nuclei will be needed to test for any drug-specific chromatin remodeling at this region. CRISPR-based functional assays demonstrated that the DAR upstream of the *Pdyn* TSS serves as a genomic enhancer that is necessary, sufficient, and specific for transcription of *Pdyn*. We were particularly interested in *Pdyn* because it encodes the dynorphin neuropeptides that are agonists for the kappa opioid receptor<sup>48</sup>. The kappa opioid receptor system has been the target of several antagonists that reduce drug-taking behaviors in pre-clinical models<sup>49-52</sup>. Identification of a novel genomic enhancer for *Pdyn* would represent a novel therapeutic target. Furthermore, *Pdyn* is regulated by dopamine<sup>53</sup> and drugs of abuse<sup>44,54-58</sup>, and polymorphisms and structural variants within the human *PDYN* gene locus are associated with drug abuse<sup>59,60</sup>. In particular, one polymorphism affects an AP-1 binding site within the *PDYN* gene promoter, which may result in less *PDYN* transcription and an increased risk for cocaine dependence<sup>59</sup>. Thus, the identification of a functional, conserved enhancer for *PDYN* in the human genome provides a novel potential therapeutic target capable of regulating stimulus-dependent changes in *PDYN* expression while leaving constitutive expression patterns unaltered. Future experiments should investigate whether this conserved enhancer element mediates reward-related behaviors and cellular adaptations.

In conclusion, we have identified and characterized temporally distinct waves of transcription and chromatin remodeling in striatal neurons. Furthermore, we found that the translation of IEGs is required for activity-dependent chromatin remodeling, particularly at genomic enhancers. Targeted analysis of LRG loci identified a genomic enhancer that is necessary, sufficient, and specific for *Pdyn* transcription. This enhancer is conserved in the human genome and represents a novel therapeutic target for modulation of the kappa opioid receptor system. Continued functional validation of putative enhancer regions within this dataset may provide additional therapeutic targets.

## Methods

### Animals

Male or female adult rats were co-housed in pairs in plastic filtered cages with nesting enrichment in an Association for Assessment and Accreditation of Laboratory Animal Care–approved animal care facility maintained between 23° and 24°C on a 12-hour light/12-hour dark cycle with ad libitum food (Lab Diet Irradiated rat chow) and water. Bedding and enrichment were changed weekly by animal resources program staff. Animals were randomly assigned to experimental groups. All experiments were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC). Timed pregnant dams were individually housed until embryonic day 18 when striatal cultures were generated as previously described<sup>17,33,47</sup>.

## Neuronal cell culture

Cells were maintained in neurobasal media supplemented with B27 and LG for 11-12 days in vitro (DIV) with half media changes DIV 1, 5-6, and 9-10. For depolarization experiments, neurons were treated with KCl dissolved in neurobasal media to a final concentration of 10mM for 1 or 4 h. To test the necessity of protein translation in mediating activity dependent transcription and chromatin remodeling, anisomycin was dissolved in DMSO and treated to a final concentration of 40μM for 30 minutes prior to depolarization. Additional stimuli outlined in [Fig. S1](#) included recombinant brain-derived neurotrophic factor (BDNF, 100ng/mL), the adenylyl cyclase activator forskolin (FSK, 20μM), and the GABA<sup>A</sup> receptor antagonist gabazine (GBZ, 5μM). All treatments were applied for 4 h followed by RNA extraction and RT-qPCR.

## HEK-293T experiments

HEK-293T cells were maintained in DMEM + 10% FBS and plated at 80,000 cells/well in 24 well plates. 24 h later cells were transfected with Fugene HD (Promega) and constructs containing dCas9-VPR and gRNA vectors targeting the conserved DAR region (500ng plasmid DNA in molar ratios sgRNA:dCas9-VPR). 48 h later, cells were lysed and RNA was extracted.

## CRISPR/dCas9 gRNA design and delivery

Guide RNAs targeting *lacZ*, the rat *Pdyn* enhancer, and conserved human *PDYN* enhancer were designed using CHOPCHOP as previously described<sup>17,32,33,47,61</sup>. CRISPR/dCas9 and gRNA constructs were packaged into lentiviral vectors and transduced as previously described<sup>17,32,61,63</sup>. gRNA sequences can be found in [Table S4](#).

## RNA extraction and RT-qPCR

RNA extractions, cDNA synthesis, and RT-qPCR were performed as previously described<sup>17,33,47,63</sup>. All RT-qPCR primers can be found in [Table S4](#).

## Western blotting

DIV11-12 cultured rat embryonic striatal neurons were treated with 40μM Anisomycin followed by 4 h of depolarization with 10mM KCl in 12 well plates. Following depolarization, media was removed, and wells were washed with 1x TBS. Cells were lysed using RIPA lysis buffer (50mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40 Substitute, 0.5% Sodium Deoxycholate, 0.1% SDS, 1X HALT protease inhibitor cocktail). Following protein separation and transfer, PVDF membrane was incubated with FOS primary antibody (Cell Signaling Cat. No. 9F6, 1:1000 in TBST) and  $\Delta$ -tubulin primary antibody (Millipore Cat. No. 05-661 1:2000 in TBST) overnight at 4°C. Following primary antibody incubation, secondary antibodies (IRDye 680RD Goat Anti-Rabbit Cat. No. 926-68071 1:10000 in TBST and IRDye 800CW Donkey Anti-Mouse Cat. No. 926-32212 1:10000 in 1:1 TBST:Intercept blocking buffer with 0.02% SDS) for 1 h at room temperature. Imaging was performed using a Licor Odyssey imager.

## RNA-Seq library preparation and analysis

RNA was extracted from cultured rat embryonic striatal neurons following stimulation (RNeasy, Qiagen) and submitted to the Genomic scorelab at the Heflin Center for Genomic Sciences at the University of Alabama at Birmingham for library preparation as previously described<sup>17,47</sup>. RNA-seq libraries were generated from experiments independent of the ATAC-seq experiments. For the RNA-seq experiment of neurons treated with vehicle or KCl for 1 h, there were 3 replicates within the KCl group and 4 replicates within the vehicle group. For the RNA-seq experiment of neurons treated with vehicle or KCl for 4 h, there were 4 replicates within each group (4 Veh, 4 KCl). 75bp paired-end libraries were sequenced using the NextSeq500. Paired-end FASTQ files were analyzed using a custom bioinformatics pipeline built with Snakemake<sup>64</sup> (v6.1.0). Briefly, read quality was assessed using FastQC before and after trimming with Trim\_Galore<sup>65</sup> (v0.6.7).

Splice-aware alignment to the mRatBn7.2/Rn7 reference assembly for cultured embryonic rat striatal neurons using the associated Ensembl gene transfer format (gtf) file (version 105) and the Hg38 reference assembly using the associated Ensembl gtf (version 99) for previously published human datasets was performed with STAR<sup>66</sup> (v2.7.9a). Binary alignment map (BAM) files were indexed with SAMtools<sup>67</sup> (v1.13). Gene-level counts were generated using the featureCounts function within the Rsubread package<sup>68</sup> (v2.6.1) in R (v4.1.1). QC metrics were collected and reviewed with MultiQC<sup>69</sup> (v1.11). Differential expression testing was conducted using DeSeq2<sup>70</sup> (v1.38.3). DEG testing *p*-values were adjusted with the Benjamini-Hochberg method<sup>71</sup>. DEGs were designated as those genes with an adjusted *p*-value < 0.05 and a  $|\log_2\text{FoldChange}| > 0.5$ . DEGs were calculated by comparing the KCl and Vehicle treatment groups at each respective timepoint. Molecular function and cellular component GO terms were identified by first characterizing upregulated DEGs specific for the 1 h or 4 h timepoint. Ensembl gene IDs for all genes were input into gProfiler<sup>18</sup> with a custom background of all expressed genes (counts > 0). Resulting *p*-values were adjusted with the Benjamini-Hochberg<sup>71</sup> method.

## ATAC-Seq library preparation and analysis

ATAC-Seq libraries were prepared as previously described<sup>47</sup>. ATAC-seq libraries were generated from experiments independent of the RNA-seq experiments. For the ATAC-seq experiment of neurons treated with vehicle or KCl for 1 h, there were 3 replicates within each treatment group (3 Veh, 3 KCl). For the ATAC-seq experiment of neurons treated with vehicle or KCl for 4 h, there were 3 replicates within each treatment group (3 Veh, 3 KCl). For the ATAC-seq experiment of neurons pre-treated with DMSO or Anisomycin, there were 4 replicates within each treatment group (4 DMSO + Veh, 4 DMSO + KCl, 4 Anisomycin + KCl). 75bp paired-end libraries were then sequenced using the NextSeq500 at the Genomics core lab at the Heflin Center for Genomic Sciences at the University of Alabama at Birmingham as previously described<sup>47</sup>. Paired-end FASTQ files were analyzed using a custom bioinformatics pipeline built with Snakemake<sup>64</sup> (v6.1.0). Read quality was assessed with FastQC before and after trimming (trimming was performed with Trim\_Galore!<sup>65</sup> v0.6.7). Particularly, Nextera adapters (5'-CTGTCTCTTATA-3') were identified and removed. For rat striatal neuron experiments, FASTQ files were then aligned using Bowtie2<sup>72</sup> (v2.4.4) to the mRatBn7.2/ Rn7 reference assembly Ensembl gene transfer format (gtf) file (version 105). Previously published human datasets were aligned to the Hg38 reference assembly using the associated Ensembl gtf (version 99). PCR duplicates were marked with using Picard<sup>73</sup> (v2.26.2). For human ATAC-seq data, encode version 4 of the Hg38 blacklist was used. PCR duplicates, in addition to reads mapping to the mitochondrial genome, were removed using SAMtools<sup>67</sup> (v1.13). BigWig files were generated with deeptools<sup>74</sup> v3.5.1. QC metrics were collected and reviewed with MultiQC<sup>69</sup> (v1.11). For ATAC-Seq libraries from cultured rat embryonic striatal neurons, peaks were called using MACS2<sup>75</sup> (v 2.1.1.20160309) callpeak with options - -qvalue 0.01 - -gsize 2626580772 - -format BAMPE. Differential accessibility analysis was performed with Diffbind<sup>76</sup> (v3.8.4). DARs were defined as regions with an adjusted *p*-value ≤ 0.05. Reads within peaks were counted using the dba.count() function with options bParallel=TRUE, summits=250, bUseSummarizeOverlaps=TRUE, and score = DBA\_SCORE\_TMM\_READS\_FULL\_CPM.

Motif enrichment was investigated using HOMER<sup>24</sup> (v4.11.1) findMotifsGenome.pl. Motif enrichment was calculated by dividing the total number of motifs identified within a defined bin and then dividing by the bin size (50bp). This number was further divided by the peak set size (5,312). This normalization ensures histograms can be compared even when generated with different bin sizes or peak sets with a significantly higher or lower number of peaks included. Dimensionality reduction of motif enrichment across all DARs was conducted using the umap package in R (v2.10.0) to calculate 10 UMAP<sup>28</sup> components with custom options min\_dist=1e-250, n\_neighbors=30, and n\_components=10. UMAP values were used to cluster points with the hdbscan() function of the dbscan<sup>29</sup> package (v1.1-11) with minPts=150.

## Pseudotime

Pseudotime calculations were performed using Monocle v3\_1.3.1<sup>77</sup> and Seurat v4.3.0<sup>78</sup>. A Seurat object containing *Drd1*-MSNs from male and female adult Sprague-Dawley rats treated with a single cocaine injection<sup>30</sup> was subject to the standard dimensionality reduction and clustering workflow using 17 principal components and a resolution value of 0.2. Annotation, counts metadata, and cell barcode information were extracted from the Seurat object and reconstructed into a Monocle v3 cds object using `new_cell_data_set()`. Rather than reclustering, the UMAP coordinates were extracted from the Seurat object and added to the Monocle object. The trajectory graph was created using `learn_graph()` and the root cell was chosen in the “inactivated” population that expressed *Drd1* and *Htr4*<sup>30</sup>. Pseudotime values for each cell were exported from the cds object and added back to the Seurat object metadata.

## Gene regulatory network reconstruction

Reconstruction of gene regulatory networks within *Drd1*-MSNs was performed using Epoch<sup>79</sup>. Dynamically expressed genes were found using `findDynGenes()`, and a *p*-value threshold of 0.05 was used to filter for significance. Transcription factors for *R. norvegicus* were downloaded from AnimalTFDB4.0<sup>80</sup> used to construct an initial static network using `reconstructGRN()`. Epoch uses a Context Likelihood of Relatedness (CLR) model to infer relationships between transcription factors (TFs) and transcription targets (TGs) using transcriptomic information and calculated pseudotime values. A process which Epoch terms as “crossweighting” was then performed to filter out indirect relationships or non-logical connections. Next, a dynamic network was extracted by fractionating the *Drd1* cells by “epochs”, which is based on pseudotime. TF to TG relationships were ranked using PageRank<sup>81</sup>.

## Single nucleus dissociation

Flash-frozen NAC tissue was added to a tube containing 500µl lysis buffer (1mM Tris-HCl pH 7.4, 1mM NAC, 0.3 mM MgCl<sub>2</sub>, 0.01% Tween-20, 0.01% Igepal in nuclease free water, 0.001% Digitonin, 0.1% BSA), homogenized using a motorized homogenizer and RNase free pestle, and incubated on ice for 5 minutes. Samples were then pipette mixed 15x and incubated on ice for an additional 10 minutes. Following lysis, 4 samples from same sex and treatment were grouped into a single sample, and 2mL chilled wash buffer (10mM Tris-HCl pH 7.4, 10mM NaCl, 3mM MgCl<sub>2</sub>, 1% BSA, 0.1% Tween-20) was added. Samples were then passed through 70µm and 40µm Scienceware Flowmi Cell Strainers. Samples were then centrifuged at 250 rcf for 5 min at 4°C. Supernatant was removed and the pellet was resuspended in 1mL of chilled wash buffer (10mM Tris-HCl pH 7.4, 10mM NaCl, 3mM MgCl<sub>2</sub>, 1% BSA, 0.1% Tween-20). 100µL of sample was then stained with 7-aminoactinomycin D and used to set a representative gate for fluorescence activated cell sorting (FACs). This gate was then used to purify nuclei further. Following FACs, samples were spun at 250 rcf for 10 min at 4°C to remove any remaining debris. Samples were then loaded into individual wells of the Chromium NextGem Single Cell Chip using 4 of the 8 available wells.

## snATAC-seq alignment and object construction

10X Genomics snATAC-seq libraries were generated from male and female Sprague-Dawley rats exposed injected with 20 mg/kg cocaine (or saline control) once daily for seven days. snATAC-seq data was aligned to the mRatBN7.2/Rn7 reference assembly using `cellranger-atac v2.1.0` and the procedure outlined by 10X Genomics<sup>82</sup>. Analysis of the `cellranger-atac` output was analyzed using Signac v1.9.0<sup>83</sup> and Seurat v4.3.0<sup>78</sup>. Counts, fragments, and barcode information for each treatment group were loaded and combined into Seurat objects as outlined by the Stuart lab website (<https://stuartlab.org/signac/>). The groups were then merged into one object based on a common set of features and the standard Signac dimensionality reduction and clustering workflow was performed using 2:30 dimensions and a resolution value of 0.2. Data was then integrated with the snRNA-seq data from the same samples. Common anchors between the two were used to predict and label the cell types present in the snATAC-seq data.

## Co-accessibility analysis

Detection of cis-co-accessible sites in the snATAC-seq data was performed using Cicero v1.3.9<sup>84</sup> and Monocle v3\_1.3.1<sup>77</sup>. The finalized snATAC-seq Seurat object was first converted into a Monocle3 cds object and then converted into a Cicero object. The Cicero connections were found using run\_cicero with the Cicero object and a dataframe containing chromosome lengths extracted from the Seurat object. Cis-coaccessible networks were then calculated using generate\_ccans(), and the produced links were added back to the starting Seurat object.

## Data availability

All relevant data that support the findings of this study are available by request from the corresponding author (J.J.D.). Sequencing data that support the findings of this study are available in Gene Expression Omnibus. Accession numbers of specific datasets are outlined below.

Bulk RNA-seq primary rat striatal neurons 1 h vehicle or KCl: GSE150499

Bulk RNA-seq primary rat striatal neurons 4 h vehicle or KCl: GSE233752

Bulk ATAC-seq primary rat striatal neurons 1 h vehicle or KCl: GSE150589

Bulk ATAC-seq primary rat striatal neurons 4 h vehicle or KCl: GSE233368

Bulk ATAC-seq primary rat striatal neurons 4 h vehicle or KCl with anisomycin: GSE233368

snATAC-seq adult rat nucleus accumbens: GSE233754

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## Author contributions

R.A.P.III and J.J.D. conceived of experiments. R.A.P.III performed all experiments. D.R. assisted with HEK293T experiments, and O.R.D. assisted with rat primary culture experiments. R.A.P.III and J.J.T. conducted the single nucleus dissociation for snATAC experiments. R.A.P.III analyzed next generation sequencing data with assistance from E.W. and L.I. R.A.P.III and J.J.D. wrote the manuscript. All authors have approved the final version of the manuscript.

## Conflicts of interest

The authors declare no competing interests.

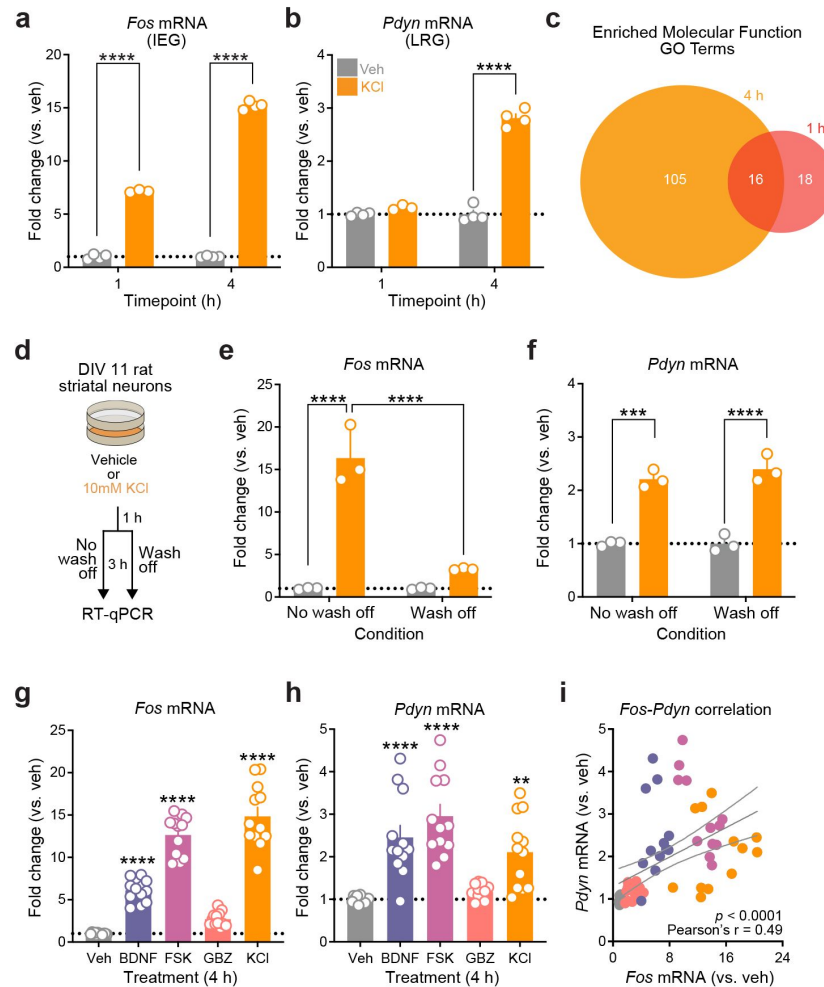


**Figure S1.**

Striatal neuron IEGs and LRGs are temporally and functionally distinct and are induced by a variety of stimuli. **a-b**, IEGs like *Fos* are activated at both 1 and 4 h after depolarization, while LRGs such as *Pdyn* are only activated after 4 h of stimulation. Two-way ANOVA with Tukey's multiple comparisons test. **c**, Venn diagram comparing molecular function GO terms enriched in 1 and 4 h DEG lists reveals largely distinct category enrichment. **d-f**, *Pdyn* induction does not require extended (4 h) stimulation, and occurs 3 h after 1 h KCl followed by media wash off. One-way ANOVA, Tukey's multiple comparisons test. **g-i**, Diverse stimuli elevate both *Fos* and *Pdyn*, and transcriptional induction is correlated across samples (One-way ANOVA, Tukey's multiple comparisons test). All treatments were applied for 4 h prior to RT-qPCR. BDNF = brain derived neurotrophic factor (100 ng/mL); FSK = forskolin (20  $\mu$ M); GBZ = gabazine (5  $\mu$ M); KCI = potassium chloride (10mM). All data are presented as mean  $\pm$  SEM. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

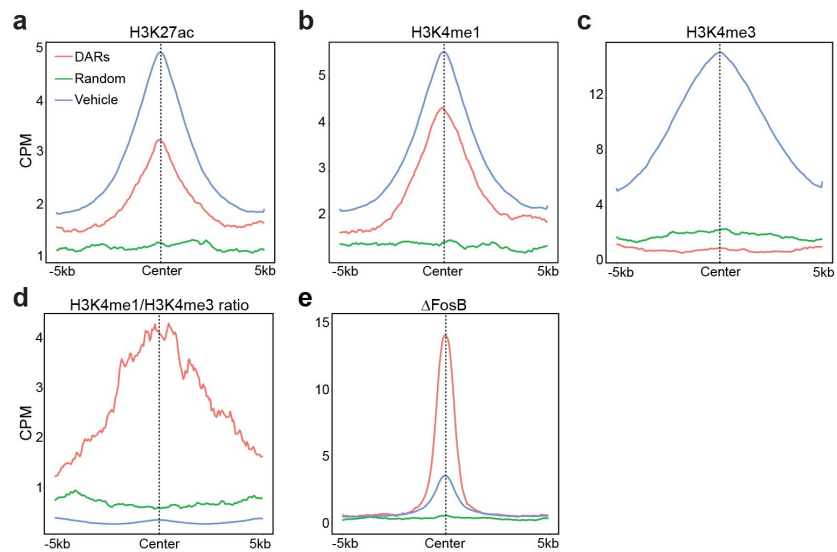


**Figure S2.**

Transcription factor binding and histone modifications in 4 h DARs, random regions, and vehicle peaks. Enrichment plots for **a**, H3K27ac, **b**, H3K4me1, **c**, H3K4me3, **d**, H3K4me1/H3K4me3, **e**,  $\Delta$ FosB. Data from Yeh, et al., 2023 *Biological Psychiatry*.

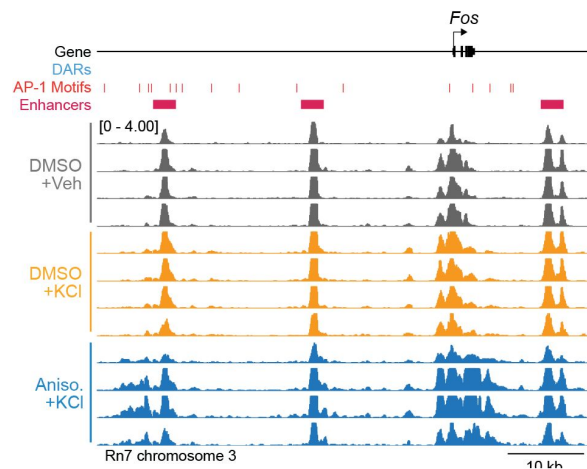


**Figure S3.**

Enhancers for *Fos* are accessible at baseline and do not undergo activity-dependent chromatin remodeling. ATAC-seq tracks at the *Fos* locus for neurons treated with DMSO + Veh, DMSO + KCl, or Anisomycin + KCl for 4 h.

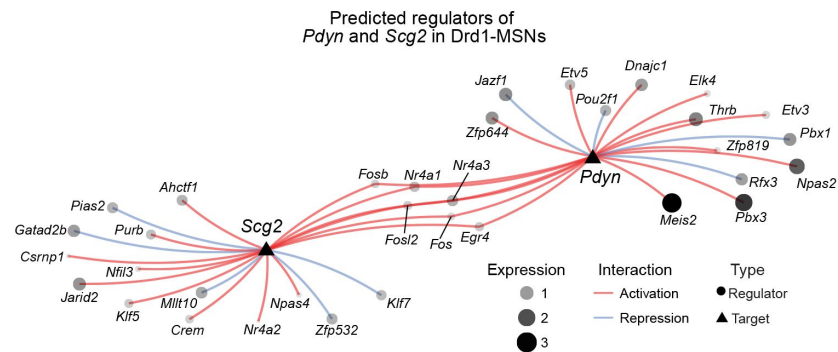


**Figure S4.**

Predicted regulators of *Pdyn* and *Scg2* in Drd1-MSNs. Reconstruction of gene regulatory networks in Drd1-MSNs predicts AP-1 family members such as *Fos* and *Fosb* regulate both *Scg2* and *Pdyn*.

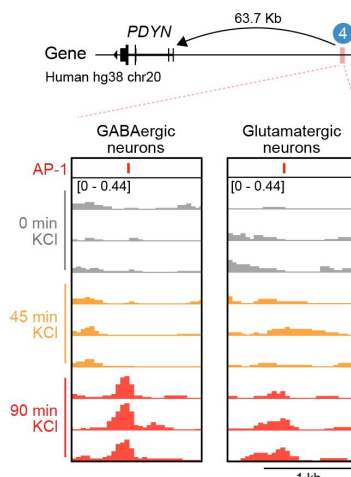


**Figure S5.**

Conserved activity-regulated *PDYN* DAR is selective for GABAergic neurons. ATAC-seq tracks from human induced GABAergic and Glutamatergic neurons treated with 55mM KCl for 0, 0.75, or 1.5 h. Data from Sanchez-Priego, et al., 2022 *Cell Reports*.

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University of California, Los Angeles, United States of America

## Reviewer #1 (Public Review):

Summary:

In this manuscript the authors use ATAC-seq to find regions of the genome of rat embryonic striatal neurons in culture that show changes in regulatory element accessibility following stimulation by KCl-mediated membrane depolarization. The authors compare 1hr and 4hr transcriptomes to see both rapid and late response genes. When they look at ATAC-seq data they see no changes in accessibility at 1hr but strong changes at 4hr. The differentially accessible sites were enriched for the AP-1 site, suggesting regulation by Fos-Jun family members, and consistent with the requirement for IEG expression, anisomycin blocked the increase in accessibility. To test the functional importance of this regulation the authors focus on a putative enhancer 45kb upstream of the activity-induced gene encoding the neuromodulator dynorphin (Pdyn). To test the function of this region, the authors recruited CRISPRi to the site, which blocked KCL-dependent Pdyn induction, or CRISPRa, which selectively increased Pdyn expression in the absence of KCL. Finally the authors reanalyze other human and rat datasets to show cell-type specific function of this enhancer correlated to Pdyn expression.

The idea that stimuli that induce expression of Fos in neurons can change accessibility of regulatory elements bound from Fos has been shown before, but almost all the data are from hippocampal neurons so it is nice to see the different cell type used here. The most interesting part of the study is the identification of the Pdyn enhancer because of the importance of this gene product in the function of striatal neurons. Overall the conclusions appear to be well supported by the data.

## Reviewer #2 (Public Review):

This study aims to characterize transcriptional and epigenetic activity-dependent striatal neuronal adaptations using rat primary cultures, a model still poorly characterized up to date. In addition, the authors aim to interrogate regulatory mechanisms that could modulate the expression of a highly-striatal enriched gene responding to neuronal activation in striatal neuronal cells, the Pdyn gene.

Among the major strengths of the article there is the generation of high quality neuronal RNA-seq and ATAC-seq data in rat striatal neuronal cells in basal level and upon neuronal activity, a experimental setup that has not been so characterized as other more common ones such as mouse hippocampal neuronal cells. In this model, the authors clearly demonstrate the need of protein translation to induce the transcriptional waves of late response genes. In addition, the functional characterization of an enhancer of the Pdyn gene might be of great interest for translational applications in which alterations of this gene might be occurring in neurological disorders.

On the other hand, the manuscript presents some limitations to be considered. One of the major points in this regard is that, at least in part, some of the conclusions reached by the

study related to the induction of particular transcriptional programs upon neuronal activation, the changes in chromatin state, and the need of protein translation for proper induction of LRGs have been already previously described in the literature, affecting the novelty of the study. However, it is needed to be mentioned, that these previous studies were not conducted using the same model (rat striatal neurons), which can make some differences in the final outputs. The other major cautionary point in the study is the selection of the time point for distinguishing early versus late response genes, as the short difference in time and the overlap of part of the transcriptional signature between them suggest that the transcriptional waves are somehow partially overlapping (also probably in part because of the recurrent stimulation of the primary cultures with KCl), which could result in missing part of the late-response genes.

Despite this, the conclusions raised in the study are well supported by the data generated in it.

In summary, the study presents a useful set of transcriptomic and epigenomic data of activity-dependent striatal neuronal programs in rats, which will be of great use for the scientific community working in this not so well characterized model. In addition, the characterization of a Pdyn distal regulatory genomic region involved in its transcriptional regulation, both at basal levels and upon neuronal activation in this particular system, can present translational relevance for striatal disorders such as Huntington's disease or other neuropsychiatric disorders.

### **Reviewer #3 (Public Review):**

This work contributes to the literature characterizing early and late waves of transcription and associated chromatin remodeling following neuronal depolarization, here in cultured embryonic striatum. While they find IEG transcription 1 h after depolarization, they find chromatin remodeling is slower (opening at the 4 h time point). While this is not the first paper to describe chromatin changes in response to neuronal activity, this paper ties previous findings all together in one place using novel sequencing analyses and visualizations. Previous work has found remodeling occurring at the 1 h time point, so the lack of differences at that early time point in the current study needs to be better understood and the "temporal decoupling" described by authors should be further explored. Differences may be due to chromatin at IEG regulatory regions already being open in embryonic tissue (here) vs generally more closed in adult tissue (previous), or due to previous studies using protocols to specifically silence neurons prior to activation. The authors next show that the chromatin remodeling that occurs at the late (4 h) stage is largely in putative regulatory regions of the genome (rather than gene bodies), and is dependent on translation, which validates and extends the prior literature. The authors then transition from genome-wide basic neuroscience to focus on a specific gene of interest, prodynorphin (Pdyn), and a putative enhancer they identify from their chromatin analysis. They target CRISPR-activating and -inhibiting complexes to the putative enhancer and demonstrate that accessibility of this locus is necessary and sufficient for Pdyn transcription. They then show that at least one PDYN enhancer is conserved from rodents to humans, and is only activity-regulated in human GABAergic but not glutamatergic neurons. Finally, the authors generate snATAC-seq and show Pdyn gene and enhancer activity is also cell-type-specific in rat striatum. The Pdyn work, in particular, is thorough and novel, and demonstrates a translational aspect of this work.

### **Author Response**

The following is the authors' response to the original reviews.

We thank all the reviewers for their comments and constructive feedback regarding our manuscript. We have made many changes to strengthen the manuscript, including addition of two new experiments (presented in Fig. S1) that help to clarify the nature and scope of activation of late response genes in striatal neurons. Our specific responses to individual reviewer comments are provided below.

### **Reviewer #1**

#### *Public review*

*Weaknesses: The timing and the location of the accessibility changes are meaningfully different from other similar studies, which should be discussed. The authors provide good data for the function of a single enhancer near Pdyn, but could contextualize this with respect to other regulatory elements nearby.*

In the revised manuscript, we have expanded our discussion of the differences between chromatin accessibility changes observed in this study and those found in prior reports in different systems. These differences are also addressed in extended detail below. Unfortunately, limitations on resources and time prevented a deeper exploration of additional candidate enhancers near the Pdyn locus. However, we believe our efforts to characterize an activity-dependent enhancer in the Pdyn locus provides a useful starting point, and future studies may seek to more completely define the contributions of nearby regulatory elements.

#### *Recommendations For The Authors*

- 1. At 1hr after stimulation in previous papers (Su 2017 which is reference #8 of Fernandez-Albert Nat Neurosci. 2019 October ; 22(10): 1718-1730.) there are large increases in accessibility directly over the IEGs, consistent with the concerted transcription of these genes following stimulation. It is surprising that the authors do not see this here, either at 1hr or at 4hr. This difference in results needs to be addressed.*

We thank the reviewer for bringing this discrepancy to our attention. Indeed, Su et al. 2017 and Fernandez-Albert et al. 2019 both describe increases in chromatin accessibility at IEG promoters. There are several experimental differences that could be contributing to differences between our study and previously published studies. Two major reasons include the developmental timepoint of the tissue/cells and the cell type/brain region that is being assayed. Su et al. assayed chromatin accessibility in ex vivo slices containing the dentate gyrus from adult mice, while Fernandez-Albert et al. assayed chromatin accessibility in forebrain principal neurons of adult mice following kainic acid injection. Bulk ATAC-Seq experiments described in the present manuscript were generated from cultured embryonic rat striatal neurons. Additionally, baseline chromatin accessibility seems to be significantly different between forebrain principal neurons studied in Fernandez-Albert et al. 2019 and the current study. For example, in Figure 3a of Fernandez-Albert et al. 2019, the Npas4 gene body is not accessible in a saline treated animal. In vehicle treated, cultured embryonic rat striatal neurons, the Fos gene body and associated enhancers are accessible at baseline (Fig. S3), and do not increase with KCl depolarization.

We have expanded our discussion of this discrepancy in the discussion section of the revised manuscript, and included additional citations addressing this difference.

1. *It is also somewhat surprising that the authors see almost no regions that show changes in accessibility at 1hr and then a very large number of differentially accessible regions at 4hr. This is quite different from the more rapid changes shown for example in Figure 7f in the human GABA neurons even though these are also studies in culture with rapid calcium channel opening. Can the authors speculate on the reason for the difference?*

Many previously published studies that use cultured neurons include a pre-treatment in which spontaneous neuronal activity is inhibited with the sodium channel blocker tetrodotoxin (SanchezPriego et al. Cell Reports, 2022; Kim et al. Nature, 2010; Malik et al. Nature Neuroscience, 2014). The Sanchez-Priego et al. Cell Reports manuscript also blocked NMDA receptor activity with the competitive NMDAR antagonist D-AP5 for 12 hours prior to depolarization. Rapid changes in chromatin accessibility observed in other studies at <1 hour timepoints could be due to prior silencing of the cells and subsequent reduction in the accessibility and transcriptional activity of IEGs. Decreased baseline accessibility and transcriptional activity of IEGs can be observed in Figure 1a of Malik et al. 2014, which displays ChIP-Seq tracks for both RNA pol II and H3K27ac. At baseline, H3K27ac and RNA pol II enrichment is low throughout the Fos locus. Subsequent depolarization of silenced neurons drives accessibility and transcription of the Fos gene and associated enhancers. In contrast, we found accessible chromatin at Fos enhancer elements at baseline (without stimulation; Fig. S3).

The experiments described in the current study do not include any pre-treatment with tetrodotoxin or D-AP5, and thus the neurons are expected to be spontaneously active. This baseline electrophysiological activity may result in increased accessibility and transcription at IEG loci, which ultimately makes it difficult to identify activity-dependent increases in IEG accessibility at timepoints <1 hour. Furthermore, a previously published manuscript from our lab (Carullo et al. Nucleic Acids Research, 2020) conducted ATAC-seq on cultured embryonic rat cortical, hippocampal, and striatal neurons and found that transcribed enhancers for IEG loci (including Fos) had decreased chromatin accessibility following depolarization when compared to vehicle treatment. These differences in experimental design (including cell type, model organism, developmental timepoint, and treatment paradigm) may all contribute to differences in the temporal dynamics of chromatin remodeling between the current manuscript and previously published studies.

1. *Experimentally it can be challenging to repress a single enhancer and show a significant effect on gene regulation which makes the repression in Fig 6c somewhat unexpected. There are several regions near Pdyn that show activity-dependent changes in accessibility in the human cells (Fig. 7e) and presumably in the rat neurons too (Fig. 5a shows a few but most of the intervening region is cut out). Did the authors target any of these other regions?*

We chose the identified regulatory element upstream of the Pdyn TSS because it met several criteria that we determined are important for characterizing LRG enhancers. These criteria are outlined in the Results: “1) located in non-coding regions of the genome, 2) inaccessible at baseline and accessible following depolarization, and 3) inaccessible when depolarization was paired with protein synthesis inhibition.” Indeed, ATAC-seq experiments presented in the current study demonstrate that thousands of genomic regions undergo reprogramming, and many of these regions meet these criteria (including additional loci near Pdyn). However, we lacked the time and resources to systematically investigate all other enhancers, and did not target any other regions within the Pdyn locus. While many enhancers may regulate a single gene, the identified enhancer seems to be particularly important for activity-dependent Pdyn gene expression. Importantly, CRISPRi-based repression of this enhancer (Fig. 6c) did not reduce basal Pdyn expression as compared to a non-targeting control, but completely blocked

stimulus-dependent induction of Pdyn transcription. We believe this is a useful starting point, and future studies may seek to more completely define the contributions of nearby regulatory elements.

*1. The authors should clarify in the methods or figure legends the number of independent replicate libraries for each experiment and were the RNA and ATAC libraries made from the same or different experiments.*

We thank the reviewer for bringing this to our attention. We have clarified the number of replicates in the methods as outlined below. Additionally, RNA and ATAC libraries were generated from different experiments, and this information is also now included in the methods.

Within the ATAC-Seq library preparation and analysis methods section: “ATAC-seq libraries were generated from experiments independent of the RNA-seq experiments. For the ATAC-seq experiment of neurons treated with vehicle or KCl for 1 h, there were 3 replicates within each treatment group (3 Veh, 3 KCl). For the ATAC-seq experiment of neurons treated with vehicle or KCl for 4 h, there were 3 replicates within each treatment group (3 Veh, 3 KCl). For the ATAC-seq experiment of neurons pre-treated with DMSO or Anisomycin, there were 4 replicates within each treatment group (4 DMSO + Veh, 4 DMSO + KCl, 4 Anisomycin + KCl).”

Within the RNA-seq library preparation and analysis methods section: “RNA-seq libraries were generated from experiments independent of the ATAC-seq experiments. For the RNA-seq experiment of neurons treated with vehicle or KCl for 1 h, there were 3 replicates within the KCl group and 4 replicates within the vehicle group. For the RNA-seq experiment of neurons treated with vehicle or KCl for 4 h, there were 4 replicates within each group (4 Veh, 4 KCl).”

## Reviewer #2

### Public review

*First of all, at a conceptual level, most of the findings related to the induction of particular transcriptional programs upon neuronal activation the changes in chromatin state, and the need for protein translation for proper induction of LRGs have been broadly characterized previously in the literature (Tyssowski et al., Neuron, 2018; Ibarra et al., Mol. Syst. Biol., 2022; and also reviewed by Yap and Greenberg, Neuron, 2018). In addition, it is not so obvious why to focus on Pdyn gene regulatory regions among the thousands of genes upregulated and with modified chromatin landscape after neuronal activation. The authors highlight three particular traits of this gene as the reason to choose it, but those traits are probably shared by most of the genes that are part of the LRGs set.*

We thank the reviewer for these comments, and have included these important publications as citations in our manuscript. With over 5,000 differentially accessible chromatin regions following KCl stimulation, it was not possible to follow up on all regulatory regions or linked genes in a rigorous way. Therefore, we selected a target candidate enhancer near the Pdyn locus for mechanistic studies. In addition to the criteria highlighted in the manuscript, we chose this locus due to decades of literature establishing the importance of prodynorphin in the striatum, and the role of this gene in human neuropsychiatric diseases. We would argue that this increases the relevance of more detailed exploration of this gene, and makes our results applicable to a broader pre-existing literature.

*At the methodological level, some attention should be put into the timings chosen for generating the data. The authors claim that these time points (1h and 4hrs) identify the*

*first (i.e. IEGs) and second (i.e. LRGs) waves of transcription. However, at 4hrs the highest over-expressed genes are still IEGs, as shown in the volcano plots of Figure 1B and 1C, showing a high overlap with up-regulated genes found at 1h (Figure 1D). This might suggest that the 4hrs time point is somewhere in between the first and second wave of transcription, probably missing some of the still-to-be-induced LRGs of the latest one.*

Given that the depolarization applied in RNA-seq and ATAC-seq experiments is continuous, it was not unexpected to find IEGs present at both 1 h and 4 h timepoints. The revised manuscript contains a new experiment (Fig. S1d-f) demonstrating that a shorter depolarization period (1 h KCl followed by a 3 h wash off period) also induces Fos mRNA, but to a much lower extent than 4 h continuous stimulation. In contrast, both short (1 h) and long (4 h) depolarization periods induce Pdyn to equivalent levels when measured at 4 h after the onset of the stimulus. These experiments support our conclusion that LRGs require a temporal delay, and not simply extended stimulation. Nevertheless, the reviewer is correct that a 4 h timepoint may potentially miss some LRGs that are induced even later. We plan to explore the full timecourse of LRG induction in future studies.

*Finally, while only probed as a suggestion, the assumption that from the data generated in this article, we can envision a mechanism by which AP-1 family of transcription factors interacts with the SWI/SNF chromatin remodeling complex is going too far, as no evidence is provided implicated SWI/SNF in the data presented in the manuscript.*

While speculative in the current context, we felt that it was important to highlight this prior literature to identify potential mechanisms that may link IEGs (specifically, AP-1 members) to chromatin remodeling machinery. We have altered this section of the discussion to emphasize that this link is speculative in the context of neuronal chromatin remodeling.

### **Recommendations For The Authors**

- 1. I couldn't find the number of replicates generated for each dataset, neither for RNA nor for ATAC-seq. It could be worth adding these data to the figure legends or in the material and methods.*

We thank the reviewer for bringing this to our attention. The number of replicates generated for each dataset are now included in the methods section (see response to Reviewer #1, comment #4 above).

- 1. In Figure 1D, Gene Ontology terms appear significant only for each of the individual datasets. While this might be expected for the 1h time-point, the 4hrs time-point comprises a big extent of the genes up-regulated at 1h as well, and it is surprising no term related to chromatin or transcription regulation appears as significant. Is this due to the fact that the analysis has been conducted with two separated lists of genes and only the top terms are shown without crossing the data? This could be misleading for the reader and maybe a comparative GO term analysis might be better such as using CluterProfiler or similar tools, that might allow for real comparison of terms enriched in each dataset.*

We thank the reviewer for pointing this out. For Figure 1d, GO term analysis was conducted with two separated gene lists, each consisting of timepoint-specific upregulated DEGs. Thus, 772 genes were included for the analysis of 4 h GO terms and 39 genes were included for the analysis of 1 h GO terms. Previously, comparisons of cellular component GO terms included in the current study only included the top 10 GO terms. The revised manuscript contains an updated analysis that compares all enriched GO terms and identifies that three of the top 10

cellular component GO terms for the 1 h gene set are also identified as significantly enriched in the 4 h gene set. We have revised the graph in Fig. 1f to reflect this updated analysis. Overall, our conclusions (that 1 h and 4 h DEG sets fall into distinct functional categories) remains supported by this analysis.

1. In Figure 3D, the graphs show the density of motifs within the DARs in units of "Motifs/Kb/peak" while the x-axis represents the peaks coordinates from -500bp to +500bp. It is not clear to me how this graph is generated and how within 1000bp the profiles can reach values of 18-20 Motifs/Kb/peak. Could this be clarified?

The motif enrichment score was calculated by identifying the number of total motifs within defined 50bp genomic bins surrounding the center of the DAR regions. HOMER builds enrichment histograms that normalize motif presence to set size (e.g., number of peaks or DARs), and also to genomic space (base pairs). While HOMER's default histogram represents motifs/bp/peak, we converted this to motifs/kb/peak for ease of interpretation. However, to avoid confusion we have returned the y axis labels to the default HOMER settings (motifs/bp/peak). The normalization and units for this graph have been clarified in the methods section.

1. In Figure 4C the newly generated ATAC-seq data is just "targeted" analyzed, showing global tendencies are maintained between the initial generated data and this one. It could be interesting, however, to see the number of DARs obtained in these conditions, especially to see if some DARs are observed in the Anisomycin condition that might be translation-independent.

The experiment described in Figure 4 was designed to both validate the 5,312 DARs and understand the role of protein translation in activity-dependent chromatin remodeling. One way to begin identifying translation-independent DARs is to compare the DMSO + Vehicle group to the Anisomycin + KCl group. With this comparison, any 4 h DAR that has increased accessibility in the Anisomycin + KCl group may be translation-independent as pretreatment with anisomycin did not prevent chromatin remodeling. After conducting this analysis, we identified a very small percentage (3.44%) of 5,312 4 h DARs that still exhibited significantly increased accessibility when pre-treated with Anisomycin. This small number is consistent with the robust effects of anisomycin on KCl-dependent remodeling shown in Fig. 4c-d. However, to confirm that these were in fact translation-independent activity-regulated DARs, we would need to perform direct comparison of chromatin accessibility between neurons pre-treated with Anisomycin and then treated with either vehicle or KCl. Since we did not include an anisomycin only group in experiments in Fig. 4, we cannot confidently claim whether this 3.4% of DARs are translation-independent. Nevertheless, we agree with the reviewer that this is an interesting avenue of future exploration.

### Reviewer #3

Public review

1. Throughout the paper, the authors emphasize a "temporal decoupling" of transcriptional and chromatin response to depolarization, based on a lack of significant chromatin changes at 1h, despite IEG transcription. However, previous publications show significant chromatin remodeling at 1h (e.g. Su et al., NN 2017 in adult dentate gyrus) or 2h (Kim et al., Nature 2010; Malik et al., NN 2014 in cultured embryonic cortical neurons). The discussion briefly mentions this contrast, but it remains difficult to conclude decisively whether there is temporal decoupling when such decoupling is not found consistently. If one is to make broad conclusions about basic neural chromatin response to depolarization, it would be ideal to know under which conditions there is temporal decoupling, or if this is a region-specific phenomenon.

Indeed, prior studies referred to in our manuscript have identified chromatin remodeling at earlier timepoints than we identified here. As addressed above (Reviewer #1, comments 1 & 2), it is possible that this discrepancy arises due to the difference in experimental model system, differences in the type of stimulation applied, pretreatment protocols used to silence neurons prior to activation, or even differences in developmental stage. Differences in each of these parameters make it difficult to make straightforward comparisons between datasets and results in this manuscript. It is possible that other cell types induce IEGs more quickly (or more robustly) in response to stimulation, which could lead to earlier chromatin remodeling. However, the common patterns of chromatin reorganization (e.g., the fact that changes are enriched at AP-1 motifs and are found in intergenic regions at putative enhancers) lend support for the idea that the transcriptional waves identified here can also be found in other cell types and in other contexts.

1. The UMAP analysis is a novel way to probe transcription factor enrichment, but it's unclear what this is actually showing. The authors sought to ask whether "DARs could be separated based on transcription factor motifs in these regions." However, the motifs present in any genomic stretch are fixed based on genomic sequence, so it seems like this analysis might be asking whether certain motifs are more likely to be physically clustered together in the genome, in activity-regulated regions (rather than certain transcription factors acting in concert, as is implied in discussion). While still potentially interesting, this analysis does not seem to give much additional insight into activity-dependent chromatin remodeling beyond the motif enrichment analysis already performed. Nevertheless, to draw stronger conclusions, it would be necessary to compare clustering to a random set of genomic regions of the same length/size to interpret the clustering here. It would also be useful to know whether the ISL1 motif is also enriched in ubiquitously accessible genomic regions in the striatum (and not just DARs).

We agree that additional analysis is needed to explore enrichment of various transcription factor motifs and activity at differently accessible regions of the genome. The motif enrichment analysis in Figure 3 demonstrated the types of motifs that were enriched in DARs (Fig. 3a-c), the overall degree of enrichment (Fig. 3c), and the distribution of those motifs across DAR sites (Fig. 3d). This analysis allowed us to understand whether motifs for cell-defining transcription factors like ISL1 are enriched uniquely in DARs, or are also found in other regions that are accessible at baseline (see direct comparisons between vehicle/baseline peaks and DARs in Fig. 3d). However, these approaches represent enrichment across all DARs as group, and do not show TF presence/absence at any specific DAR. The UMAP analysis presented in Figure 3e allowed identification of DAR clusters based on the presence or absence of specific transcription factor motifs, and allowed us to represent specific DARs in a reduced two-dimensional space. Because this analysis identifies the existence of distinct motifs within single DARs, it allowed us to speculate as to the possibility of transcription factor cooperation within DARs, or the meaning of DAR clusters that appear to be defined by

specific motifs (e.g., KLF10 in Fig. 3f). Given the information that this adds to the initial analyses, we argue that its inclusion in the manuscript is useful and potentially informative for generating follow-up hypotheses.

*1. The authors identify late-response gene enhancers by 3 criteria. However, only Pdyn was highlighted thereafter. How many putative DARs met these three criteria in striatum? Only Pdyn?*

As illustrated in Figures 2 and 4, nearly all of the DARs in our dataset met these criteria, which included presence in non-coding genomic regions, increase in accessibility following stimulation, and prevention of chromatin accessibility changes by protein synthesis inhibition. We did not mean to indicate that the Pdyn locus was unique in this way. In addition to the criteria highlighted in the manuscript, we chose this locus due to decades of literature establishing the importance of prodynorphin in the striatum, and the role of this gene in human neuropsychiatric diseases. We would argue that this increases the relevance of more detailed exploration of the regulator mechanisms that control expression of this gene, and makes our results applicable to a broader pre-existing literature. The revised manuscript includes additional experiments that examine Pdyn expression changes in response to different stimuli, which help to justify the focus on this gene from the beginning of the manuscript.

#### *Recommendations For The Authors*

*1. Figure 1 volcano plots show a scatter primarily in the up-regulated portion at both the 1-h and 4-h time points. However, the Venn diagrams show largely similar numbers of up- and downregulated genes at the 4-h time point. Is the clustering of down-regulated genes tighter/more overlapping? If so, semi-translucent volcano dots or some acknowledgment of the visual discrepancy would be useful.*

We thank the reviewer for bringing this to our attention. Down-regulated genes are clustering tighter on the volcano plot due to smaller fold changes. This visual discrepancy is acknowledged by the numeric indicators of up- and down-regulated genes in the upper left-hand corner of the volcano plot.

*1. Methods for RNA and ATAC seq analysis align to human genome Hg38, rather than rat?*

RNA- and ATAC-Seq analyses from rat neurons were aligned to the mRatBn7.2/Rn7 rat genome. RNA- and ATAC-Seq analyses from human neurons were aligned to the Hg38 human genome. We have updated the methods to make this clear.

*1. The introduction states that different classes of neurons induce distinct LRGs. Please add a citation. Citations are also needed for the last statement WRT consequences of chromatin remodeling near LRGs not being concretely linked to LRG transcription.*

We thank the reviewer for pointing this out. The revised manuscript now includes additional citations supporting each of these statements.

*1. Specify somewhere in Methods that DEGs were compared to vehicle for both 1-h and 4-h (and not 4 vs 1 h).*

We thank the reviewer for bringing this to our attention. We have updated the methods to include: “DEGs were calculated by comparing the KCl and Vehicle treatment groups at each

respective timepoint.”

1. In Figure 2E, why are the enrichments exactly opposite, especially given these are two different types of input (all baseline peaks vs DARs)?

Odds ratios were calculated by comparing baseline peaks (i.e., ATAC-seq peaks identified in vehicle treated cells) to KCl-induced DARs. This allowed us to identify the enrichment of DARs in specific genomic annotations in comparison to the genomic features that are accessible at baseline, rather than making comparisons to random probe sets or genomic space dedicated to these distinct annotations. This analysis identified that relative to baseline peaks, DARs are significantly depleted in coding regions of the genome and enriched in non-coding regions of the genome. However, given this analysis we agree that it does not make sense to graph both the vehicle (baseline) and DARs on this graph, given that enrichment of each set is determined relative to the other (creating the reciprocal enrichment in this panel). We have updated Fig. 2e to only include points for 4 h DARs.

1. Some references are off. One that I noted was "...chromatin remodeling in the mouse dentate gyrus following 1 h of electricoconvulsive stimulation" should be Su et al 2017 not Malik 2014. For the statement that IEGs are critical regulators of non-neuronal IEGs, the authors may want to add Hrvatin 2017 ref.

We thank the reviewer for bringing this to our attention. We have revised the manuscript to include the correct citation for this claim, and also to include the Hrvatin, et al reference.

1. It would be helpful for the authors to write out the whole gene name for Pdyn somewhere.

We have updated the text to include the gene name for Pdyn, both in the abstract and also in the introduction of the manuscript.

1. Figure 5f: For ease, please include what is high vs low in the figure caption in addition to the main text.

We thank the reviewer for bringing this to our attention. We have updated the figure caption and main text to include what is high vs low in Pseudotime estimates in Fig. 5f.

1. How are the tracks ordered in Fig8c?

Tracks within Fig. 8c demonstrate snATAC-seq signal at the Pdyn gene locus for transcriptionally distinct cell types within the NAC. The tracks are ordered by cluster size (nuclei number) in the snATAC-seq dataset.