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Neuronal RNA-binding protein HuD regulates addiction-related gene expression and behavior

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Abstract

The neuronal RNA-binding protein HuD is involved in synaptic plasticity and learning and memory mechanisms. These effects are thought to be due to HuD-mediated stabilization and translation of target mRNAs associated with plasticity. To investigate the potential role of HuD in drug addiction, we first used bioinformatics prediction algorithms together with microarray analyses to search for specific genes and functional networks upregulated within the forebrain of HuD overexpressing mice (HuD_{OE}). When this set was further limited to genes in the Knowledgebase of Addiction Related Genes database (KARG) that contains predicted HuD-binding sites in their 3' untranslated regions (3' UTR), we found that HuD regulates networks that have been associated with addiction-like behavior. These genes included *Bdnf* and *Camk2a*, two previously validated HuD targets. Since addiction is hypothesized to be a disorder stemming from altered gene expression causing aberrant plasticity, we sought to test the role of HuD in cocaine conditioned place preference (CPP), a model of addiction-related behaviors. HuD mRNA and protein were upregulated by CPP within the nucleus accumbens (NAc) of wild type C57BL/6J mice. These changes were associated with increased expression of *Bdnf* and *Camk2a* mRNA and protein. To test this further, we trained HuD_{OE} and wild type mice in CPP and found that HuD_{OE} mice showed increased cocaine CPP compared to controls. This was also associated with elevated expression of HuD target mRNAs and proteins, CaMKII α and BDNF. These findings suggest HuD involvement in addiction-related behaviors such as cocaine conditioning and seeking, through increased plasticity-related gene expression.

Keywords

RNA binding protein; HuD; post-transcriptional regulation; Nucleus Accumbens; CPP; CaMKII α ; BDNF

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Conflict of Interest

The authors declare no conflict of interest.

Introduction

Post-transcriptional mechanisms affect gene expression at multiple levels, including the control of mRNA stability or ability of recently transcribed mRNA to be translated into functional protein (Bevilacqua *et al.* 2003). This set of regulatory mechanisms may be even more integral to neuronal physiology because many neuronal proteins have a variety of isoforms generated through alternative splicing, the neuronal structure requires long-distance transport of mRNAs, and *de novo* protein synthesis can occur away from the cell body (Lee & Irizarry 2003; Lipscombe 2005; Sutton & Schuman 2006; Hengst & Jaffrey 2007). Given the simultaneous influence over mRNA processing, stability and protein translation, it is not surprising that post-transcriptional regulation plays a role in many behaviors (Dracheva *et al.* 2009; Im *et al.* 2010; Lin *et al.* 2011; Tan *et al.* 2013; Smith *et al.* 2014; Schmidt *et al.* 2014; Tapocik *et al.* 2014; Bastle, Oliver, *et al.* 2017; Yan *et al.* 2017).

HuD is a neuronal RNA binding protein (RBP; (Perrone-Bizzozero & Bolognani 2002) that stabilizes specific target mRNAs, such as those encoding BDNF and CaMKII α , through direct interaction with their 3'UTRs (Bolognani *et al.* 2010; Allen *et al.* 2013; Sosanya *et al.* 2015). HuD is localized to the dendritic compartment of hippocampal neurons (Bolognani *et al.* 2004), where it has been implicated in activity-dependent synaptic tagging controlling CamkII α mRNA localization and translation (Sosanya *et al.* 2015). Additionally, neuronal activity regulates HuD expression, localization, and association with plasticity associated mRNAs such as *Homer1a* and *Camk2a* (Tiruchinapalli *et al.* 2008). Recent studies indicate that HuD also plays a role in translation (Fukao *et al.* 2009) and that PKC-mediated phosphorylation of HuD increases local translation of BDNF (Vanevski & Xu 2015), suggesting that HuD may be involved in protein synthesis-dependent forms of plasticity. Additionally, HuD can regulate alternative RNA processing and localization these mRNA transcripts (Hinman & Lou 2008; Sosanya *et al.* 2015). Therefore, HuD is involved in nearly every level of post-transcriptional regulation. Moreover, HuD has been associated with hippocampal-dependent learning (Pascale *et al.* 2004; Bolognani *et al.* 2004; Bolognani, *et al.* 2007) and seizures (Bolognani, Tanner, *et al.* 2007), suggesting it may be involved in other activity-dependent regulatory networks. A single RBP, such as HuD, can associate with a number of target mRNAs via binding to specific nucleotide sequences in the 3' untranslated regions (3'UTRs). In fact, HuD has been predicted to bind about 20% of the mouse forebrain transcripts (Bolognani *et al.* 2010). Thus, HuD and related RBPs have been termed “master switches” of gene regulation (Deschênes-Furry *et al.* 2006; Keene 2007).

Substance use disorders, and their animal model cognate, addiction-like behaviors, are aberrant forms of learning that have been hypothesized to arise from similar molecular and electrophysiological processes as those found in other forms of associative learning and memory (Alkon *et al.* 1991; Kelley 2004; Hyman 2005; Hyman *et al.* 2006; Koob & Volkow 2010). The encoding of environmental cues predicting reward-availability is a feature shared by both natural and drug-induced forms of learning and memory. These strong associations with cues are thought to be involved in the chronic relapsing state of patients with substance use disorders (Childress *et al.* 1999). Drug-conditioned place preference (CPP) is an animal model useful for studying learning and motivation acquired through associations between

drug effects and environmental cues present during the drug experience. Acquisition and expression of CPP is thought to recruit protein-synthesis dependent mechanisms (Kuo *et al.* 2007; Robinson & Franklin 2007), which are critical for forming the drug-cue associations that motivate approach and contact with the cues (i.e., a model of conditioned drug-seeking behavior; (Tzschentke 2007). Since HuD mRNA targets have been suggested to play a role in general plasticity and learning, as well as cocaine-induced behaviors (Graham *et al.* 2007; Anderson *et al.* 2008), we hypothesized that HuD regulation of mRNAs, such as *Camk2a* and *Bdnf*, may play a role in cocaine-CPP.

Methods

Animal husbandry

Transgenic mice overexpressing HuD (HuD_{OE}) under the *Camk2a* promoter were generated and backcrossed to a C57BL/6J background for >20 generations (Bolognani, *et al.* 2006). Control animals were either wild type littermates or standard C57BL/6 mice. Animals for these studies were 2 month-old males that were group housed, maintained on a 12 h reverse light/dark cycle and tested during the dark cycle. Experiments were performed in accordance with NIH Animal Welfare guidelines under protocols approved by the Institutional Animal Care and Use committees at the University of New Mexico.

Bioinformatics analyses and microarray studies

Initial bioinformatics analyses were used to predict the percentage of genes in the Knowledgebase of Addiction Related Genes database (KARG; <http://karg.cbi.pku.edu.cn>; Li *et al.* 2008) that contain recognition motifs for HuD binding (Bolognani *et al.* 2010). Briefly, to search for these motifs, we downloaded all the 3' UTR from the genes in the mouse KARG datasets (both the microarray-based set and the total gene expression and genetic dataset) using EMSEMBL BioMart and used a Perl script to search for the following sequences, allowing for one mismatch: [CG][CT][CT]TC[CT][CT]TC[TC]C[TC]C, [TG]TTTGT[TT][TG][GT]TTT, and TTTTTTTTT[TA]AAA, for motifs 1, 2 and 3, respectively as described in Bolognani *et al.* 2010. The percent of genes in KARG with predicted HuD binding motifs 1, 2 and 3 were compared to those in the whole mouse genome using Chi-squared tests.

To identify the *in vivo* regulatory program induced by HuD, either directly or indirectly, RNA samples from 2 month old male transgenic mice overexpressing HuD and control littermates were run on Affymetrix 430 2.0 microarrays (n = 3/group). The forebrain, which includes the cerebral cortex, basal ganglia, hypothalamus, thalamus, subthalamus, and hippocampus, was isolated by removal of the olfactory bulb and cerebellum from the entire brain. RNA was extracted from this tissue using a standard Trizol isolation technique (Thermo Fisher Scientific, Waltham, MA, USA). Microarray data was analyzed using MultiExperiment Viewer software (MeV_4_9_0, <http://www.tm4.org>). Molecules that were upregulated by a fold change of 1.25 with p<0.05 were selected as a set of transcripts potentially regulated by HuD. The fold change cutoff was based on two different criteria. First, the threshold was based on the minimal level of global forebrain HuD mRNA upregulation found on the same microarrays relative to wild type littermates. In addition,

since post-transcriptional regulators such as RBPs and microRNAs fine-tune the levels of expression of specific target genes, changes in gene expression are likely to be smaller than for those induced by transcription factors. In previous studies, we used a similar 25% cutoff when analyzing the effect of a microRNA miR-495 on another set of ARGs (Bastle, Oliver et al, 2017). Within this set of potential HuD-regulated genes, putative direct HuD targets were further identified by searching for HuD binding sites found within the 3'UTR as described above. Next, the list of putative direct HuD targets was cross-referenced with the list of genes in the entire mouse KARG dataset with evidence scores ≥ 2 . High confidence molecules are designated in the database through evidence score numbers, i.e., the number of evidences of their association with addiction from categories such as microarray data, low-throughput evidence and genetics. Thus, as in a previous study (Bastle, Oliver et al, 2017), we selected genes where their association with addiction was supported by at least two independent sets of evidence. ARGs with evidence scores ≥ 2 were cross-analyzed with the identified molecules regulated by HuD and the resulting set of 173 molecules are listed in Table 1.

qRT-PCR and Western blot analyses

For mRNA analyses, brains from HuD_{OE} mice were isolated and quickly frozen in isopentane cooled at -40°C by a methanol-dry ice bath. They were then dissected using a brain matrix and the nucleus accumbens was punched using a 1.25 mm brain punch (Harris Unicore™). Total RNA was isolated and mRNAs quantified using Power SYBR Green PCR Master Mix (Life Technologies) as described previously (Bullock *et al.* 2009), with primers designed against mouse HuD (Mansfield & Keene 2012) and selected target mRNAs (*Bdnf-L*: Forward TGGCCTAACAGTGTTCGAG, Reverse GGATTTGAGTGTGGTCTCC; *Camk2a*: Forward TATCCGCATCACTCAGTACCTG, Reverse GAACTGGACGATCTGCCATTT); and compared to a reference transcript (*Gapdh*: Forward TGTGATGGGTGTGAACCACGAGAA, Reverse GAGCCCTTCCACAATGCCAAAGTT). Relative expression was determined using the comparative 2^{-C_t} method (Livak & Schmittgen 2001). A no reverse transcriptase (RT) reaction was run for each sample and none of the no-RT controls amplified. qPCR experiments were replicated on 2-3 separate occasions. Data collected from each run were averaged together.

For protein analysis, NAc tissue was homogenized with lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40) supplemented with fresh protease inhibitors (cOmplete™ Mini, Roche, Indianapolis, IN, # 4693159001). Lysates were pre-cleared by centrifugation at $14,000 \times g$ and then used for protein determination. Aliquots containing 35 μg of total protein were diluted 1:1 in 2 $\times$ Laemmli Sample Buffer (Sigma, # S3401) and run on 4-15% SDS polyacrylamide gels (Bio-Rad, #4568086). Western blots were performed as previously described (Tanner *et al.* 2008) using antibodies to CaMKII α (1:2000; Santa Cruz, Dallas, TX, USA, # sc-13141), mature BDNF (1:1000; Icosagen, Össu, Ülenurme, Tartumaa, Estonia, #327-100), proBDNF (1:1000; Alomone, Jersusalem, Israel, #AGP-032), HuD (1: 2000 Santa Cruz, Dallas, TX, USA, # sc-28299), or GAPDH (1:5000; Santa Cruz, Dallas, TX, USA # sc-32233). Membranes were then incubated for 1 h in either secondary goat anti-mouse HRP (1:5000; Santa Cruz, # sc-2005) or goat anti-guinea pig

HRP (1:5000; Santa Cruz, # sc-2438), respectively, and were developed with standard chemiluminescent reagents and procedures (NEL103001EA, Perkin Elmer, Waltham, MA, USA). Each sample *n* is exactly one animal with both hemispheres pooled together. Specific bands that correspond to the correct molecular weight of the target (~14 kDa for mature BDNF, ~38 kDa for proBDNF, ~38 for GAPDH, ~50 for CaMKII α) were quantified using densitometric analysis in ImageJ and then standardized by pixel density to the Coomassie Brilliant Blue stain of total protein as described previously (Tanner *et al.* 2008). Westerns were replicated 2-3 times and data were averaged together.

Conditioned place preference studies

For CPP, 2 month old Male C57Bl/6J mice (*n* = 20/group) underwent training as previously described (Allan *et al.* 2001). The conditioning apparatus is a set of two 20×20×20 Plexiglass chambers connected by a 9×10×20 cm anteroom, with the walls either entirely black or vertical black and white stripes in addition to either textured plastic or metal lattice floors to allow for multidimensional discrimination between chambers. Experiments were performed under red light at 30-34 lux. On the first day, animals received a saline injection and were allowed to roam throughout the apparatus for 10 minutes to habituate to the chambers. Time spent in each chamber was recorded in the habituation session with an Ethovision XT8 system calibrated for C57Bl/6J mice on the chamber background. Data were recorded in the habituation session to determine if the animals inherently preferred either chamber, confirming these mice showed no preference for either chamber. Conditioning to non-contingent injections took place over the next three days, and on each day animals were confined to one of the chambers for 30 min, and 5h later were confined to the other chamber for 30 min. Mice were returned to their home cages between sessions. In one experiment, wild type mice were randomly assigned to either a conditioned group that received cocaine immediately before placement into the conditioned stimulus (Cs+) chamber and saline immediately before placement into the other (Cs-) chamber, or a control group that received saline immediately before placement into either chamber. In the subsequent experiment, wild type mice were compared to HuD_{OE} mice and all were cocaine-conditioned as above. The chamber assigned as the Cs+ was randomly assigned assuring unbiased equal numbers assigned to each chamber in each group. The session during which conditioned mice received cocaine was counterbalanced across AM/PM sessions. Finally, on the 5th day, animals were given a saline injection and allowed to freely explore the entire apparatus for 20 minutes. Using the Ethovision XT8, preference was calculated by time spent in CS+ versus CS-. The first 5 minutes of the test day was not analyzed to remove the effects of the aversive injection, such as post-injection grooming. Animals were sacrificed 1 h following the saline injection.

Statistical analyses

Behavioral and biochemical measures were analyzed using Student *t* tests using SPSS 24.0 (IBM, Armonk, NY, USA).

Results

Analyses of HuD regulated addiction-related targets

Previously, it has been shown that HuD regulates a number of genes associated with memory, learning, and plasticity through stabilization and translation of the mRNA transcript (Bolognani *et al.* 2010; Allen, Bird, *et al.* 2013; Sosanya *et al.* 2015; Tiruchinapalli *et al.* 2008; Vanevski & Xu 2015). Due to the multiple sets of transcripts regulated by HuD, the entire regulatory program induced by this RBP has not been fully determined. Since we hypothesized that HuD may regulate mRNAs associated with addiction-related behaviors, we sought to identify predicted targets of HuD in a database of addiction-related genes (KARG; <http://karg.cbi.pku.edu.cn>, Li *et al.* 2008), including those identified by expression patterns in microarray studies. The percentage of genes containing any of the three HuD binding motifs in their 3' UTRs in the mouse KARG database was compared with those containing the same motifs in the entire mouse genome. These analyses (Figure 1A) revealed that the KARG database set was significantly enriched in genes containing any of the three consensus HuD binding motifs described in forebrain neurons (** $p < 0.001$ two-tailed χ^2 test; (Bolognani *et al.* 2010) in their 3' UTRs. To identify which of the predicted targets were regulated by this RBP *in vivo*, we used Affymetrix 430 2.0 microarrays to analyze forebrain samples from transgenic animals overexpressing HuD and control littermates ($n = 3/\text{group}$). Transcripts that were upregulated by a fold change of 1.25 and $p < 0.05$ relative to control mice were selected as putative HuD-regulated targets. We then used KARG to refine our list to addiction-related transcripts with evidence scores ≥ 2 , which indicate that at least two independent publications support the association with drug addiction. The presence of predicted HuD binding sites in this subset was confirmed upon 3' UTR analyses as described before (Bolognani *et al.* 2010 and Supplementary Table 1). From these bioinformatics analyses, we found 173 HuD-regulated genes were previously identified as ARGs (Table 1). Finally, we used Ingenuity Pathway Analysis (IPA) to identify pathways, networks, and functions associated with this set of genes. Many of the HuD-regulated ARGs have been implicated in behavior, neurological disease, cell morphology, and psychological disorders (Fig. 1B). Focusing on the 74 molecules specifically associated with behavior (Table 1), we found that these molecules were enriched in top biological functions associated with addiction such as cognition, memory, locomotion, neuritogenesis, and synaptic transmission (Fig. 1C). Additionally, the top network from these HuD-regulated and behavior-associated ARGs contained many key molecules associated with addiction-related plasticity including those involved in dopamine and glutamate receptor signaling as well as ERK/MAPK and PKA signaling (complete network in Table 1; selections shown in Fig. 1D). Thus, we concluded that HuD induces a regulatory program that controls the expression of many genes associated with drug addiction.

Regulation of HuD targets during cocaine CPP

Since these HuD-regulated transcripts were previously identified as ARGs and associated with behavior, we sought to determine whether HuD and its targets were regulated during cocaine CPP. A group of 16 C57Bl/6J male mice were trained, as previously described, with alternating cocaine or saline injections each paired with a specific chamber, respectively, to form an association between the cocaine unconditioned stimulus (US) and the environmental

conditioned stimulus (CS+ or in the case of Saline, CS-). A control group of 16 mice were trained in a similar fashion with saline paired with both chambers, thus removing the associative learning between CS and US. On the test day, animals received a saline injection and were allowed to freely roam between the chambers. As described earlier, CPP was measured by time spent in the CS+ versus the CS- chamber, thus scores >1 indicate preference for the CS+ chamber while scores ~1 suggest no preference for either chamber. As expected, mice trained with cocaine and saline showed CPP for the cocaine paired chamber (Fig. 2A; $t(28.47) = 4.05$, $p = 0.0004$, with Welch's correction). Animals were sacrificed 1 h following the start of the test day session and brains were flash frozen for RNA and protein analyses. We focused on the NAc because this region is necessary and sufficient, specifically the shell, for cocaine CPP as determined by lesion and region-specific cocaine injection experiments (Liao *et al.* 2000; Sellings *et al.* 2006). We found that NAc HuD mRNA and protein was significantly increased in those animals trained with cocaine (Fig. 2B, C; $t(10) = 2.5$, $p = 0.03$; $t(10) = 2.7$, $p < 0.02$). This suggests that NAc HuD upregulation is associated with cocaine CPP.

Given that CPP was associated with an upregulation in NAc HuD, we expected that HuD ARG targets may be upregulated as well. We assessed the expression of two well validated HuD targets, *Bdnf* and *Camk2a*, which also have the highest evidence scores in KARG (*Bdnf*=16 and *Camk2a*=7, Li *et al.* 2008). As shown in Figures 2D and E, we found that *Camk2a* mRNA and CaMKII α protein were both upregulated within the NAc after cocaine CPP training ($t(5) = 2.8$, $p = 0.04$; $t(11) = 3.5$, $p = 0.005$). The other validated HuD target and ARG, *Bdnf*, can be transcribed from many promoters (Lu, 2003). However, at the mRNA level, differential poly(A) site usage leads to two different variants via alternative splicing. One variant is characterized by a shorter 3'UTR (*Bdnf*-S) while the other has a longer 3'UTR (*Bdnf*-L; Timmusk *et al.* 1993). Since *Bdnf*-S and the initial segment of *Bdnf*-L are identical, we could only reliably quantify total *Bdnf* mRNA (*Bdnf*-Pan) from *Bdnf*-L. Thus, to fully evaluate the regulation of *Bdnf* mRNA, we assessed both variants. As such, we found *Bdnf*-L and *Bdnf*-Pan mRNA were both upregulated by cocaine CPP training (Fig. 2F, H; $t(10) = 2.8$, $p = 0.02$; $t(11) = 2.2$, $p = 0.047$). BDNF protein also exists in two different forms, the unprocessed proBDNF and the smaller, processed mature BDNF (matBDNF; Leibrock *et al.* 1989). These two forms of protein have different receptors and cellular effects, so it is again important to assess both forms of BDNF (Zanin *et al.* 2017). Both proBDNF and matBDNF were upregulated by cocaine CPP training within the NAc (Fig. 2G, I; $t(6) = 3.8$, $p = 0.009$; $t(6) = 2.8$, $p = 0.03$). Taken together, this suggests that two independently-validated HuD ARG targets that have been previously shown to be associated with addiction-related behaviors are upregulated by cocaine CPP and associated with the upregulation of HuD after CPP training.

To confirm our microarray analyses and further dissect the role of HuD-induced upregulation of *Camk2a* and *Bdnf* *in vivo* specifically in the NAc, we used HuD^{OE} animals to determine if overexpression without training or drug treatment was sufficient to elicit a similar effect on these two validated targets. Analysis of HuD expression in the NAc of naïve HuD^{OE} mice revealed that both the mRNA (Fig. 3A; $t(7) = 2.6$, $p = 0.04$) and HuD protein (Fig. 3B; $t(7) = 3.2$, $p = 0.01$) were overexpressed within this region. We also found that *Camk2a*, *Bdnf*-L, and *Bdnf*-Pan mRNA were concomitantly increased in HuD^{OE} mice

(Fig. 3C, E, and G; $t(6) = 2.5$, $p = 0.049$; $t(6) = 2.6$, $p = 0.04$; $t(6) = 2.6$, $p = 0.04$). Additionally, NAc CaMKII α , pro- and mat-BDNF proteins were increased (Fig. 4D, F, and H; $t(5) = 9.9$, $p = 0.0002$; $t(11) = 2.2$, $p = 0.049$; $t(5) = 2.7$, $p = 0.04$). This suggests that in the absence of CPP training or cocaine treatment, animals overexpressing HuD within the NAc show increased levels of HuD and its plasticity associated targets.

Increased ARG expression and cocaine CPP in HuD_{OE} mice

To test the functional role of enhanced levels of HuD, we trained HuD_{OE} mice and wild type littermates in the same cocaine CPP procedure described above. Notably, although all groups received cocaine during conditioning, HuD_{OE} animals showed increased CPP behavior compared to wild type controls (Fig. 4A; $t(25) = 2.6$, $p = 0.02$). First, we confirmed that HuD_{OE} animals indeed had elevated HuD in the NAc, as found in Fig. 3A and 3B in naive animals. Although we did not detect increased expression of HuD mRNA, we did find increased HuD protein expression in HuD_{OE} animals that underwent cocaine CPP (Fig. 5A, B, $t(9) = 4.7$; $p = 0.0002$; $t(5) = 3.5$, $p = 0.02$). Additionally, HuD_{OE} animals showed significantly higher expression of HuD ARG target mRNA and protein (Fig. 5C–H, $t(5) = 2.9$; $p = 0.03$; $t(5) = 3.2$, $p = 0.02$; $t(4) = 4.0$, $p = 0.02$; $t(8) = 3.8$, $p = 0.005$; $t(7) = 2.5$, $p = 0.04$; $t(4) = 3.0$, $p = 0.04$). To determine the specificity of HuD overexpression, we assessed the expression of GAPDH which is not predicted to be targeted by HuD. We found that *Gapdh* mRNA and protein were not significantly different between controls and HuD_{OE} animals (Fig. 5I, J; $t(12) = 0.04$, $p = 0.97$; $t(7) = 0.9$, $p = 0.4$). Overall, this data suggests that HuD regulates cocaine CPP by inducing a regulatory program within the NAc associated with increased expression of two selected targets involved in CPP behavior.

Discussion

Overall, our study is the first to identify the neuronal RNA-binding protein HuD as a post-transcriptional regulator of ARG expression and addiction-related behaviors. HuD overexpression induced global alterations including multiple predicted direct HuD-regulated transcripts containing HuD binding sequences in their 3' UTRs. Many of these targets were found to be enriched in the KARG and formed regulatory networks associated with behavior. In agreement with these observations, we found that HuD was upregulated by cocaine CPP training and overexpression caused increased cocaine CPP behavior along with upregulation of two well-studied, direct HuD targets CaMKII α and BDNF. HuD_{OE} animals that showed increased cocaine CPP behavior also were found to have increased expression of these targets within the NAc, a critical brain region associated with CPP and addiction in general. Since the encoding of cues indicative of drug exposure are required for CPP behavior and are cited as a major cause of relapse in human patients, post-transcriptional regulation involving HuD could play a role in therapeutics targeting relapse to drugs of abuse.

Our data suggests that HuD regulates multiple networks enriched in genes associated with addiction-related behaviors. For example, ERK/MAPK signaling, which was a central node in a network significantly regulated by HuD_{OE}, has been found to be integral for CPP to other drugs (Gerdjikov *et al.* 2004; Miller & Marshall 2005; Mizoguchi *et al.* 2004).

Additionally, elevated ERK signaling within other brain regions, such as subregions of the amygdala, have been found to be necessary for reactivity to cues that elicit drug seeking during withdrawal (Lu *et al.* 2005; Lu *et al.* 2006; Thiel *et al.* 2012). Since cue-induced reinstatement of drug-seeking is proposed to model human drug-relapse behavior, this suggests that HuD may be involved in reward reinstatement behavior.

To assess the regulatory impact of HuD on behavior, we analyzed the expression of two direct targets of HuD, BDNF and CaMKII α (Tiruchinapalli, *et al.*, 2008; Allen, Bird, *et al.* 2013; Sosanya *et al.* 2013; Sosanya *et al.* 2015). Although HuD is predicted to regulate other 72 ARG mRNAs, as proof of principle, we focused the validation on the top two ARG genes, as they have the highest evidence scores in KARG and been highly associated with behavioral and addiction-related processes. For example, these two genes regulate molecular processes involved in the learning and memory of discrete spatial cues (Silva *et al.* 1992; Falkenberg *et al.* 1992; Linnarsson *et al.* 1997; Cho *et al.* 1998; Mu *et al.* 1999; Mizuno *et al.* 2000; Gorski *et al.* 2003; Cirulli *et al.* 2004; Poulsen *et al.* 2007; Nakajo *et al.* 2008; McGauran *et al.* 2008; Achterberg *et al.* 2014). Since visual spatial cues are critical in CPP behavior (Cunningham *et al.* 2006), and these molecules have been associated with CPP for other drugs of abuse, this suggested that HuD may regulate cocaine-CPP through direct targeting of CaMKII α and BDNF (Lu *et al.* 2000; Sakurai *et al.* 2007; Bahi *et al.* 2008; Rosen *et al.* 2015).

Although overexpression of HuD was found to facilitate cocaine CPP learning, previous work from our laboratory on the role of HuD in other learning associated behaviors suggests that its constitutive overexpression disrupts more complex learning and retention of spatial memories (Bolognani, *et al.* 2007). This apparent discrepancy may be due to the valence of the hedonic stimulus in drug-induced behaviors versus stress-induced behaviors such as contextual fear conditioning (CFC) or Morris water maze (MWM). Secondly, this may be due to differences in the regions recruited in these tasks. Indeed, researchers studying the role of NR2B containing NMDARs found that ifenprodil inhibition blocked the acquisition of morphine-induced CPP without affecting the acquisition of MWM demonstrating a delineation between drug and non-drug associated spatial learning (Ma *et al.* 2011). Finally, temporal and spatial aspects of HuD expression and activity may play an important role in these findings. The influence of post-transcriptional regulators in spatial learning seems to follow a bidirectional response, where both overexpression and knockout of a regulatory gene impairs spatial learning, thus, suggesting that a specific expression pattern of downstream plasticity-associated genes is required for proper learning and memory (Paradee *et al.* 1999; Peier 2000). Adding further complication, these plasticity-associated genes themselves exhibit discretely regulated spatial and temporal expression. For example, while hippocampal *Bdnf* mRNA was upregulated at specific time points of MWM training (Kesslak *et al.* 1998), many drugs of abuse acutely and chronically upregulate BDNF within the NAc (Filip *et al.* 2006; Graham *et al.* 2007; Bastle, Oliver *et al.* 2017). Additionally, BDNF is upregulated during abstinence from drug, which may contribute to “incubation of craving” (Li & Wolf 2015). Thus, overexpression of plasticity-associated genes such as BDNF and CaMKII α may cause biphasic learning responses as well (Mayford *et al.* 1996).

In conclusion, we have determined that HuD plays an important role in cocaine CPP behavior, suggesting that regulation of mRNA stability and protein translation mediated by this RBP is involved in controlling reward seeking. While further testing will be required to delineate the exact function of HuD in the molecular underpinnings of this behavior and the role of this RBP in the regulation of other ARGs, our work suggests that regulation of ARG expression mediated by HuD is a novel regulatory pathway involved in addiction-related behaviors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. HuD regulates addiction-associated molecular networks

Predicted HuD targets were identified *in silico* by selecting mRNAs that contained 1 HuD binding motif(s) within the 3'UTRs as described by Bolognani *et al*, (2010). (A) Predicted HuD targets were found to be enriched within the KARG datasets for genetic and microarray evidence compared to transcripts containing the same motifs in the entire mouse genome (***) $p < 0.001$ two-tailed χ^2 test). Next, *in vivo* regulated, addiction-related targets were identified using microarrays. A set of transcripts that a) were upregulated by a fold change of 1.25 and $p < 0.05$ in HuDOE compared to control littermate forebrain tissue, b) were present in the KARG database and c) contained HuD binding sites in the 3' UTR as described in (A). (B) Molecules fitting these criteria were associated with CNS functions and diseases, such as behavior. mRNAs associated with behavior were clearly organized into biological functions associated with addiction (C) and formed a top molecular network (D) implicated in addiction-associated behaviors.

Figure 2. Cocaine CPP training is associated with elevated levels of NAc HuD and two validated HuD-targets

A) C57Bl/6J mice trained with 15 mg/kg cocaine show increased preference for the cocaine-paired conditioned stimulus (Cs+) compared to animals that received saline paired with both chambers ($n = 20/\text{treatment}$). Cocaine CPP training was associated with an increase in NAc HuD mRNA (B) and protein (C; $n = 5-7/\text{treatment}$). Cocaine trained animals showed elevated *Camk2a*, *Bdnf-L*, and *Bdnf-Pan* mRNA (D, F, H) as well as CaMKIIα, pro-BDNF, and mature-BDNF protein (E, G, I with representative blots). $n = 3-8/\text{treatment}$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t-test cocaine versus saline. Data are Means $\pm$ SEM.

Fig 3. Naïve HuDOE animals show increased NAc HuD and validated HuD targets

Male HuDOE mice and littermate controls were sacrificed around 2 mo. of age and the NAc was dissected. HuDOE mice show increased NAc HuD mRNA (A) and protein (B) compared to controls. Following elevated HuD expression, previously validated, direct HuD targets were elevated in this region as well. *Camk2a*, *Bdnf-L* and *Bdnf-Pan* mRNA was increased within the NAc (C, E, G) as well as *CaMKIIα*, pro-BDNF, and mature-BDNF protein (D, F, H). $n = 3-6/\text{genotype}$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Student's t-test cocaine versus saline. Error bars indicate $\pm$ SEM.

Fig 4. HuD^{OE} animals show increased cocaine CPP

Male HuD^{OE} mice and littermate controls trained with 15 mg/kg cocaine in the same CPP-induced protocol as Fig. 2 except both groups were trained with cocaine. HuD^{OE} mice show increased preference for the cocaine-paired Cs+ compared to littermate controls (n = 13-14/genotype). * $p < 0.05$, Student's t-test HuD^{OE} versus control littermates. Error bars indicated $\pm$ SEM.

Fig 5. HuD_{OE} animals show increased cocaine CPP-induced HuD target expression within the NAc

The NAc was harvested 1hr following initiation of the test day in the CPP protocol. HuD_{OE} animals did not show an increase in *HuD* mRNA (A) but did show elevated HuD protein within this region (B). HuD_{OE} animals showed elevated *Camk2a*, *Bdnf-L*, and *Bdnf-Pan* mRNA (C, E, G) as well as CaMKIIα, pro-BDNF, and mature-BDNF protein (D, F, H with representative blots to the right). No significant change in GAPDH mRNA or protein (I, J), which is not a predicted HuD target. $n = 3-5/\text{treatment}$. * $p < 0.05$, ** $p < 0.01$ Student's t-test HuD_{OE} versus control littermates. Error bars indicated $\pm$ SEM.

Table 1

HuD regulated Addiction-related genes (ARGs)

Putative HuD ARG target mRNA upregulated in HuD_{OE}	<i>Acot12, Acot2, Adarb1, Adcy8, Adcyap1, Adrb2, Aff1, Ank1, Ap2a2, Apod, Arf4, Atp6v1b2, Atp6v1e1, Bche, Bcl2, Bcl2l11, Bdnf, Bmp7, Cacng1, Calm3, Camk2a, Ccl12, Cd44, Cdh1, Cdkn1a, Cebpg, Chn2, Chrna3, Cnr1, Cnr2, Cpd, Creb1, Crem, Csk, Ddh2, Dld, Dnajb14, Dpysl2, Drd4, Dsc2, Dusp4, Edil3, Egfr, Eif4e, Elavl4, Fgf2, Fkbp5, Foxp1, Fyn, Gap43, Gdnf, Gnaq, Gnb5, Gpm6a, Gria2, Grip1, Gsn, H2-D1, Hhex, Hint1, Hmgb2, Hspb1, Igf1, Ikbkb, Il10ra, Il16, Ipo11, Itgb1, Itpkb, Jun, Kalrn, Kcmf1, Kcna1, Kcnc1, Kcnj6, Klk6, Kmo, Ldb2, Limk1, Lims1, Lrch4, Mapk1, Mapk10, Mapk14, Mapk8, Matr3, Mbp, Mme, Mpdz, Mrpl17, Myt1, Myt1l, Ncam1, Ndst1, Ndufs4, Nedd4, Nf1, Nfasc, Nfia, Nfkb, Nr4a3, Nsf, Ntrk3, Oprd1, Oprm1, Pdk4, Pdxk, Phf1, Pml, Ppm1b, Ppp1cc, Ppp1r14c, Ppp1r2, Ppp2cb, Ppp2r2c, Ppp2r5c, Ppp3cb, Ppp3r2, Prkca, Prrx1, Psmb2, Ptdss2, Pten, Ptk2b, Ptprk, Rac2, Rad23b, Rad51, Ran, Rb1, Rgs12, Rgs9, Rhobtb3, Rhou, Rps6ka5, Sall3, Samhd1, Scn2b, Seh1, Serpina3n, Sesn2, Sfpq, Sh2d5, Slc6a1, Smarcd1, Sox9, Ssx2ip, Syn2, Syp, Syt11, Tacr1, Tacstd2, Taf7, Thrb, Tiam1, Timp2, Tlk2, Tmed10, Tmod2, Tmpo, Tmprss2, Tnfrsf9, Tph1, Trib2, Trim59, Trp53inp2, Ubtg, Ugcg, Uqcrc2, Vamp3, Vnn1, Xpo1, Ywha, Zbtb16, Zwint</i>
HuD ARG targets associated with behavior	<i>Adarb1, Adcy8, Adcyap1, Adrb2, Apod, Arf4, Bche, Bcl2, Bdnf, Camk2a, Cdkn1a, Chrna3, Cnr1, Cnr2, Creb1, Crem, Csk, Ddh2, Drd4, Dusp4, Eif4e, Elavl4, Fgf2, Foxp1, Fyn, Gdnf, Gnaq, Gria2, Hint1, Igf1, Ikbkb, Itgb1, Jun, Kalrn, Kcna1, Kcnc1, Kcnj6, Limk1, Mapk1, Mapk10, Mapk14, Mapk8, Mme, Ncam1, Ndufs4, Nedd4, Nf1, Nfasc, Nfkb, Nr4a3, Ntrk3, Oprd1, Oprm1, Pml, Ppp1cc, Ppp1r14c, Ppp3cb, Prkca, Pten, Rad23b, Rb1, Rgs9, Rps6ka5, Sall3, Scn2b, Sfpq, Slc6a1, Syn2, Tacr1, Thrb, Timp2, Tmod2, Tph1, Trib2</i>
Top HuD regulated network associated with behavior	<i>Adcy, Adrb, Adrb2, Bdnf, Calcineurin Protein(S), Camk2a, Cnr2, Cofilin, Drd4, Erk1/2, F Actin, G Protein, G Protein Alpha1, Gnaq, Gpcr, Gria2, Kcnj6, L-type Calcium Channel, Limk1, Nmda Receptor, Nr4a3, Oprd1, Oprm1, Pka, Pkg, Plc, Pp1 Protein Complex Group, Pp2a, Ppp1cc, Ppp3cb, Prkca, Proinsulin, Syn2, Tacr1, Trib2</i>

Shown are candidate HuD target mRNAs that were significantly upregulated (fold change 1.25 and $p < 0.05$) in HuD_{OE} animals containing potential HuD binding sites in their 3' UTR as defined by (Bolognani *et al.*, 2010) and that were identified as ARG in the KARG database, suggesting that they may play a role in addiction-related processes.