CELLULAR NEUROSCIENCE

A long noncoding eRNA forms R-loops to shape emotional experience–induced behavioral adaptation

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Emotional experiences often evoke neural plasticity that supports adaptive changes in behavior, including maladaptive plasticity associated with mood and substance use disorders. These adaptations are supported in part by experience-dependent activation of immediate-early response genes, such as *Npas4* (neuronal PAS domain protein 4). Here we show that a conserved long noncoding enhancer RNA (lnc-eRNA), transcribed from an activity-sensitive enhancer, produces DNA:RNA hybrid R-loop structures that support three-dimensional chromatin looping between enhancer and proximal promoter and rapid *Npas4* gene induction. Furthermore, in mouse models, *Npas4* lnc-eRNA and its R-loop are required for the development of behavioral adaptations produced by chronic psychosocial stress or cocaine exposure, revealing a potential role for this regulatory mechanism in the transmission of emotional experiences.

Emotional experiences, which we define here as physiological arousal in specific contexts that shapes cognitive processing, often lead to behavioral responses that enable an organism to adapt to its environment (*1*, *2*). These emotional stimuli trigger the expression of neuronal activity–induced immediate-early response genes (IEGs), which initiate transcriptional programs that support cellular, synaptic, and behavioral plasticity (*3*). IEGs are critical for adaptive behavioral responses after emotional experiences, and their dysregulation mediates maladaptive behaviors associated with neuropsychiatric disorders, including mood and substance use disorders (SUDs) (*4*–*6*). Rapid and accurate IEG induction is regulated by complex cell signaling and epigenetic mechanisms acting on conserved genomic enhancers that can be located far from the gene's proximal promoter (*7*). However, the nature of this regulation remains elusive. Recent advancements in sequencing technology revealed that a vast majority of the mammalian genome (~80%) is dedicated to regulation of gene expression, and the ENCODE (Encyclopedia of DNA Elements) project showed that ~60% of the human genome produces non-protein-coding RNAs, including long noncoding RNAs (lncRNAs) (>200 nucleotides) (*8*, *9*). These lncRNAs are differentially expressed in patients with disrupted reward-associated behavior, such as major depressive disorder, schizophrenia, and SUDs (*10*, *11*). A subclass of lncRNAs transcribed from genomic enhancers, called enhancer RNAs (lnc-eRNAs), influence gene expression through various mechanisms (*9*, *12*–*15*). Lnc-eRNAs can form DNA:RNA hybrid three-stranded structures known as R-loops (*16*, *17*). R-loops can influence various transcriptional, translational, and repair mechanisms (*18*). However, the physiology and function of R-loops in the brain remain largely unexplored.

Emotional experiences, including exploration of novel environments, use of psychoactive substances, or psychosocial stress, trigger the rapid and transient expression of IEGs (*1*) in brain regions such as the nucleus accumbens (NAc) (*6*, *19*), hippocampus (*20*), and medial prefrontal cortex (mPFC) (*5*). One such IEG, *Npas4*, encodes the transcription factor neuronal PAS domain protein 4 (NPAS4), which drives cell type–specific transcriptional programs that support behavioral adaptations (*21*–*23*). Here we demonstrate that an lnc-eRNA, and its associated R-loops, functions to regulate activity-induced *Npas4* gene expression in response to emotional experiences.

A conserved *Npas4* enhancer region produces an lnc-eRNA

Npas4's putative enhancer element is located ~3 kb upstream of the transcription start site, and it mediates neuronal activity–dependent induction of $Npas4^{mRNA}$ (*6*) (Fig. 1A and fig. S1). It is highly conserved in vertebrates, including humans, and associates with various transcriptional regulators, including CCCTC-binding factor (CTCF), which promotes three-dimensional (3D) chromatin looping (*24*) (fig. S1). Indeed, our chromosome conformation capture (3C) assay in the NAc and mPFC of mice confirmed that the *Npas4* enhancer interacts with its promoter through a 3D loop, which increases transiently after emotional experiences, such as cocaine conditioning or social defeat stress, paralleling *Npas4* expression (Fig. 1, B and C) (*6*). Like other IEG enhancers (*9*, *13*, *14*), it is associated with RNA polymerase II (RNAPII), which suggests RNA transcription (fig. S1). Using total RNA sequencing (RNA-seq) of adult mouse NAc or mPFC samples from home-caged animals and at 15 min after social defeat stress or cocaine conditioning in a novel context, we detected mRNAs and ncRNAs that map to the *Npas4* genomic region (Fig. 1A). These emotional experiences increased minus-strand *Npas4* mRNA expression in the mPFC and NAc, respectively (Fig. 1A), as previously observed (*5*, *6*). In all conditions in both NAc and mPFC, we found a low abundance of a nonannotated, ~2.2-kb lnc-RNA transcript mapping to the positive strand of the enhancer region (Fig. 1A). Subsequent analysis of published total RNA-seq datasets of cultured primary mouse neurons (*13*) and human postmortem PFC samples (*25*) (Fig. 1A) confirmed the transcription of $Npas4^{eRNA}$, extending the findings from mice to humans.

$Npas4^{eRNA}$ is required for $Npas4^{mRNA}$ expression

To test the function of $Npas4^{eRNA}$, we altered its expression using a combination of a viral-mediated RNA-interference (RNAi) approach and single-guide RNA (sgRNA)–targeted recruitment of CRISPR-transcriptional activator, CRISPRa [enzymatically dead Cas9 (dCas9)–VPR) (*26*) (Fig. 2, A and B). CRISPRa recruitment by sgRNA-E1 and sgRNA-E1′ to the *Npas4* enhancer increased the expression of both $Npas4^{eRNA}$ and $Npas4^{mRNA}$ in Neuro2A cells (Fig. 2B). $Npas4^{eRNA}$-shRNA (short hairpin RNA) not only reduced $Npas4^{eRNA}$ to basal expression but also reduced $Npas4^{mRNA}$ by ~50% (Fig. 2B). In contrast, $Npas4^{mRNA}$-shRNA decreased $Npas4^{mRNA}$ expression by ~50% but had no effect on $Npas4^{eRNA}$ expression (Fig. 2B). This suggests a unidirectional necessity of *Npas4* lnc-eRNA for full $Npas4^{mRNA}$ expression. Furthermore, recruitment of CRISPRa directly to the *Npas4* promoter increased $Npas4^{mRNA}$ expression, but in this context, reducing $Npas4^{eRNA}$ had no effect on $Npas4^{mRNA}$ (fig. S2, A and B), highlighting the specific role of $Npas4^{eRNA}$ on enhancer function. Similarly, injection of neurotropic AAV2-$Npas4^{eRNA}$-shRNA in NAc and mPFC of adult mice reduced $Npas4^{eRNA}$ expression and cocaine conditioning– or social defeat stress–induced up-regulation of $Npas4^{mRNA}$ expression (Fig. 2C and fig. S2C) without altering $cFos^{mRNA}$ expression, suggesting that the noted reduction of $Npas4^{mRNA}$ is specific and not caused by an indirect effect of reduced neuronal activity. Finally, AAV2-mediated overexpression of $Npas4^{eRNA}$ in the NAc increased $Npas4^{mRNA}$ expression but not $cFos^{mRNA}$ in home-caged mice (Fig. 2D). Taken together, these data suggest that $Npas4^{eRNA}$ is both necessary and sufficient to specifically augment $Npas4^{mRNA}$ expression.

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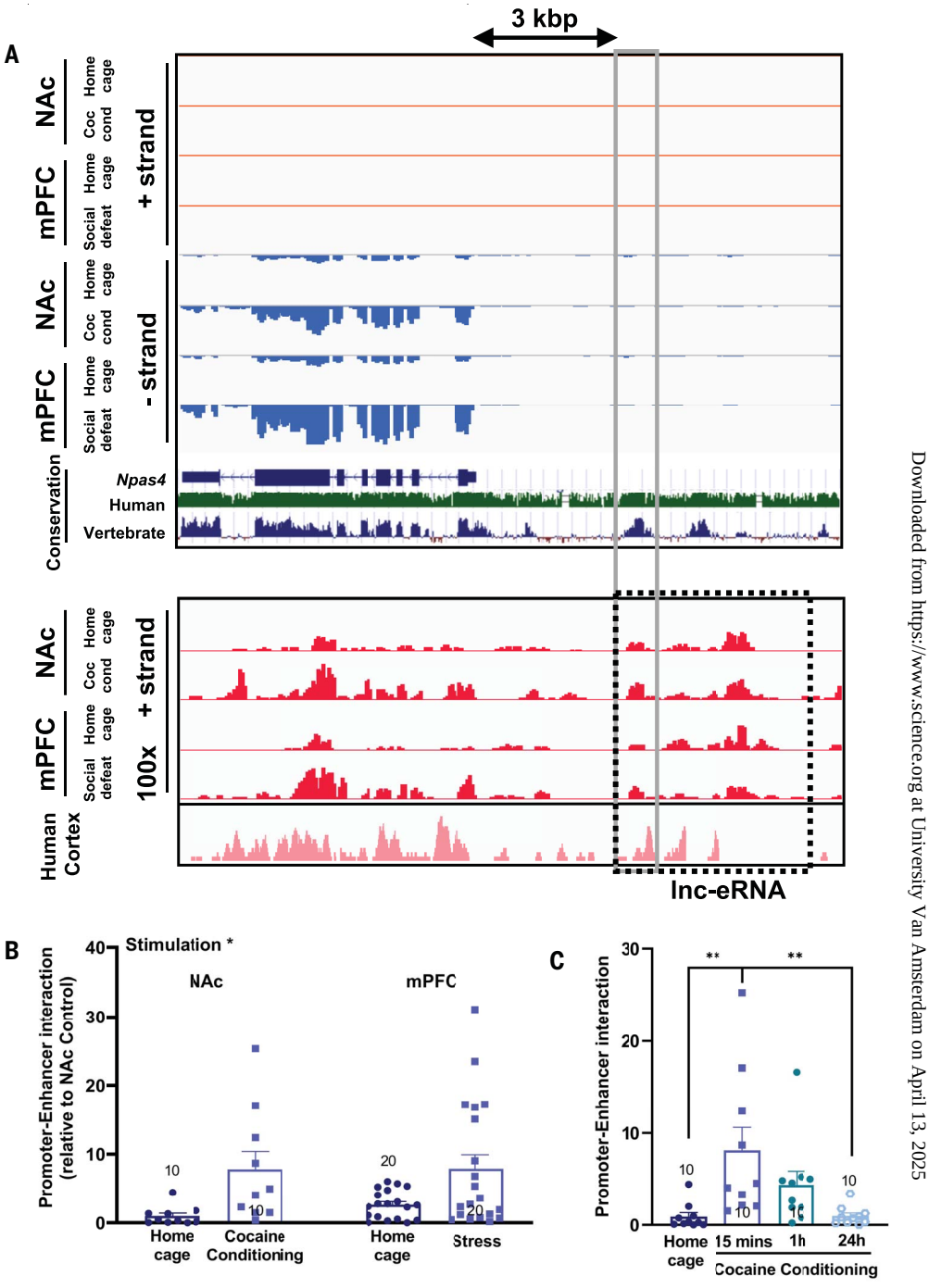

Fig. 1. *Npas4* conserved enhancer produces a long noncoding enhancer RNA. (**A**) Genomic landscape of the *Npas4* enhancer and gene coding region combined with total RNA-seq data from NAc of mice subjected to cocaine conditioning ("Coc cond") or home-caged controls, as well as from mPFC of mice subjected to social defeat stress or home-caged controls. Additionally, human and vertebrate conservation are included. On the lower part, the positive strand is visualized at 100× scale in comparison to the data on top, together with total RNA-seq data from the human cortex. The enhancer region is indicated with a gray rectangle, and *Npas4*^{eRNA} is indicated with a dashed black rectangle. kpb, kilo–base pairs. (**B**) 3C assay shows the *Npas4* enhancer-promoter interaction in home-caged control mice. Cocaine conditioning and social defeat stress increase this interaction in the NAc and mPFC, respectively (n = 10 to 20 mice per group). (**C**) 3C assay shows the time course of *Npas4* enhancer-promoter interaction in the NAc (n = 10 mice per group). Data display the mean ± SEM. Statistical analyses were performed using a one- or two-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test; *P < 0.05, **P < 0.001. Detailed statistical analyses are provided in table S1.

Npas4^{eRNA} and *cFos*^{eRNAs} form DNA:RNA hybrid R-loops at their enhancers

To explore the mechanism by which *Npas4*^{eRNA} regulates *Npas4*^{mRNA} expression, we examined its sequence composition. *Npas4*^{eRNA} was rich in GC (>60%), and some areas displayed a substantial GC skew [(G − C)/(G + C)] compared with the surrounding regions (Fig. 3A). The GC skew led us to speculate that *Npas4*^{eRNA} might form DNA:RNA hybrids (R-loops) (*27*). Using a DNA:RNA hybrid R-loop immunoprecipitation (DRIP) assay (fig. S3, A and B) with cultured striatal or cortical neurons (fig. S3, C and D) or mouse NAc or mPFC tissues (Fig. 3, B and C), we detected enrichment in R-loops in the *Npas4*^{eRNA} coding regions. Treatment with ribonuclease H1 (RNaseH1), an endonuclease specifically degrading RNA in R-loops (*28*), confirmed the specificity of this enrichment (Fig. 3, B and C, and fig. S3, C and D). Dot blot analysis in cortical neurons demonstrated that KCl stimulation increased R-loops, supporting the formation of activity-dependent R-loops (fig. S3E). We detected a basal, stimulation-independent R-loop within the *Npas4*^{eRNA} coding region (fragment A; Fig. 3, B and C, and fig. S3, C and D) as well as activity/experience-inducible R-loops (fragments B and C; Fig. 3, B and C, and fig. S3, C and D) that correlated with the transient kinetics of *Npas4*^{mRNA} expression (Fig. 3, D and E). R-loops were not detected between the enhancer and promoter (fragment α; Fig. 3, B and C, and fig. S3, C and

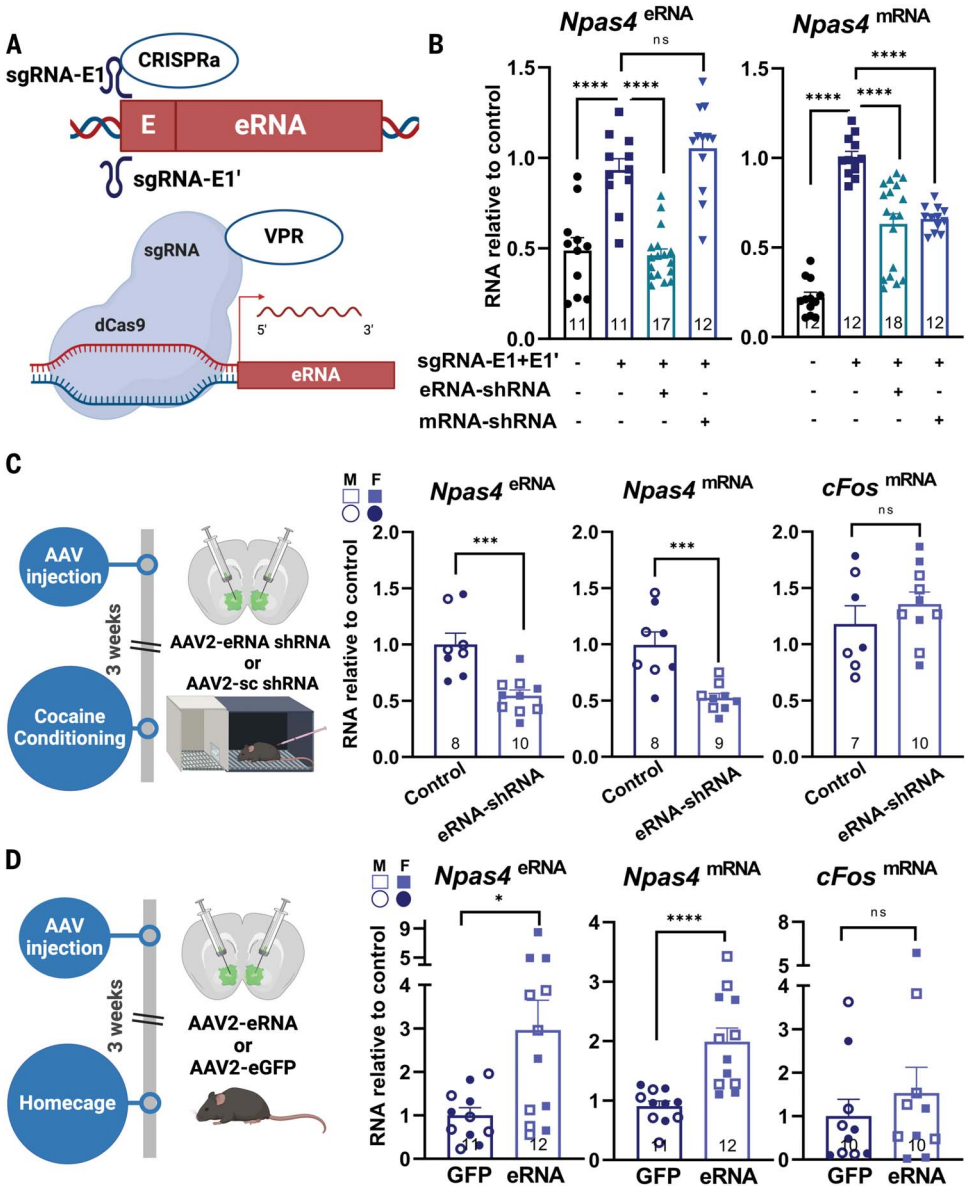

Fig. 2. *Npas4*^{eRNA} is required for *Npas4*^{mRNA} expression. (**A**) Experimental design showing CRISPRa recruitment to the *Npas4* enhancer region using sgRNAs (sgRNA-E1 and sgRNA-E1'). VPR, VP64-p65-Rta. (**B**) CRISPRa + sgRNA-E1+E1' increases *Npas4*^{mRNA} and *Npas4*^{eRNA}, *Npas4*^{eRNA}-shRNA reduces *Npas4*^{eRNA}, and CRISPRa-induced *Npas4*^{mRNA} in N2A cells (n = 11 to 18 biological replicates for each group). (**C**) *Npas4*^{eRNA}-shRNA reduces *Npas4*^{eRNA} and cocaine conditioning induced *Npas4*^{mRNA} in the NAc of mice compared with scrambled control; levels of *cFos* are similar across conditions (n = 7 to 10 mice per group). (**D**) *Npas4*^{eRNA} overexpression increases *Npas4*^{eRNA} and *Npas4*^{mRNA} in the NAc of home-caged mice compared with green fluorescent protein (GFP) overexpression; levels of *cFos* are not changed across conditions (n = 10 to 12 mice per group). Data plots show the mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by a Tukey's post hoc multiple comparison test and Mann-Whitney U test; *P < 0.05, ***P < 0.001, ****P < 0.0001, nonsignificant (ns) P > 0.05. Detailed statistical analyses are provided in table S1.

D) nor at 4 kb upstream of the *Npas4* enhancer outside of the *Npas4*^{eRNA} coding region (fig. S3, F and G). In contrast to *Npas4*, R-loops found at the actin beta (*Actb*) gene, which exhibits constitutive gene expression, demonstrated no changes in the NAc or mPFC in response to the emotional experiences (fig. S3H). The IEG *Fos* is regulated by multiple enhancers and lnc-eRNAs (fig. S4A) (*9*, *13*), and two of these enhancer regions (2 and 5) had substantial GC enrichment and GC skew (fig. S4B). Consequently, our DRIP analysis in the NAc and mPFC showed enrichment of R-loops at enhancers 2 and 5 but not at enhancer 1 (fig. S5, A and B). In comparison with home-caged behavior, cocaine conditioning or social defeat stress increased *Fos* enhancer 2 R-loops in the NAc and mPFC, respectively (fig. S5, A and B). In addition, cocaine conditioning decreased the *Fos* enhancer 5 R-loop, whereas stress increased it (fig. S5, A and B). This suggests that R-loops at IEG enhancers are dynamically and differentially regulated by emotional experiences to support rapid IEG expression. Similar to *Npas4*, R-loop expression at *Fos* enhancers in the NAc mirrored the dynamics of *Fos*^{mRNA} expression after cocaine conditioning (fig. S5, C and D). Together, our data reveal the presence of R-loops at eRNA-transcribing regions that can be either constitutive or dynamically regulated by emotional experiences.

R-loops at the *Npas4* enhancer regulate activity-induced *Npas4*^{mRNA} expression

To test the function of the *Npas4*^{eRNA}-region R-loops in regulating *Npas4*^{mRNA} expression, we used a CRISPR-mediated approach combining sgRNA-mediated targeting of a fusion protein comprising dCas9 and enzymatically active RNaseH1 (CRISPR-H1) to degrade genomic locus-specific R-loops (Fig. 4A). We validated this lentivirus-mediated R-loop–degrading system (fig. S6) and used a dCas9 fused with an enzyme-dead RNaseH1 mutant (*29*) (CRISPR-H1d) as a negative control. In cultured primary neurons, we found that CRISPR-H1 targeted to either the proximal (sgRNA-E1) or distal (sgRNA-E2) eRNA-transcribing region reduced depolarization-induced *Npas4*^{mRNA} expression (fig. S7). Similarly, NAc infusion of Lenti-CRISPR-H1 combined with sgRNA-E1 or sgRNA-E2 reduced cocaine conditioning–induced *Npas4*^{mRNA} expression in male and female mice (Fig. 4, A to C). It also decreased

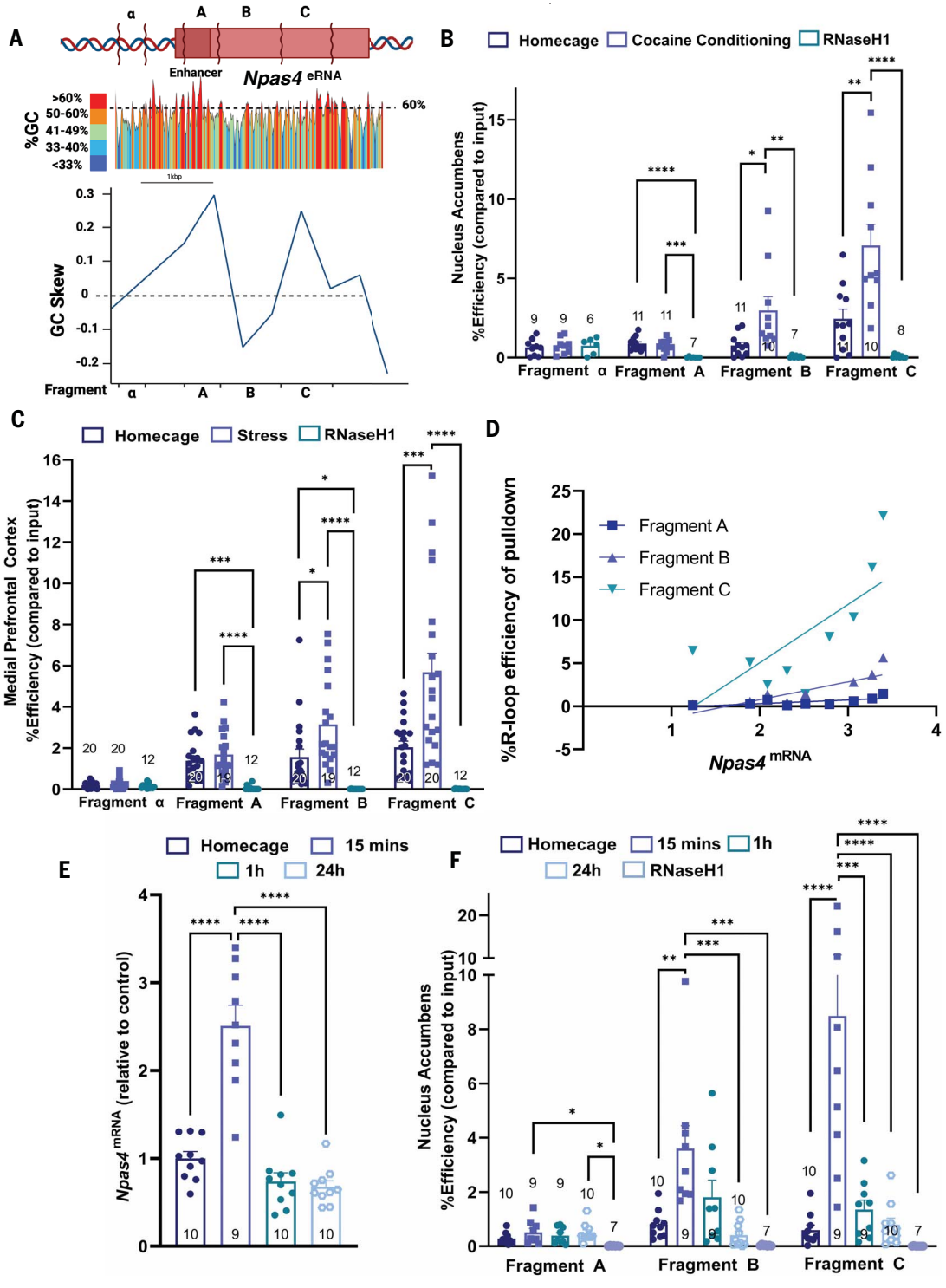

Fig. 3. *Npas4*^{eRNA} forms DNA:RNA hybrid R-loops at the enhancer. (**A**) Diagram showing genomic fragments of the *Npas4* enhancer region after enzymatic digestion with a %GC (guanine-cytosine) plot (calculated and adapted from SnapGene) as well as their GC skew. (**B** and **C**) DRIP-qPCR assay showing the percent enrichment of genomic fragments over the input in the NAc of mice with or without cocaine conditioning (B), the mPFC of mice with or without social defeat stress (C), and RNaseH1 pretreatment around *Npas4* enhancer. The DRIP-qPCR assay shows enrichment of fragments A, B, and C in comparison to RNaseH1 control, with fragments B and C showing increased enrichment after cocaine conditioning (B) and social defeat stress (C) compared with the home-caged controls. Fragment α does not show the increased enrichment compared with RNaseH1 negative control (n = 6 to 20 mice per group). (**D**) Correlation analyses of enrichment fragments A, B, and C with *Npas4*^{mRNA} expression in the NAc after 15 min of cocaine conditioning (n = 9 or 10 mice per group). (**E** and **F**) Time course of *Npas4* mRNA expression and R-loops in the NAc after cocaine conditioning. *Npas4*^{mRNA} is increased in the NAc of mice after 15 min of cocaine conditioning compared with those in the home cage. At 1 and 24 hours after cocaine conditioning, *Npas4*^{mRNA} expression is similar to home-caged controls (n = 9 or 10 mice per group) (E). The DRIP-qPCR shows enrichments of fragment A compared with RNaseH1 control, and fragments B and C show increased enrichment 15 min after cocaine conditioning compared with mice in the home cage. At 1 and 24 hours after cocaine conditioning, the enrichments are similar to home-caged controls (n = 7 to 10 mice per group) (F). Data show the mean ± SEM. Statistical analyses were performed using a one-way ANOVA followed by Tukey's post hoc multiple comparison test; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Detailed statistical analyses are provided in table S1.

Npas4^{eRNA} expression (fig. S8, A and B) but without changing *cFos*^{mRNA} expression (fig. S8C) or the amounts of a low-abundance (<10%) *Npas4*^{mRNA} isoform containing a long 5′ untranslated region (long-5′UTR *Npas4*^{mRNA}) spanning the *Npas4*^{eRNA} coding region (fig. S8, A to C), suggesting that the targeted recruitment of CRISPR-H1 to the *Npas4* enhancer does not degrade RNAs synthesized in the vicinity. Moreover, sgRNA-mediated targeting of CRISPR-H1 to the *Npas4* protein-coding region (sgRNA-CR) or regions between the *Npas4* promoter and

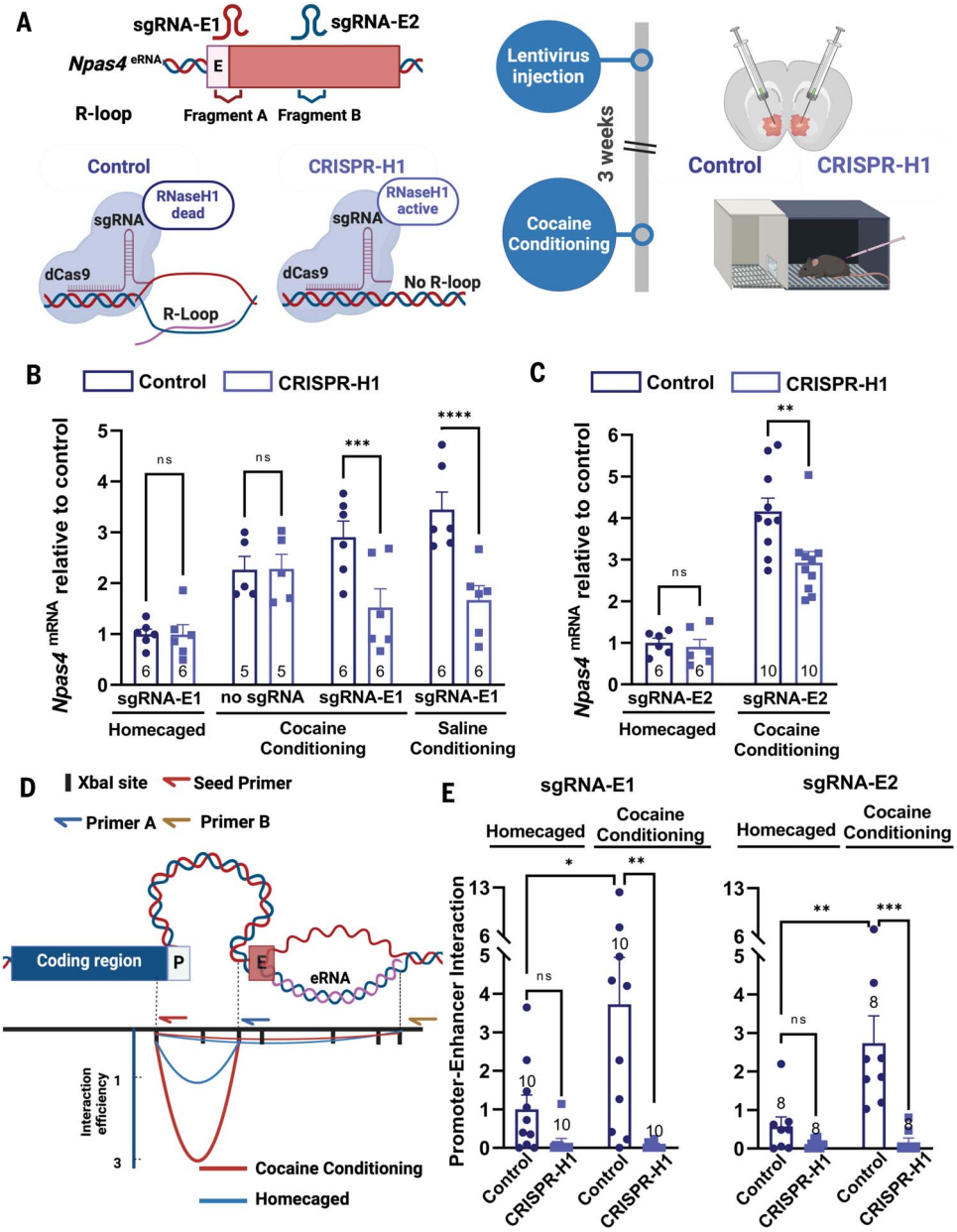

Fig. 4. R-loops at the *Npas4* enhancer regulate activity-induced *Npas4*^{mRNA} expression and promoter-enhancer 3D chromatin looping. (**A**) (Left) Experimental design using dCas9-RNaseH1 active (CRISPR-H1) or enzymatically dead (control) with sgRNAs targeting the constitutive R-loop at the *Npas4* enhancer (sgRNA-E1) or the activity-inducible R-loop (sgRNA-E2) to degrade R-loops in a locus-specific manner. (Right) Diagram showing experimental strategy to assess R-loop function in the NAc after cocaine conditioning. (**B** and **C**) CRISPR-H1 recruited with sgRNA-E1 (B) and sgRNA-E2 (C) in the NAc reduces cocaine and saline conditioning-induced *Npas4*^{mRNA} expression compared with the enzymatically dead control [n = 5 or 6 mice per group (B), n = 6 to 10 mice per group (C)]. (**D**) Schematic of the experimental strategy to detect 3D looping through a 3C method with primers. (**E**) 3C analysis shows that *Npas4* promoter-enhancer interaction in the NAc of control mice (CRISPR-H1d + sgRNA-E1/E2) is increased after 15 min of cocaine conditioning, whereas CRISPR-H1 + sgRNA-E1/E2 eliminates the promoter-enhancer interaction in basal and cocaine-conditioned animals (n = 8 to 10 mice per group). Data display the mean ± SEM. Statistical analyses were performed using two-way ANOVA followed by Tukey's, Bonferroni, or Sidak post hoc multiple comparison test; *P < 0.05, ** P < 0.01, ***P < 0.001, ****P < 0.001, nonsignificant P > 0.05. Detailed statistical analyses are provided in table S1.

enhancer (sgRNA-E3) did not alter *Npas4*^{mRNA} expression (fig. S6B), further confirming that the reduction of *Npas4*^{mRNA} induced by CRISPR-H1 plus sgRNA-E1/E2 was not caused by direct *Npas4*^{mRNA} degradation. Together, these data reveal that R-loops formed at the *Npas4*^{eRNA}-transcribing region are critical for stimulus-dependent induction of *Npas4*^{mRNA} expression in vitro and in vivo.

R-loops at the *Npas4* enhancer regulate activity-induced promoter-enhancer 3D chromatin looping

As 3D chromatin loops between distal regulatory elements and proximal promoters can support gene expression (*30*), we sought to test whether the *Npas4*^{eRNA}-associated R-loops are involved in the formation of the chromatin loop between the *Npas4* promoter and enhancer. We thus used a 3C assay in NAc tissues isolated from home-caged or cocaine-conditioned (15 min, 7.5 mg/kg; intraperitoneal injection) adult male and female mice (Fig. 4D). By using a seed primer and primer A (Fig. 4D), we detected an increase in the *Npas4* promoter-enhancer interaction after cocaine conditioning (Figs. 1, B and C, and 4E), and CRISPR-H1 targeted with sgRNA-E1 or sgRNA-E2 (Fig. 4E, left and right, respectively) nearly eliminated both the basal and cocaine conditioning–induced promoter-enhancer interaction. We failed to detect an interaction between the *Npas4* promoter and a genomic region 4.0 kb away from the *Npas4* enhancer using seed primer and primer B [no detection by quantitative polymerase chain reaction (qPCR)]. These data reveal that *Npas4*^{eRNA}-associated R-loops are required for basal and stimulus-induced 3D chromatin looping of the *Npas4* promoter and enhancer to regulate experience-dependent *Npas4* expression.

Npas4^{eRNA} and its R-loops are required for behavioral adaptations produced by cocaine or chronic stress experiences

To test the importance of the *Npas4*^{eRNA} on positive or negative emotion–induced behavioral adaptation, we used cocaine conditioned

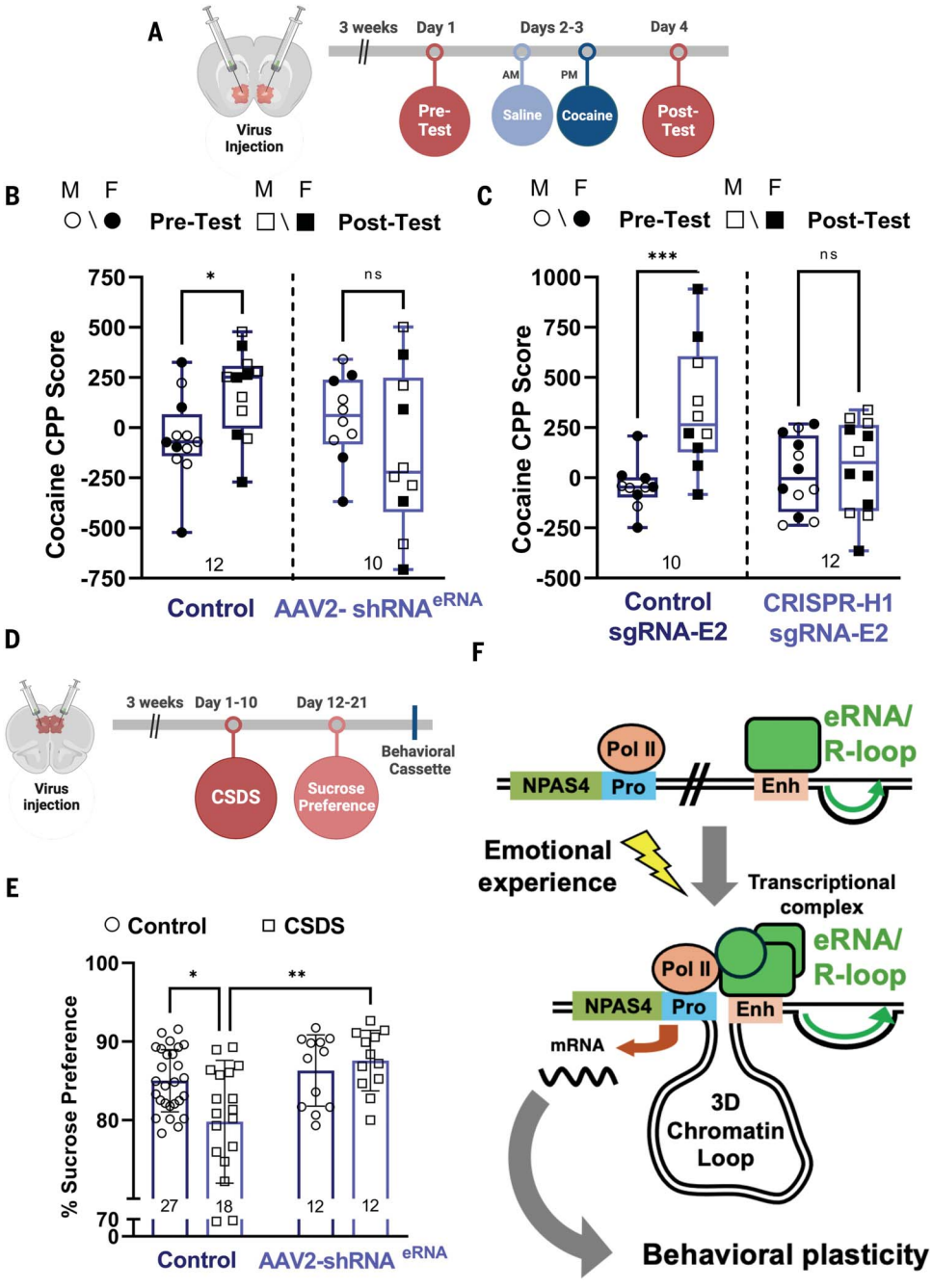

Fig. 5. *Npas4*^{eRNA} and its R-loop are required for behavioral adaptation produced by cocaine or chronic stress experiences. (**A**) Diagram showing the experimental timeline of cocaine CPP with adult male and female mice that received AAV2-*Npas4*^{eRNA} shRNA, control shRNA, or Lenti-dCas9-RNaseH1 (CRISPR-H1) or enzymatically dead (control; CRISPR-H1d) together with Lenti-sgRNA-E2 in the NAc. (**B**) Male and female mice with control-shRNA in the NAc showed an increased CPP score after cocaine conditioning, whereas mice with *Npas4*^{eRNA} shRNA had a similar CPP score before and after conditioning (n = 10 to 12). (**C**) Male and female mice with control lentivirus in the NAc showed an increased CPP score after cocaine conditioning, but those with CRISPR-H1 + sgRNA-E2 failed to develop cocaine CPP (n = 10 to 12). (**D**) Diagram showing the experimental timeline of CSDS followed by behavioral test battery including sucrose preference assay with adult male mice that received AAV2-*Npas4*^{eRNA} shRNA or control shRNA in the mPFC. (**E**) Control mice show decreased sucrose preference after CSDS, whereas mice with *Npas4*^{eRNA} shRNA in the mPFC fail to reduce their sucrose preference after CSDS (n = 12 to 27). (**F**) Working model illustrating how the *Npas4*^{eRNA} forms R-loops that prime for emotional experience–induced enhancer-promoter chromatin looping, rapid *Npas4* mRNA expression, and behavioral plasticity. Statistical analyses were performed using two-way ANOVA followed by Sidak or Tukey's post hoc multiple comparison test; *P < 0.05, **P < 0.01, ***P < 0.001, nonsignificant P > 0.05. Detailed statistical analyses are provided in table S1.

place preference (CPP) to measure the formation of cocaine-context associations (Fig. 5A) (*31*), and chronic social defeat stress (CSDS) followed by sucrose preference testing to measure chronic stress–induced anhedonia-like behavior (Fig. 5D) (*32*, *33*). Unlike controls, which successfully develop cocaine CPP, male and female mice expressing AAV2-*Npas4*^{eRNA}-shRNA in NAc failed to develop CPP (Fig. 5B and figs. S8D and S9A), which is similar to the effects of *Npas4*^{mRNA} reduction in the NAc (*6*). The *Npas4*^{eRNA}-shRNA in the NAc did not affect the expression of the low-abundance *Npas4*$^{5'UTR}$ mRNA isoform (fig. S9, B and C). However, to rule out the possibility that the *Npas4*^{eRNA}-shRNA blocks cocaine CPP through regulation of the *Npas4*$^{5'UTR}$, we designed and validated an AAV2-*Npas4*$^{5'UTR}$-shRNA specifically degrading its corresponding RNA without affecting *Npas4*^{eRNA} expression (fig. S9, C to E). Unlike the reduction of *Npas4*^{eRNA} (Fig. 5B) or *Npas4*^{mRNA} (*6*), *Npas4*$^{5'UTR}$-shRNA had no effect on cocaine CPP (fig. S9D). To test the *Npas4* enhancer R-loops, we infused Lenti-CRISPR-H1 combined with sgRNA-E1 in the NAc, and, similar to *Npas4*^{eRNA}-shRNA, we observed that targeted degradation of the *Npas4* enhancer R-loops blocked cocaine CPP (Fig. 5C). Finally, we tested the role of *Npas4*^{eRNA} in the CSDS-induced behavioral adaptations in mice with *Npas4*^{eRNA}-shRNA in the mPFC. After 10 days of CSDS followed by a battery of behavioral tests (Fig. 5D and fig. S10), we found that AAV2-*Npas4*^{eRNA}-shRNA blocked CSDS-induced reduction of sucrose preference (Fig. 5E). Together, these data confirm that *Npas4*^{eRNA} and associated R-loops are required for behavioral adaptations after both positive and negative emotional experiences (Fig. 5F).

Discussion

How emotional experiences trigger the rapid induction of IEGs, such as *Npas4*, to support or oppose neural circuit adaptations remains an important topic in neuroscience. We show here that a highly conserved *Npas4* enhancer element produces a nonannotated lnc-eRNA necessary for the rapid induction of *Npas4*^{mRNA} transcription in NAc and mPFC after an emotional experience. Moreover, the *Npas4*^{eRNA} appears to regulate *Npas4*^{mRNA} expression and behavioral adaptations through the formation of R-loops at the *Npas4* enhancer and the facilitation of experience-dependent promoter-enhancer 3D looping leading to rapid *Npas4*^{mRNA} expression.

Despite their discovery decades ago (*34*, *35*), the functions and mechanisms of action of lnc-eRNAs or R-loops in the context of behavioral adaptations remain unclear. Our findings show that an lnc-eRNA forms R-loops in the adult mouse brain, which are necessary for full experience-dependent induction of the IEG *Npas4*. Genome-wide analyses in non-neuronal cells revealed ~15,000 R-loop loci in the genome, mostly at regulatory domains (*27*). R-loop formation is highly correlated with transcriptional activity (*16*, *27*), suggesting a role in promoting transcription, analogous to our findings with *Npas4*. Paradoxically, R-loops can also inhibit transcription by stalling RNAPII (*36*, *37*). R-loops can influence transcription by multiple means, including the recruitment of epigenetic modifiers (*18*, *38*), transcriptional activators (*16*, *18*), and RNAi machinery to regulatory sites (*18*, *39*). Although we cannot rule out the involvement of these processes, we found that the *Npas4* enhancer R-loops are required for experience-induced promoter-enhancer 3D chromatin looping. Published assay for transposase-accessible chromatin with sequencing (ATAC-seq) revealed open chromatin at the *Npas4* enhancer in both neurons and astrocytes, but the *Npas4* promoter was closed in astrocytes (*40*). Our single-nucleus RNA-seq of mPFC tissues (*5*) also shows that *Npas4*^{mRNA} is neuron-restricted, consistent with previous reports (*21*, *23*, *41*). R-loops could form in cis (like our *Npas4*^{eRNA} R-loop) or in trans (*18*, *27*, *42*), potentially explaining the sufficiency of our viral-mediated overexpression of the *Npas4*^{eRNA} in increasing endogenous *Npas4*^{mRNA} expression (Fig. 2D). However, as there is ongoing brain activity in home-caged animals, whether *Npas4*^{eRNA} overexpression increases *Npas4*^{mRNA} independent of cellular activity was not directly tested. Considering that *Npas4*^{mRNA} is highly responsive to neuronal activity, and *Npas4*^{eRNA} is not, it seems likely that neuronal activity is still needed for the *Npas4*^{eRNA} overexpression effects in vivo.

Recent studies reveal that synaptic activity can induce DNA double-strand breaks (DSBs) at gene regulatory regions of IEGs (*43*–*45*). R-loops have been linked to DSBs in non-neuronal cells (*46*), but our initial analysis at the *Npas4* promoter-enhancer region in NAc tissue after cocaine conditioning revealed no clear evidence of DSBs. However, we only assessed a single time point, and only a subpopulation of neurons induces *Npas4* mRNA and protein. Future studies could determine whether the *Npas4*^{eRNA} and enhancer R-loops are involved in regulating DSBs in activated NAc neurons.

Prior studies have identified activity-dependent induction of eRNAs at IEG enhancers of *Fos* and *Arc* (*9*, *13*, *14*) that recruit the transcriptional activator CBP or decoy the negative regulator elongation factor, NELF (*14*). In contrast, *Npas4*^{eRNA} is not up-regulated by neuronal activity, is expressed constitutively in low amounts, and appears to prime the *Npas4* enhancer for rapid promoter-enhancer 3D looping and *Npas4*^{mRNA} expression after emotional experiences by bypassing the need for up-regulating eRNAs first. However, we cannot rule out the possibility that *Npas4*^{eRNA} is rapidly and transiently generated.

Similar to what we observed for the *Npas4* enhancer, we also detected R-loops at *Fos* enhancers 2 and 5, where multiple transcriptional and epigenetic regulators, including CTCF, are enriched. Previous studies have demonstrated neuronal activity–induced expression of eRNAs and formation of 3D chromatin loops between the *Fos* promoter and these enhancers (*9*). Our DRIP analysis demonstrated that cocaine conditioning and social defeat stress bidirectionally and differentially regulate R-loops on enhancers 2 and 5, indicating enhancer, stimuli, and brain region–specific regulatory mechanisms of R-loop formation. Investigating the function of R-loops on other activity-regulated IEGs might reveal a conserved role for enhancer R-loops in promoting their rapid expression in neurons.

Our findings here manipulating *Npas4*^{eRNA} in these brain regions highlight the importance of *Npas4* in emotional experience–induced behavioral adaptations. Although we cannot rule out the possibility that *Npas4*^{eRNA} and associated R-loops might also regulate other genes, the parsimonious explanation is that *Npas4*^{eRNA} and R-loops mediate the CPP and CSDS behavioral adaptations through regulation of *Npas4*^{mRNA} expression. Behavioral adaptation can produce maladaptive changes in brain function that support neuropsychiatric conditions (*1*, *4*). Understanding the critical molecular players that mediate maladaptive plasticity in the brain through in vivo CRISPR-based epigenetic modifiers (*47*–*49*) could reveal new strategies for therapeutic development. Our findings of *Npas4* lnc-eRNA and its R-loops reveal valuable insights into the complex mechanism of IEG induction and emphasize the critical role of *Npas4* in regulating behavioral adaptions associated with SUDs and chronic stress.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S10
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MDAR Reproducibility Checklist

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