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Distinct Roles of CREB Within the Ventral and Dorsal Hippocampus in Mediating Nicotine Withdrawal Phenotypes

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Addiction to nicotine and the inability to quit smoking are influenced by genetic factors, emphasizing the importance of understanding how genes and drugs of abuse mechanistically impact each other. One well-characterized protein responsible for regulating both response to drugs and gene expression is the transcription factor CREB (cAMP-responsive element binding protein). Previous work indicates that hippocampal-specific alterations in CREB signaling and synaptic plasticity may underlie certain nicotine withdrawal phenotypes. However, the structure of the hippocampus possesses dorsal and ventral subregions, each differing in behavioral, anatomic and gene expression characteristics. This study examines the effects of CREB deletion specifically in the ventral or dorsal hippocampus of animals chronically treated with saline, nicotine, or undergoing 24 h withdrawal. After region-specific viral injections of AAV-GFP or AAV-CRE in $CREB^{loxP/loxP}$ animals, behavioral testing measured anxiety levels, using the Novelty-Induced Hypophagia test, and cognition, using a contextual fear conditioning paradigm. Deletion of CREB in the ventral, but not dorsal, hippocampus resulted in amelioration of nicotine withdrawal-induced anxiety-like behavior in the Novelty-Induced Hypophagia test. In contrast, CREB deletion in the dorsal hippocampus resulted in learning and memory deficits in fear conditioning, whereas CREB deletion in the ventral hippocampus showed an enhancement in learning. Gene expression analysis showed differential treatment- and region-dependent alterations of several CREB target genes that are well-known markers of neuroplasticity within the hippocampus. Collectively, these data provide persuasive evidence towards the distinct roles of CREB within the dorsal and ventral hippocampus separately in mediating select nicotine withdrawal phenotypes.
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INTRODUCTION

Last year marked the 50th Anniversary of the Surgeon General Report on Smoking and Health, yet nearly 20% of Americans continue to smoke (CDC, 1988). Nicotine, one of the main addictive psychopharmacological ingredients found in tobacco, is believed to mediate dependency on cigarettes. While acute nicotine produces modest reinforcing effects (Mansvelder *et al*, 2002), chronic nicotine use results in neuroadaptive changes (Tiffany *et al*, 2004). During abstinence from chronic nicotine use, cognitive and affective withdrawal (WD) symptoms emerge and these symptoms are likely due to chronic nicotine's neuroadaptive effects (De Biasi and Salas, 2008). WD symptoms are the predominant driving factors to relapse to smoking, accounting for why 80% of smokers attempting to quit, fail (Nishino *et al*, 2014). Therefore, more mechanistic understanding of the neural correlates underpinning these symptoms may lead to better treatment options for nicotine dependence.

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Previous studies suggest the involvement of CREB (cAMP-responsive element binding protein)-dependent transcription in the molecular mechanism of dependence on multiple drugs of abuse, including nicotine (Nestler, 2005). In human studies, there is an observed correlation between the number of cigarettes smoked per day and CREB expression (Lenz *et al*, 2010). In adult mice, CREB activation is necessary for nicotine reward (Walters *et al*, 2005). These findings suggest a possible role for CREB in mediating the neuroplasticity changes that characterize nicotine dependence (Kutlu and Gould, 2016). Furthermore, CREB may be required for behaviors that manifest during abstinence as well, as altered CREB phosphorylation (pCREB) (Pandey *et al*, 2001) as well as changes in CREB-DNA binding (Turner *et al*, 2014) have been observed during nicotine WD. Previous work from our lab has shown that these effects are region-specific; in the hippocampus, both pCREB and CREB binding to target genes can be correlated with nicotine WD phenotypes (Turner *et al*, 2014). However, whether CREB activity in the hippocampus is necessary for nicotine WD -induced behaviors is unknown.

Supporting data in human (Picciotto *et al*, 2002; Pomerleau *et al*, 2005) and animal models (Costall *et al*, 1989; Jackson *et al*, 2008) link hippocampal function with cognitive and affective nicotine WD impairments, both reliable determinants for nicotine WD. Functional imaging

studies in smokers show that activation of this brain region can be correlated with both cognitive and affective WD symptoms (Froeliger *et al*, 2010; McClernon and Gilbert, 2004). Additionally, these studies report a correlation between hippocampal volume and successful quit attempts (Froeliger *et al*, 2010). However, the hippocampus is not a homogenous structure, but instead can be divided into dorsal and ventral regions, each mediating different behaviors (Fanselow and Dong, 2010). The dorsal hippocampus mediates spatial navigation as well as learning and memory formation (Fanselow and Dong, 2010). The ventral hippocampus contributes to anxiety and affective responses and is known to have bidirectional connectivity with the amygdala (Fanselow and Dong, 2010). This dissociation is also important in nicotine-associated phenotypes. For example, Wilkinson *et al* (2013) demonstrated that following chronic nicotine administration, nicotinic acetylcholine receptors in the dorsal, but not ventral, hippocampus, mediate nicotine WD deficits observed in contextual fear conditioning (Nishino *et al*, 2014). Reciprocal studies from Turner *et al* (2013) showed that microinjecting nicotinic compounds specifically into the ventral hippocampus could ameliorate anxiety-like nicotine WD symptoms in mice (Turner *et al*, 2013). Previous studies from our lab show that hippocampal CREB signaling and the concurrent synaptic plasticity changes may underlie nicotine WD phenotypes in mice (Turner *et al*, 2014) (Turner *et al*, 2013). However, the hippocampal specificity of these effects is unknown. Therefore, this study examines how region-specific CREB deletion in either the dorsal or ventral hippocampus impacts 24 h WD behavioral phenotypes and what possible CREB targets may be responsible.

MATERIALS AND METHODS

Animals

Male and female $CREB^{loxP/loxP}$ mice bred in-house were 8–10 weeks of age at the beginning of microinjection surgeries. These mice were originally generated as described in Gundersen *et al* (2013). Mice were maintained on a 12 h light–dark cycle (lights on at 07 : 00 hours), with *ad libitum* food and water. All behavioral procedures were conducted during the hours of 09 : 00–17 : 00 hours.

Drugs and Administration

(−)-Nicotine tartrate (MP Biomedicals, Solon, OH) was dissolved in 0.9% saline. Nicotine was administered subcutaneously via osmotic minipumps (Alzet model 2002; DURECT Corporation, Cupertino, CA) at a dose of 18 mg/kg per day for 12 days. This dose, reported as freebase weight and based on previous work (Turner *et al*, 2010, 2011, 2013, 2014), corresponds to plasma levels of ~0.3 μM following chronic treatment for 12 days (Matta *et al*, 2007), a concentration similar to that observed in human smokers consuming an average of 17 cigarettes a day (plasma levels between 0.06 and 0.31 μM) (Matta *et al*, 2007).

Osmotic Minipump Surgeries

Pump implantation was performed as described previously (Davis *et al*, 2005). Briefly, mice were anesthetized with 5% isofluorane and pumps were implanted subcutaneously. Twelve days after pump implantation, a second, similar surgery was performed to remove pumps and induce spontaneous WD.

Adenoassociated Virus Production

The University of Pennsylvania Vector Core generated, purified, and quantified neuron-selective adenoassociated virus (AAV) constructs expressing CRE recombinase (AAV-CRE; AAV2/9.CMV.PI.CRE, titer 2.84×10^{13} genome copies (gc)/ml) and enhanced green fluorescent protein (AAV-GFP; AAV2/9.CMV.eGFP, titer 3.74×10^{13} gc/ml). Each expression cassette contained AAV2 terminal repeats flanking the cytomegalovirus (CMV) promoter-PI-CRE recombinase and CMV promoter-enhanced GFP (eGFP) packaged into AAV9.

Stereotaxic Surgery

Surgery was performed on adult mice 8–10 weeks old as described previously (Turner *et al*, 2013). After anesthesia with isofluorane, mice were secured in a stereotaxic frame (Stoelting, Wood Dale, IL). Holes were drilled bilaterally into the skull at the injection sites. Stereotaxic coordinates were measured from the skull surface as follows: ventral intrahippocampal injections were AP −2.9, ML ± 3.0, and DV −3.8; dorsal intrahippocampal injections were AP −2.1, ML ± 1.4, and DV −2.0. After surgeries, mice remained in their home cage for an additional 4 weeks until the beginning of Novelty-Induced Hypophagia (NIH) training.

NIH Test

The NIH test was performed as described previously (Turner *et al*, 2010). Briefly, during training mice were exposed daily to a highly palatable food (Reese's peanut butter chips; Nestle, CA) and latency to consume was measured. NIH testing occurred on the last 3 days of treatment, consisting of presentation of food in the home environment (Home Day 1, 2) or in the novel environment (Novel Day). On Novel Test Day, mice were removed from the home cage and placed in an empty standard cage with no bedding that had been wiped with Pine Sol (1 : 10) to emit a novel odor and placed in a white box with bright illumination (2150 lux). Latency to consume was recorded on all days via a blinded observer.

Fear Conditioning

Fear conditioning occurred in Plexiglas chambers ($26.5 \times 20.4 \times 20.8\ cm^3$) housed in sound-attenuating boxes (Med Associates, VT). The floor of each chamber consisted of metal bars connected to a shock generator and scrambler (Med Associates; Model ENV-414). Shock administration was controlled using the LabView software. All chambers were cleaned with 70% ethanol before and after behavioral procedures. A modified delay fear conditioning training procedure that used a one 15 s CS (context)–US (foot shock) pairing was performed similar to previously described

methods (Gould *et al*, 2004). Freezing was sampled for 1 s every 10 s (Gould and Wehner, 1999). On training day, mice were placed into chambers and baseline freezing was scored for 120 s. Then, three 0.57 mA foot shocks were delivered with an intershock interval of 45 s. Mice remained in the chambers for an additional 30 s before returning to their home cages. The next day, mice were returned to the chambers and contextual freezing was scored for 5 min.

Experimental Design

Previous studies demonstrate that an $>80\%$ neuronal knockdown of CREB immunoreactivity was accomplished 8 weeks postinjection using these procedures (Gundersen *et al*, 2013). Therefore, all behavioral testing occurs $\geqslant 8$ weeks postviral injections (Figure 1a).

Animals from each group ($N=8$–10) were stereotactically injected with either AAV-GFP or AAV-CRE virus into the ventral or dorsal hippocampus, followed by a 4-week recovery period in their home cage. NIH training occurred during weeks 5–6. After NIH training, osmotic minipumps were implanted for a 2-week administration period. On week 9, NIH testing began. Following Home Day 1 testing, half of the animals had their minipumps removed to initiate WD. 24 h later, animals were placed in the novel environment and tested. That afternoon, the same cohort of animals also underwent fear-conditioning training. The third and final day of testing consisted of animals undergoing NIH Home Day 2 testing in the morning and then fear conditioning testing that afternoon. Following behavioral testing, animals were immediately killed and the dorsal and ventral hippocampal tissues were microdissected and used for quantitative PCR (qPCR) analysis.

qPCR

qPCR was performed as described previously (Cleck *et al*, 2008) on ventral or dorsal hippocampal samples across all treatment groups. Briefly, RNA was isolated using the RNeasy Mini Kit (Qiagen) and qPCR reactions were assembled using Thermo Scientific Maxima SYBR Green master mix along with 100 nM primers (Eurofins). The mRNA levels were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and target genes were normalized to the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or hypoxanthine phosphoribosyltransferase (*HPRT*). All gene expression values were normalized to their respective AAV-GFP saline-treated controls. Additionally, CRE recombinase activity is shown as both normalized expression (Figures 3ai and ci) and average raw cycle threshold (CT) values (Figures 3aii and cii). Primer sequences are shown in Table 1.

Nicotinic Acetylcholine Receptor Binding

[^{3}H]Epibatidine binding was performed as described previously (Turner *et al*, 2011). Briefly, cortical homogenates were incubated with 2 nM [^{3}H]epibatidine (Perkin-Elmer, USA) for 2 h at RT. Bound receptors were separated from free ligand by vacuum filtration over GF/C glass-fiber filters (Brandel, MD) and the filters were then counted in a liquid scintillation counter. Nonspecific binding was determined in the presence of 300 μM nicotine, and specific binding was defined as the difference between total binding and nonspecific binding.

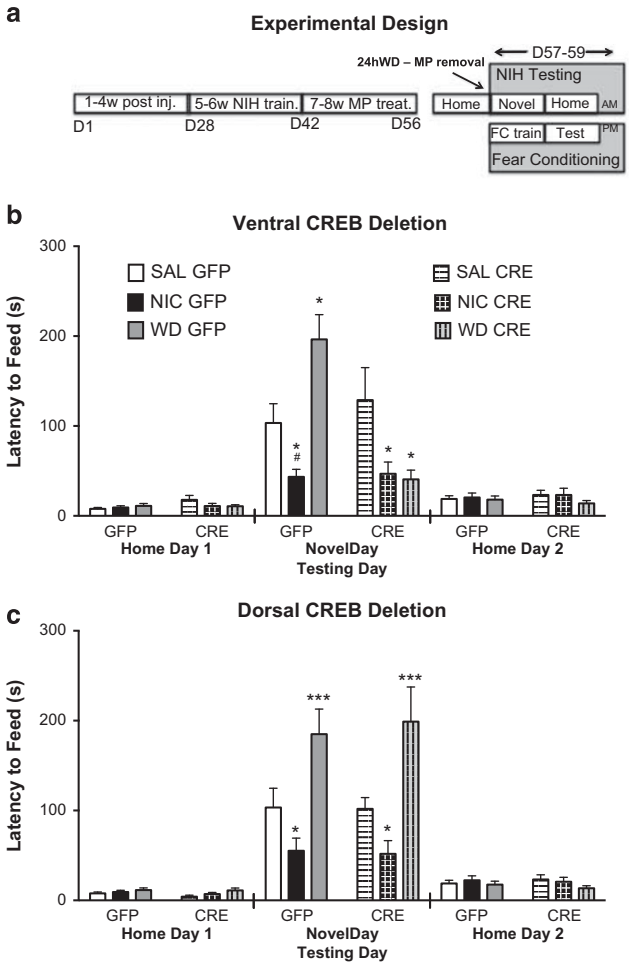

Figure 1 Experimental design setup and Novelty-Induced Hypophagia (NIH) behavior of animals with CREB (cAMP-responsive element binding protein) deleted in either the ventral or dorsal hippocampus. (a) Day 1 AAV-GFP or AAV-CRE injections into the ventral or dorsal hippocampus. Day 28 beginning of NIH training. Day 42 implantation of osmotic minipumps (saline (SAL) or nicotine (NIC)). Day 56 removal of osmotic minipumps in withdrawal (WD) animals only. Day 57–59 NIH testing (AM). Day 58 fear conditioning training. Day 59 fear conditioning testing (PM). (b) Nicotine treatment attenuates latency to feed in both the AAV-GFP and AAV-CRE groups within the ventral hippocampus. AAV-GFP animals undergoing 24 h withdrawal display increased latency to feed compared with both saline and nicotine counterparts, whereas AAV-CRE animals undergoing 24 h withdrawal display a reduction in latency to feed. (c) Nicotine treatment attenuates latency to feed in both the AAV-GFP and AAV-CRE groups within the dorsal hippocampus. Both AAV-GFP and AAV-CRE groups undergoing 24 h withdrawal result in an increase in latency to feed compared with both saline and nicotine treatment groups. $n=6$–8 per group ($^*p<0.05$, $^{***}p<0.0005$ viral effect; $^{\#}p<0.05$ treatment effect). AAV, adenoassociated virus; GFP, green fluorescent protein.

Data Analysis

Statistical analyses were performed with the GraphPad Prism 6.0 software package (GraphPad Software, CA). Except

Table 1 qPCR Primers

Gene name (CT)	Forward strand (5′3′)	Reverse strand (5′3′)
GAPDH (29)	AACTTTGGCATTGTGGAAGG	GGATGCAGGGATGATGTTCT
HPRT (21)	CAAAGCCTAAGATGAGCGCAAG	TTACTAGGCAGATGGCCACAGG
CRE recombinase (27)	GAACGAAAACGCTGGTTAGC	CCCGGCAAAACAGGTAGTTA
CREB (26)	CATTGCCCCTGGAGTTGTTATG	TTCTCTTGCTGCCTCCCTGTT
ARC (23)	GAACATCAAGAAGCCCCTTTC	TTGCCTTTCAGACACTTGGTT
GRIN1 (23)	CCACCCTCACTTTTGAGAACA	ATCAGTGGGATGGTACTGCTG
JNK1 (24)	TCTTGGGTGTTTCCTGTGAAC	AACCAGAAGTGCCCAGAAAGT
$BDNF_{total}$ *(25)*	AGGCAAACAATCGCTTCATCT	CAGGCTAACTCGAAAGGAACG
$BDNF_{exon\ 4}$ *(25)*	CTCCGCCATGCAATTTCCAC	GCCTTCATGCAACCGAAGTA

Abbreviations: ARC, activity-related cytoskeleton; BDNF, brain-derived neurotropic factor; CT, cycle threshold; CREB, cAMP-responsive element binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRIN1, glutamate ionotrophic NMDA type subunit 1; HPRT, hypoxanthine phosphoribosyltransferase; JNK1, Jun-N terminal kinase 1; qPCR, quantitative PCR.

where noted, results were analyzed using two-way repeated-measures ANOVA followed by Sidak's multiple comparison tests. Because group data were collapsed for the CREB and CRE qPCR data and the fear conditioning test day data, results were instead analyzed with a Student's *t*-test. All data are expressed as mean ± SEM.

RESULTS

CREB Deletion in the Ventral, but not Dorsal, Hippocampus Ablates Nicotine WD-Induced Anxiety-Like Phenotype in the NIH Test

Ventral hippocampal CREB deletion. In humans, nicotine WD is often characterized by an increase in anxiety. To model this preclinically, we used a well-validated model of anxiety-like behavior in rodents, the NIH test (Merali *et al*, 2003). Presentation of food in the home environment 24 h before or 24 h following testing in the novel environment showed no differences between the experimental groups (Figure 1b, Home Day 1 or 2). In contrast, the latency to feed in the novel environment (Novel Day) was significantly elevated in all animals compared with the home day environment, as well as a significant effect of treatment and interaction (main effect of day, $F(2,106) = 44.38$, $p < 0.0001$; main effect of treatment $F(6,53) = 6.973$, $p < 0.0001$; interaction $F(12,106) = 7.486$, $p < 0.0001$) (Figure 1b). In GFP-injected animals (AAV-GFP), chronic treatment with nicotine significantly attenuated the latency to feed in the novel environment compared with saline-treated controls ($p < 0.01$). AAV-GFP-injected animals undergoing 24 h WD displayed increased latency to feed compared with both their saline ($p < 0.01$) and nicotine-treated ($p < 0.01$) counterparts. In animals with ventral hippocampal CREB deletion (AAV-CRE), nicotine treatment also resulted in an anxiolytic response compared with saline controls ($p < 0.01$). However, unlike the GFP-injected animals, the 24 hWD AAV-CRE group did not result in an anxiogenic response on Novel Test Day and maintained a significant anxiolytic response compared with saline ($p < 0.01$), suggesting that nicotine WD-related anxiety involves a ventral hippocampal CREB-mediated mechanism.

Dorsal hippocampal CREB deletion. Data collected from animals with dorsal hippocampal injections with either the AAV-GFP or AAV-CRE across all treatments again showed no differences in behavior during Home Day testing between the experimental groups (Figure 1c, Home Day 1 or 2). Latency to feed in the novel environment (Novel Day) was significantly higher in all animals compared with the home day environment, accompanied by a significant effect of treatment and interaction between groups (main effect of day, $F(2,120) = 72.99$, $p < 0.0001$; main effect of treatment, $F(6,60) = 6.257$; $p < 0.0001$; interaction, $F(12,120) = 7.217$, $p < 0.0001$) (Figure 1c). On Novel Day, chronic nicotine treatment in the AAV-GFP-injected control animals again showed a reduced latency to feed compared with the saline-treated AAV-GFP controls ($p < 0.05$), whereas the 24 hWD animals displayed an increased latency to feed compared with both the saline ($p < 0.01$) and nicotine-treated ($p < 0.01$) animals within the GFP group. In AAV-CRE-injected animals, nicotine treatment resulted in an anxiolytic response compared with saline controls ($p < 0.05$), similar to ventral AAV-CRE mice. However, in contrast to those animals, mice undergoing 24 hWD with dorsal CREB deletion displayed a significant increase in latency to feed compared with their saline ($p < 0.01$) and nicotine ($p < 0.01$) AAV-CRE counterparts, demonstrating an anxiogenic-like response typical of what is observed during nicotine WD in control animals.

These effects were not attributable to alterations in appetitive behavior as there are no differences in the latency to feed in the home environment on Home Day 1 or 2 ($p > 0.05$) (Figures 1b and c). No sex effects were observed between viral or treatment groups in the NIH test.

CREB Deletion in the Dorsal Hippocampus *Impairs* Fear Conditioning, while Ventral Hippocampal CREB Deletion *Enhances* Fear Conditioning

Fear conditioning is a well-established cognitive behavioral paradigm that is a validated test of learning and memory in rodents (Kim and Jung, 2006). Animals learn to associate the attributes of the context with an aversive foot shock, leading to the formation of a specific memory and resulting in a freezing response. Figure 2 shows the effects of ventral or

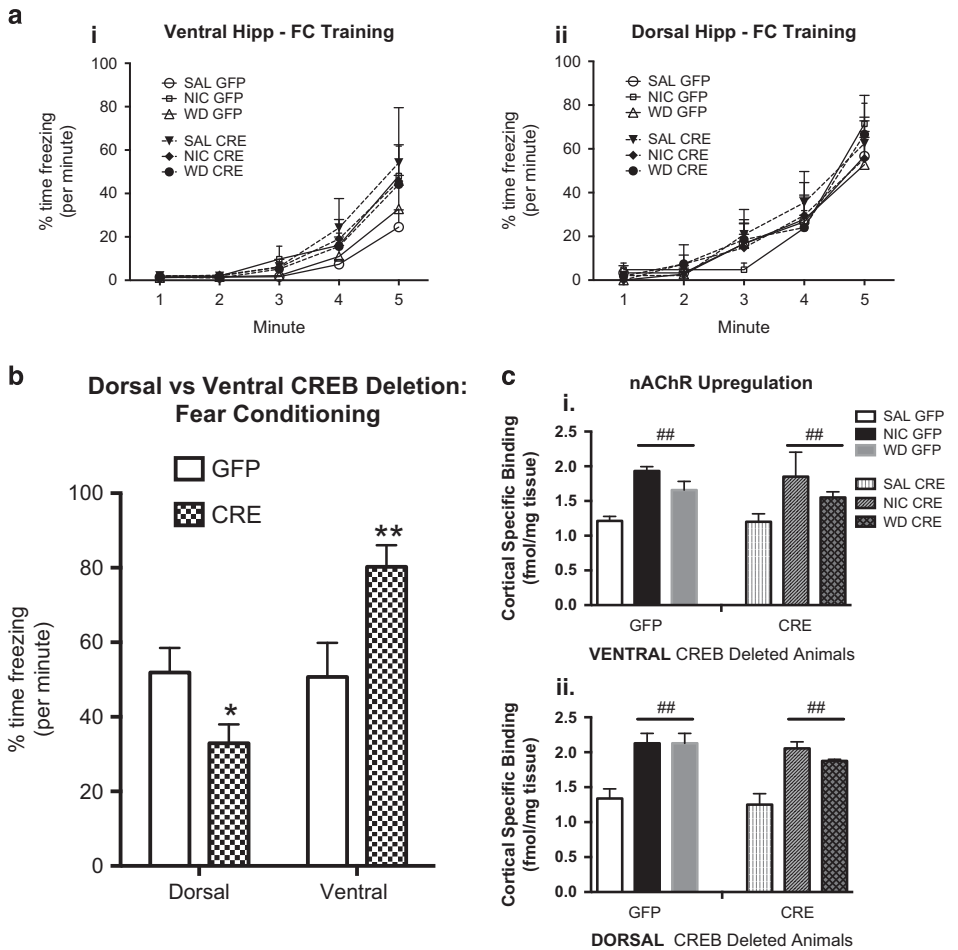

Figure 2 The effects of CREB (cAMP-responsive element binding protein) deletion in either the ventral or dorsal hippocampus in animals subjected to fear conditioning (FC). (a, i and ii)Training of animals during saline (SAL), nicotine (NIC), or withdrawal (WD) treatment that were injected with AAV-GFP or AAV-CRE into the ventral or dorsal hippocampus had no deficits during training of fear conditioning ($n = 8$–10) treatment group. (b) Animals injected with CRE in the dorsal hippocampus show an impairment in fear conditioning with a reduced percent time freezing compared to GFP control animals, whereas animals injected with CRE in the ventral hippocampus show an enhancement in fear conditioning with an increased percent time freezing compared with GFP control animals ($n = 16$–20 per group). (c, i and ii) [^{3}H]Epibatidine radioligand binding using cortical tissue from ventral or dorsal hippocampal CREB treatment groups show nicotinic acetylcholine receptor (nAChR) upregulation during chronic nicotine and withdrawal compared with saline controls ($n = 6$–8 per treatment group) (*$p < 0.05$ viral effect, **$p < 0.01$ viral effect, $^{\#\#}p < 0.01$ treatment effect). AAV, adenoassociated virus; GFP, green fluorescent protein.

dorsal CREB deletion on animals treated with saline, nicotine, or 24 hWD on fear conditioning. There were no differences between any of the groups during training (Figure 2a). On testing day, our data showed no drug treatment effects (saline/nicotine/24 hWD) within any of the groups; therefore, all drug treatment groups were combined for analysis and a *t*-test was performed comparing the effects of virus in each subregion. Dorsal hippocampal CREB deletion significantly reduces percent time freezing ($p < 0.05$), suggesting impairment in cognition (Figure 2b). In contrast, ventral hippocampal CREB deletion significantly increases percent time freezing ($p > 0.01$), suggesting an enhancement of fear conditioning (Figure 2b). No sex effects were observed between viral or treatment groups in fear conditioning. To confirm treatment efficacy, we performed cortical [^{3}H]epibatidine radioligand binding to evaluate nicotinic acetylcholine receptor (nAChR) upregulation, a canonical response to chronic nicotine treatment (Turner *et al*, 2011). These data show that there was a main effect of nicotine treatment in both the ventral (Figure 2ci, $F(2,38) = 7.092$, $p = 0.0024$) and dorsal experimental animals (Figure 2cii, $F(2,31) = 21.71$, $p < 0.0001$), confirming nicotine delivery in these animals.

Increased CRE Recombinase Expression Decreases CREB Levels within the Ventral and Dorsal Hippocampus

Administration of AAV-CRE disrupts *Creb1* expression by excising exon 10/11 via flanking loxP sites (Gundersen *et al*, 2013). This excision occurs predominantly in neurons because of the selective infection of these cells by the AAV2/9 serotype (Cearley and Wolfe, 2006). qPCR data indicate a significant increase in mRNA expression levels of CRE recombinase in ventrally (Figure 3ai, $p < 0.01$) and dorsally injected animals (Figure 3ci, $p < 0.01$). This was echoed by a decreased in mean CT value (Figure 3aii, $p < 0.01$ and Figure 3cii, $p < 0.01$) and corresponded with significant reduction in CREB expression (Figure 3bi,

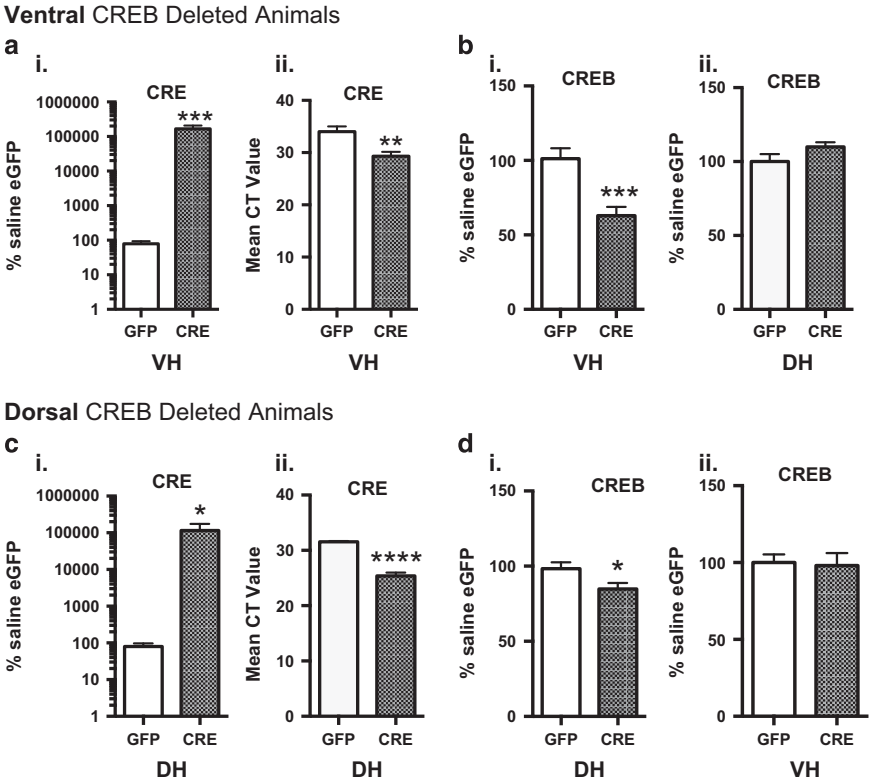

Figure 3 CRE recombinase and CREB (cAMP-responsive element binding protein) mRNA expression levels in the ventral (VH) and dorsal hippocampus (DH) via quantitative PCR (qPCR) analysis. (a, i) CRE expression is significantly increased in animals following AAV-CRE injections into the ventral hippocampus compared with GFP control animals. (ii) Mean CT values for CRE expression are significantly lower in CRE-injected animals compared with that of GFP controls. (b, i) Animals receiving CRE injections into the ventral hippocampus have significantly decreased CREB expression in the ventral hippocampus. (ii) There is no significant change in CREB expression in the dorsal hippocampus of ventral CREB-deleted animals compared with GFP controls. (c, i) CRE expression is significantly increased in animals following AAV-CRE injections into the dorsal hippocampus compared with GFP control animals. (ii) Mean CT values for CRE expression are significantly lower in CRE-injected animals compared with that of GFP controls. (d, i) Animals receiving CRE injections into the dorsal hippocampus have significantly decreased CREB expression in the dorsal hippocampus. (ii) There is no significant change in CREB expression in the ventral hippocampus of dorsal CREB-deleted animals compared with GFP control animals. $n = 16$–20 per group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0005$, ****$p < 0.0001$). AAV, adenoassociated virus; eGFP, enhanced green fluorescent protein.

$p < 0.01$ and Figure 3di, $p < 0.05$). Additionally, this reduction was restricted to the injection site, as CREB levels were not significantly different from GFP controls in the uninjected portion of the hippocampus (Figure 3bii, $p = 0.8530$ and Figure 3dii, $p = 0.17$).

Decreased CREB Levels Modulate the Expression of CREB Target Genes during Saline, Nicotine, and 24hWD

Ventral CREB deletion. To evaluate how CREB signaling may result in these behavioral effects, we examined mRNA expression of five well-documented CREB target genes in the ventral and dorsal hippocampus using qPCR. Figure 5 shows alterations in mRNA expression levels of CREB target genes specifically in the *ventral* hippocampus of animals that received viral injections of either AAV-GFP or AAV-CRE within that structure. No significant changes were observed from CREB deletion within the ventral hippocampus in activity-related cytoskeleton (ARC) protein (Figures 4a, $p > 0.05$) or glutamate ionotropic NMDA type subunit 1 (GRIN1) receptor (Figures 4b, $p > 0.05$) mRNA expression levels. However, Jun-N terminal kinase 1 (JNK1) expression within the ventral hippocampus displayed a significant interaction and main effect of treatment (main effect of treatment $F(2,23) = 3.743$, $p = 0.0391$; interaction $F(2,23) = 5.077$, $p = 0.0149$) (Figure 4c). Saline-treated AAV-CRE animals had a significant increase in expression compared with the saline ($p < 0.05$) and nicotine ($p < 0.01$) AAV-GFP animals, as well as compared with nicotine- ($p < 0.05$) and 24 hWD- ($p < 0.05$) treated AAV-CRE groups, suggesting that at baseline CREB occupancy at this promoter site may impede activation of the gene by other transcription factors. A similar trend was observed in mRNA levels of total brain-derived neurotropic factor ($BDNF_{total}$), which displayed a significant interaction effect ($F(2,24) = 6.161$, $p = 0.0069$) (Figure 4d). The AAV-CRE saline-treated group had a significant increase in expression compared with GFP saline control ($p < 0.01$) and the AAV-CRE nicotine ($p < 0.05$) and 24 hWD ($p < 0.05$) treatment groups. While these effects may be due to inhibitory CREB occupancy, these effects could also be due to other BDNF variants being expressed. For example, $BDNF_{exon4}$ contains a well-described CRE site (Tao *et al*, 1998). Expression of the $BDNF_{exon4}$ shows a significant interaction ($F(2,22) = 7.271$, $p = 0.0038$) (Figure 4e) between viral and treatment groups. Viral knockdown of CREB in saline animals results in a reduction

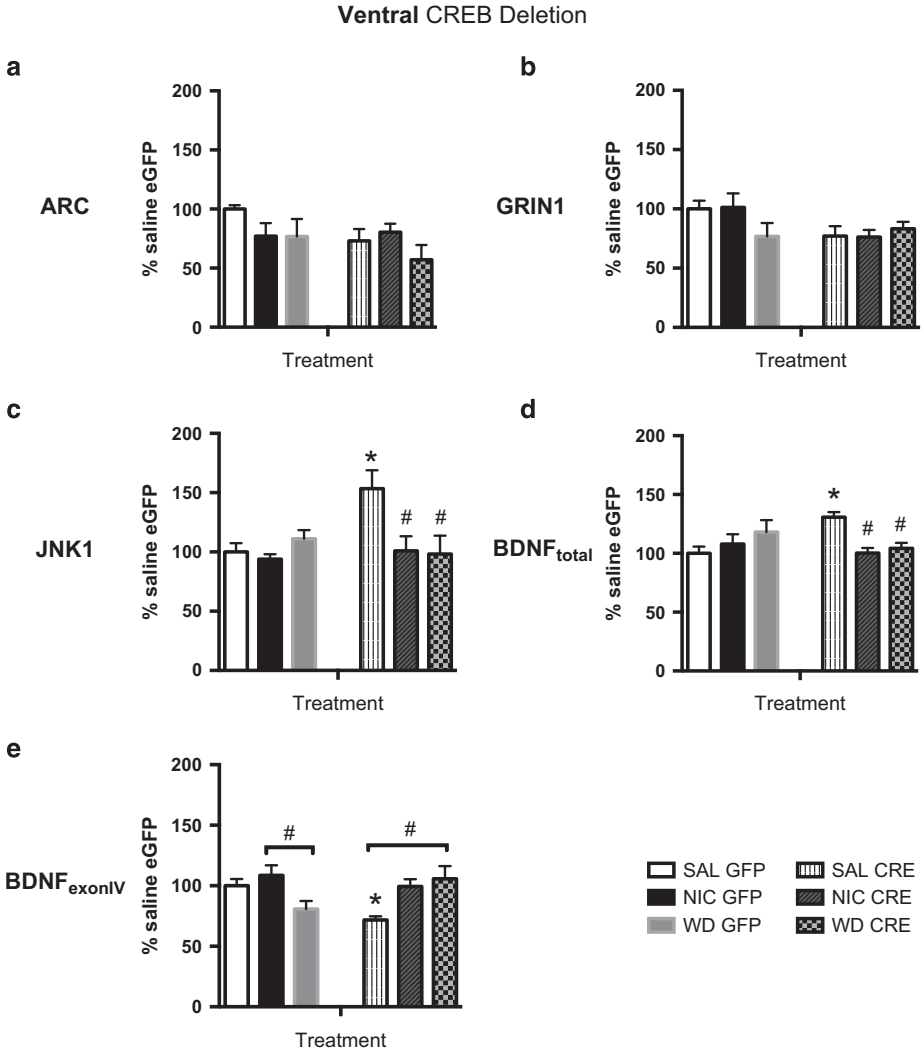

Figure 4 Quantitative PCR (qPCR) analysis of alterations in mRNA expression of CREB (cAMP-responsive element binding protein) target genes after injection of AAV-GFP or AAV-CRE into the ventral hippocampus. (a) Activity-related cytoskeleton (ARC) mRNA expression shows no significant changes across treatment or virus conditions within the ventral hippocampus. (b) Glutamate ionotropic NMDA type subunit (GRIN1) mRNA expression shows no significant changes across treatment or virus within the ventral hippocampus. (c) Jun-N terminal kinase 1 (JNK1) expression within the AAV-CRE saline (SAL)-treated group shows a significant increase when compared with both saline- and nicotine (NIC)-treated AAV-GFP groups, as well as nicotine- and 24 h withdrawal (WD)-treated AAV-CRE groups. (d) Total brain-derived neurotropic factor ($BDNF_{total}$) expression levels within the AAV-CRE saline-treated animals are significantly increased compared with their GFP saline controls and AAV-CRE nicotine and 24 h withdrawal treatment counterparts. (e) $BDNF_{exon\ 4}$ mRNA expression within the AAV-GFP group is significantly reduced during 24 h withdrawal when compared with nicotine-treated animals. AAV-CRE saline animals have significantly reduced expression compared with saline and nicotine AAV-GFP controls, as well as to their nicotine and 24 h withdrawal AAV-CRE counterparts. $N = 8–10$ per group. ($^{*}p < 0.05$ viral effect; $^{\#}p < 0.05$ treatment effect.) AAV, adenoassociated virus; GFP, green fluorescent protein.

of $BDNF_{exon4}$ expression ($p < 0.05$). Furthermore, while 24 hWD resulted in a significant decrease in exon 4 expression in AAV-GFP mice ($p < 0.05$), chronic nicotine and 24 hWD increased $BDNF_{exon4}$ expression in AAV-CRE mice relative to their saline controls ($p < 0.05$, $p < 0.01$, respectively). Saline-treated animals within the AAV-CRE group had significantly reduced expression compared with both saline ($p < 0.05$) and nicotine ($p < 0.05$) GFP controls (Figure 4e).

Dorsal CREB deletion. Figure 5 shows mRNA expression of the same CREB target genes, but in animals that received *dorsal* viral injections. ARC expression showed a significant main effect of CREB deletion ($F(1,2) = 9.795$, $p = 0.0053$) (Figure 5a). Expression levels of GRIN1 displayed no significant effects of CREB deletion within the dorsal hippocampus (Figure 5b). JNK1 mRNA expression analysis showed a significant main effect of treatment ($F(2,20) = 5.625$, $p = 0.0115$) (Figure 5c). qPCR analysis further showed a main effect of virus when observing expression patterns of both $BDNF_{total}$ ($F(1,26) = 29.93$, $p < 0.0001$)] and $BDNF_{exon4}$ ($F(1,21) = 7.667$, $p = 0.0115$) (Figures 5d and e), with a significant increase in AAV-CRE animals compared with their GFP controls.

DISCUSSION

Despite over half a century of research investigating the basic general function of the hippocampus, there is still much

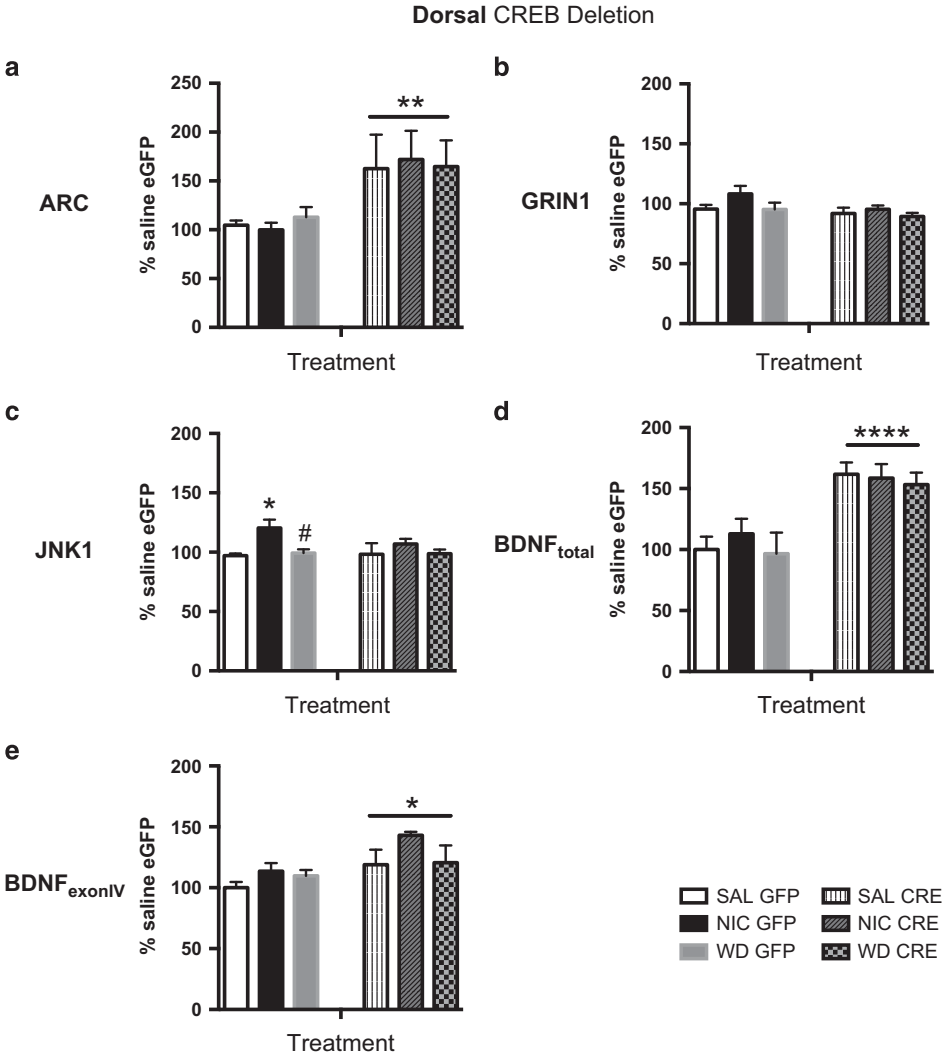

Figure 5 Real-time-quantitative PCR (RT-qPCR) analysis of alterations in mRNA expression of CREB (cAMP-responsive element binding protein) target genes after injection of AAV-GFP or AAV-CRE into the dorsal hippocampus. (a) Activity-related cytoskeleton (ARC) expression shows a significant main effect of viral CREB deletion across all treatments. (b) Glutamate ionotropic NMDA type subunit 1 (GRIN1) mRNA expression shows no significant differences in expression between AAV-GFP and AAV-CRE groups. (c) Jun-N terminal kinase 1 (JNK1) expression shows an increase in expression within the AAV-GFP group during nicotine treatment, compared with saline (SAL) and 24 h withdrawal (WD). (d) Total brain-derived neurotropic factor ($BDNF_{total}$) expression levels show a significant main effect of viral CREB deletion during all treatments. (e) $BDNF_{exon\ 4}$ expression shows a significant main effect of viral CREB deletion during all treatments. $n = 8$–10 per treatment group (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ viral effect). AAV, adenoassociated virus; GFP, green fluorescent protein.

debate over how this once thought to be unitary structure is now divided into separate subregions, each with differing molecular and functional domains. The present study builds upon this idea by identifying CREB's hippocampal-specific roles in mediating distinct nicotine WD-related behaviors. Our results demonstrate a double dissociation between viral and regional effects in mediating functional CREB-dependent behaviors during nicotine WD. In addition, gene expression analysis showed a differential regulation of CREB target genes between the viral and hippocampal region groups, suggesting differences in molecular organization and function underlying the observed phenotypes. Therefore, we show here the first evidence that CREB regulation of nicotine WD phenotypes is differentially modulated within the dorsal and ventral hippocampus separately through transcriptionally driven adaptations.

Early studies by Swanson and Cowan (1977) found the ventral and dorsal hippocampus to have distinct input and output connections (Swanson and Cowan, 1977). In the dorsal hippocampus, the CA1 region contains the greatest density of place cells, which code for spatial location by sending projections to the dorsal parts of the subiculum and other subcortical regions (Swanson and Cowan, 1977). Behavioral studies underscore this: spatial memory depends on the dorsal, not the ventral, hippocampus in a variety of spatial navigation tasks, including the Morris water maze and radial arm maze (Morris, 1981; Pothuizen *et al*, 2004). Our findings in contextual fear conditioning demonstrate that dorsal hippocampal CREB is essential for the encoding of long-term memory, which is often enhanced during nicotine treatment and impaired during WD (Gould and Wehner, 1999). The absence of nicotine treatment effects

during contextual fear conditioning in our study was not unexpected, as previous studies have shown that nicotine WD impairs hippocampal-dependent contextual learning in C57BL6 mice, but not in 129SvEv;C57Bl/6J F1 hybrid mice (Wilkinson *et al*, 2013), which are the parent strain of the $CREB^{loxP/loxP}$ mice used in this study. However, to confirm treatment efficacy, we also used [^{3}H]epibatidine to quantify cortical nAChR density in all treatment groups. Upregulation of nAChRs, a hallmark of nicotine treatment (Schwartz and Kellar, 1983), was observed in nicotine-treated animals. In contrast to the necessary role of dorsal CREB expression in fear conditioning, our findings also suggest that CREB expression in the ventral hippocampus can actively impede spatial memory encoding, as deletion of CREB selectively in the ventral hippocampus results in enhanced recall. Other studies have observed similar trends in the dissociation between these two regions using a water maze performance task (Richmond *et al*, 1999), as well as a conditioned place preference task (Ferbinteanu and McDonald, 2001), both demonstrating that lesions to the dorsal hippocampus results in an impairment of the task, while lesions to the ventral hippocampus enhance it.

In contrast to fear conditioning, CREB deletion in ventral, but not dorsal, hippocampus alters affective symptoms associated with nicotine WD. Lesions within the ventral hippocampus have been shown to impact specifically anxiety behaviors in conflict and hyponeophagia paradigms like the NIH test (Bannerman *et al*, 2002). Our data showing that disrupting CREB activity in the ventral hippocampus prevents expression of nicotine WD anxiety-like behavior in the NIH supports these previous findings, and also provides persuasive evidence that CREB activity in this region is integral to nicotine WD-induced anxiety. In contrast, mice with dorsal hippocampal CREB deletion displayed normal anxiogenic responses during WD, further demonstrating this dichotomy between dorsal and ventral hippocampal regions.

While CREB is generally regarded as a transcriptional activator, multiple studies have demonstrated that CREB can also act as a transcriptional repressor, due to competition for cofactors, such as CBP, or due to dimerization with other members of the ATF/CREB family (Vincent and Struhl, 1992; Walker *et al*, 1996). This perhaps explains the apparent induction of certain genes as a function of CREB deletion. CREB targets many genes within the brain, but five well-described CREB targets with known roles in neuroplasticity are ARC, GRIN1, JNK1, and BDNF and our analysis of these genes suggests that CREB may mediate nicotine WD-induced anxiety and cognitive impairments by altering their transcription. For example, changes in ARC and JNK1 are correlated with contextual fear conditioning and consolidation of memories (Huff *et al*, 2006) (Kenney *et al*, 2012) (Guzowski *et al*, 2000), and our own findings support the roles of these genes in memory formation (Figure 5a). Perhaps, the most persuasive evidence for CREB target genes mediating these responses relates to our findings with BDNF and its exon 4 variant, which possesses a well-documented CRE site that is highly responsive to neuronal activity (Tao *et al*, 1998). BDNF is the most common neurotrophin within the brain and is involved in activity-dependent synaptic plasticity (Huang and Reichardt, 2001). Studies with acute nicotine, which reduces BDNF expression (Nishino *et al*, 2014), have been shown to enhance fear conditioning (Nishino *et al*, 2014). Here we show the inverse, where an increase in $BDNF_{total}$ and $BDNF_{exon4}$ as a result of dorsal CREB knockdown corresponds with an impairment in fear conditioning. In addition to its role in cognition, impairments in BDNF signaling have also been associated with numerous neuropsychiatric disorders (Martinowich *et al*, 2007). Decreased $BDNF_{exon4}$ expression within the ventral hippocampus of 24 hWD control animals corresponds with increased anxiogenic effects of nicotine WD in the NIH task. Both this reduction in $BDNF_{exon\ 4}$ and the concordant anxiogenic behavior is absent in 24 hWD animals with ventral CREB deletion (Figure 5e), suggesting that BDNF mRNA levels may decrease during negative affective states. A similar observation has previously been found in human studies, where BDNF expression is lower in patients diagnosed with anxiety disorders compared with control patients with no diagnosis (Suliman *et al*, 2013). Therefore, CREB-mediated differential regulation of $BDNF_{exon\ 4}$ may be a mechanism for both the expression of anxiety-like behavior during nicotine WD and the consolidation of contextual memories.

WD phenotypes, such as impaired cognition and affect, directly impact relapse to smoking. While both of these WD phenotypes rely upon hippocampal function, our results demonstrate a dichotomy between nicotine's transcriptionally driven neuroplasticity effects in the ventral or dorsal hippocampus, which in turn differentially mediate the nicotine WD symptoms. This highlights how region-specific CREB-mediated plasticity can impact discrete nicotine WD behavioral responses. This has major implications in understanding the basic mechanisms whereby gene expression governs distinct behavioral domains, depending upon the specific region in which it occurs. Furthermore, these mechanisms may open opportunities for more targeted therapeutics. For example, *activation* of BDNF may be a potential target for individuals suffering from WD-related cognitive impairments, whereas *reduction* of BDNF signaling may be a viable option for smokers presenting with primarily affective symptoms. Therefore, these aspects emphasize the importance for smoking cessation drug discovery efforts to be cognizant of such complexities, and also show how these same complexities may offer opportunities for more personalized approaches.

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