Changes in neuronal immunofluorescence in the C- versus N-terminal domains of hnRNP H following D1 dopamine receptor activation

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Highlights

- We investigated immunofluorescence and localization of hnRNP H following dopamine receptor activation in primary rat cortical neurons.
- Activation of D1 dopamine receptor increased nuclear immunofluorescence of C-terminal but not the N-terminal domain of hnRNP H with no change in either nuclear or cytoplasmic levels of hnRNP H protein.
- D1 dopamine signaling could alter interaction of hnRNP H with other proteins.

Abstract

RNA binding proteins are a diverse class of proteins that regulate all aspects of RNA metabolism. Accumulating studies indicate that heterogeneous nuclear ribonucleoproteins are associated with cellular adaptations in response to drugs of abuse. We recently mapped and directly validated heterogeneous nuclear ribonucleoprotein H1 (*Hnrnph1*) as a quantitative trait gene underlying differential behavioral sensitivity to methamphetamine. The molecular mechanisms by which hnRNP H1 alters methamphetamine behavior are unknown but could involve pre- and/or post-synaptic changes in protein localization and function. Methamphetamine initiates post-synaptic D1 dopamine receptor signaling indirectly by binding to pre-synaptic dopamine transporters and vesicular monoamine transporters of midbrain dopaminergic neurons which triggers reverse transport and accumulation of dopamine at the synapse. Here, we examined changes in neuronal localization of hnRNP H in primary rat cortical neurons that express dopamine receptors that can be activated by the D1 or D2 dopamine receptor agonists SKF38393 and (-)-Quinpirole HCl, respectively. Basal immunostaining of hnRNP H was localized primarily to the nucleus. D1 dopamine receptor activation induced an increase in hnRNP H nuclear immunostaining as detected by immunocytochemistry with a C-domain directed antibody (epitope near the glycine-rich domain) but not with an N-domain specific antibody. Although there was no change in hnRNP H protein in the nucleus or cytoplasm, there was a decrease in *Hnrnph1* transcript following D1 receptor stimulation. Taken together, these results suggest that D1 receptor activation increases availability of the hnRNP H C-terminal epitope, which could potentially reflect changes in protein-protein interactions. Thus, D1 receptor signaling could represent a key molecular post-synaptic event linking *Hnrnph1* polymorphisms to drug-induced behavior.

Keywords

hnRNP; RNA binding protein; psychostimulant use disorder; methamphetamine; addiction

1. Introduction

Heterogeneous nuclear ribonucleoproteins (hnRNPs) represent a large family of RNA binding proteins with multifunctional roles in RNA biogenesis and metabolism, both in the nucleus and the cytoplasm [1,2]. In the nucleus, hnRNPs regulate transcription, splicing, and mRNA stability [1–4]. In the cytoplasm, hnRNPs can repress or enhance translation by binding to 3'- and 5'-untranslated regions of mRNAs [1,2,5]. Several hnRNPs are present in both the nucleus and cytoplasm and bind to mRNA targets to regulate bidirectional transport [6,7]. In response to neuronal stimulation, hnRNPs can also regulate synaptic plasticity through shuttling crucial mRNAs encoding synaptodendritic proteins, kinases, and cytoskeletal elements to dendritic processes [4,8–10].

Immunopurification and mass spectrometry of HeLa cells identified over 20 hnRNPs labeled from A through U [11]. hnRNP H1, H2, and F regulate mRNA alternative splicing and polyadenylation [3,12–14]. hnRNP H1 and H2 share a 96% protein sequence homology and have yet to be functionally differentiated [6]. hnRNP H contains three quasi-RNA recognition motifs (qRRMs), and two glycine-rich domains, GY and GYR [7,15]. The qRRMs recognize and bind to poly-G runs of varying lengths or GGG triplets within RNA sequences [15,16]. The glycine-rich GYR domain is critical for the nuclear localization of hnRNP H [7], while both GYR and GY domains facilitate protein-protein interactions [5].

We used quantitative trait locus (QTL) mapping, positional cloning, and gene editing to identify *Hnrnph1* as a quantitative trait gene underlying reduced sensitivity to the locomotor stimulant properties of methamphetamine (MA) in mice [17]. Based on transcriptome analysis of the QTL within the striatum, we hypothesized that the mechanism could involve a deficit in mesolimbic dopaminergic neuron development. Dopaminergic neurons in the ventral midbrain project to the basal forebrain and prefrontal cortex where MA binds to pre-synaptic dopamine transporters and vesicular monoamine transporters and induces reverse transport, increased synaptic dopamine, and activation of synaptic D1 Gs and D2 Gi/Go-coupled receptors [18,19]. hnRNP H could potentially act in presynaptic dopaminergic neurons or in postsynaptic forebrain neurons to influence the acute behavioral response to MA and activity-dependent neurobehavioral plasticity. In the present study, we utilized primary rat cortical neurons as an *in vitro* model to examine changes in localization of hnRNP H following dopamine receptor activation.

2. Materials and Methods

2.1. Primary rat cortical neuron culture

Primary cortical neurons were dissected from the neocortex of E18 Sprague-Dawley embryos (Charles River Laboratories, Wilmington, MA, USA) and grown in defined media as described [20]. Dissociated neurons were plated on Poly-L-lysine coated dishes (18k cells/cm^2) and placed in an incubator (37ºC; 5% CO_2) for attachment to coverslips/dish surface. Neuronal cultures were grown for 8 days *in vitro* (DIV8) until harvesting unless specified otherwise (DIV16).

2.2. Neuronal treatment and immunocytochemistry

For the control treatment, 1 mL of conditioned media was replaced with 1 mL of warmed neurobasal media. For the experimental treatments, 1 mL warmed neurobasal media was mixed with potassium chloride (KCl; Boston BioProducts, Ashland, MA, USA), the D1 dopamine receptor agonist SKF38393 (Sigma Aldrich, St. Louis, MO, USA), the D1 dopamine receptor

antagonist SCH23390 (Sigma Aldrich) plus SKF38393 or the D2 dopamine receptor agonist (-)-Quinpirole HCl (Quinpirole; Sigma Aldrich). Final treatment concentrations were 1 µM SKF38393, 1 µM Quinpirole and 10 nM SCH23390 and cells were incubated for 1.5 h. Neurons were washed with 1 x PBS at room temperature, fixed with 4% paraformaldehyde, permeabilized, and blocked with 0.1% Triton-X and 1% Bovine Serum Albumin (BSA), and incubated with primary antibody that is specific for the C-terminus or N-terminus of hnRNP H in 1% BSA overnight at 4°C. Neurons were washed with 1X PBS and incubated with fluorophore-conjugated antibody: Alexa Fluor 594 secondary antibody (C-domain: Donkey anti-Rabbit; N-domain: Donkey anti-Goat in 1% BSA). A complete list of antibodies is provided in Tables S1 and S2. Processed coverslips were then treated with ProLong Diamond Antifade Mountant with DAPI (ThermoFisher, Waltham, MA, USA) and mounted onto glass slides. Slides were imaged on a Zeiss AxioObserver microscope with the 20X objective (unless specified otherwise) under uniform settings. 20 serial images were taken per condition and fluorescence was quantified using ImageJ under a uniform threshold range. For each neuronal culture batch, fluorescent intensities of the images from respective control or experimental culture plates were averaged and then normalized to the mean of the control condition to calculate the average fold changes. Statistical significance was assessed via unpaired Student's t-test ($p<0.05$). All treatments were replicated at least three times using separate pregnant dams.

2.3. Nuclear/cytoplasmic fractionation and immunoblotting

Following D1 dopamine receptor stimulation with SKF38393, neurons were fractionated using Pierce™ NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Waltham, MA, USA). The fractioned samples were quantified with the Bradford assay. For immunoblotting, 15 ug of protein for each sample was denatured in Laemmli buffer (Bio-Rad, Hercules, CA, USA), heated at 70ºC for 10 minutes, and then was loaded into a 4-15% mini-PROTEAN TGX gel (Bio-Rad) to be separated by SDS-PAGE. The separated proteins were then transferred to a nitrocellulose membrane, washed with 1X Tris-Buffered saline with 0.1% Tween-20, blocked with 5% BSA, and probed with C-domain or N-domain hnRNP H antibody overnight at 4°C. Fractionation efficiency was assessed through immunologically probing for CREB (a nuclear marker) and HSP90 (a cytoplasmic marker). Membranes were then washed and incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (HRP) and imaged via chemiluminescence photo-detection. Following densitometry analysis (using ImageJ), the quantified values for the hnRNP H bands were normalized to their respective loading control bands (CREB for nucleus fractions and HSP90 for cytoplasmic fractions) to generate final comparable 'arbitrary unit' (AU) protein level values. AU values were averaged and normalized to the control group to calculate fold-change. Two-way ANOVA was used to assess interaction between cellular compartment and treatment.

3. Results

3.1. Increase in hnRNP H nuclear immunofluorescence at the C-terminus but not the N-terminus follwing D1 dopamine receptor activation in primary rat cortical neurons.

We previously reported an increase in nuclear fluorescence of hnRNP H following 2 h of KCl stimulation using ICC with an antibody against the hnRNP H C-domain [21]. We replicated this result following 1.5 h of KCl stimulation via ICC against the hnRNP H C-domain (GY; **Fig. S1A, B**). Strikingly however, KCl stimulation followed by ICC against the hnRNP H N-domain (qRRM 1-2; **Fig. S2A**) revealed no change in nuclear fluorescence of hnRNP H (**Fig. S1C**). Taken together, our results suggest that the differential detection of hnRNP H by these two antibodies

at the C versus N terminus could reflect activity-dependent changes in hnRNP H cellular function.

We next asked whether dopamine receptor activation would induce an increase in hnRNP H fluorescence by treating neurons with either D1 or D2 dopamine receptor agonists. Similar to depolarization with KCl, neurons treated with the D1 dopamine receptor agonist SKF38393 showed an increase in nuclear fluorescence as detected with the C-domain antibody. Furthermore, this increase was reversed by co-treatment with the D1 dopamine receptor antagonist SCH23390 (**Fig. 1B, C**). In contrast, there was no increase in nuclear immunofluorescence with the N-domain antibody following D1 dopamine receptor stimulation (**Fig. 1D**)**.** Neurons treated with the D2 receptor agonist, Quinpirole-HCl, did not show any change in hnRNP H nuclear immunofluorescence with the C-domain antibody (**Fig. S2**), although this null result could potentially be explained by lower expression of D2 versus D1 dopamine receptors. In support, we found that *Drd1* transcript abundance was higher than *Drd2* in these DIV8 primary rat cortical neurons (**Fig. S3A**). D1 dopamine receptor activation did not significantly influence transcription of *Drd1* or *Drd2* (**Fig. S3B**). We also detected D1 dopamine receptor protein (**Fig. S3C**). To determine whether the D1-mediated increase in C-terminal staining of hnRNP H was dependent on the age of the neurons, we also treated DIV16 primary rat cortical neurons with SKF38393 and found that the effect of treatment on C-terminal hnRNP H immunofluorescence to be still significant (**Fig. S4)**.

Our findings were dependent on an hnRNP H antibody that recognizes the epitope at the C-terminus. This epitope is located in a region between residues 400 to 488 of the human hnRNP H1 as indicated by the numbering provided in entry NP_005511.1 (Bethyl Laboratories, Montgomery, TX, USA). To provide further evidence that this region was responsible for the D1-mediated increase in C-terminal staining of hnRNP H, we conducted immunoadsorption with a blocking peptide which binds to the C-terminal end of the hnRNP H protein prior to primary antibody incubation for ICC. As expected, we observed a complete blockade of D1-induced increase in nuclear immunofluorescence of hnRNP H (**Fig. S5**), which is consistent with increased hnRNP H epitope availability following D1 dopamine receptor stimulation.

Using co-ICC of the neuron-specific marker NeuN (e.g. RBFOX3) and hnRNP H, we determined that hnRNP H immunofluorescence was localized to neurons (**Fig. S6A**). In addition to neuronal cells (approximately 60%), we also identified a population of DAPI-stained nuclei in both control and treated (1 uM SKF38393) that did not colocalize with hnRNP H or NeuN (approximately 40%; **Fig. S6B**). NeuN is frequently used as a marker for differentiated, post-mitotic neurons but in contrast to nestin (another neuronal marker), NeuN is not expressed in undifferentiated neuronal precursors [22]. Thus, we are underestimating the total number of neurons which we previously estimated to be approximately 80% using nestin in this neuronal culture system by the Russek lab. No significant difference in the number of NeuN/hnRNP H-positive or non-NeuN staining cells was found between treatments (**Fig. S6B**).

3.2. Changes in Hnrnph1 and Hnrnph2 transcription following D1 dopamine receptor stimulation in primary rat cortical neurons.

D1 receptor activation induced a small yet significant decrease in *Hnrnph1* mRNA levels, and a trend in the same direction with *Hnrnph2* mRNA (**Fig. 2A**). In contrast, KCl treatment had no effect on transcription of either *Hnrnph1* or *Hnrnph2* (**Fig. S7A**). Interestingly, although not detectable via ICC, immunoblot revealed the presence of a small amount of hnRNP H protein in

the cytoplasmic fraction compared to the nuclear fraction ("Cy" vs. "Nu"; **Fig. 2B & Fig. S7B**). Furthermore, there was no significant change in hnRNP H protein in the nucleus or cytoplasm following D1 dopamine receptor stimulation (**Fig. 2B**). Importantly, neither the C- nor the N-domain directed antibody displayed any non-specific binding and banding at baseline or in response to SKF38393 treatment (**Fig. S8A & B**), therefore further supporting specificity of hnRNP H detection. In addition, immunoprecipitation of C-term hnRNP H antibody in primary rat cortical neurons followed by immunoblotting with the N-term hnRNP H antibody demonstrated that the antibodies are capable of recognizing the same protein (**Fig. S8C**).

3.3. Blue native gel analysis of changes in hnRNP H-associated protein complexes following D1 dopamine receptor activation in rat cortical primary neurons

To test whether changes in protein complexes containing hnRNP H were associated with the D1 dopamine receptor-induced C-domain-specific increase in immunofluorescence staining in hnRNP H and thus, whether this could potentially explain the increase in epitope availability, we conducted native gel electrophoresis and immunoblotted with the hnRNP H (C-term) antibody. There were no visible changes in the banding pattern or intensity of the bands that represent hnRNP H complexes (**Fig. S9**). The current results do not support a change in hnRNP H protein complexes in response to D1 receptor activation that could account for the increase in epitope availability at the C-terminus of hnRNP H.

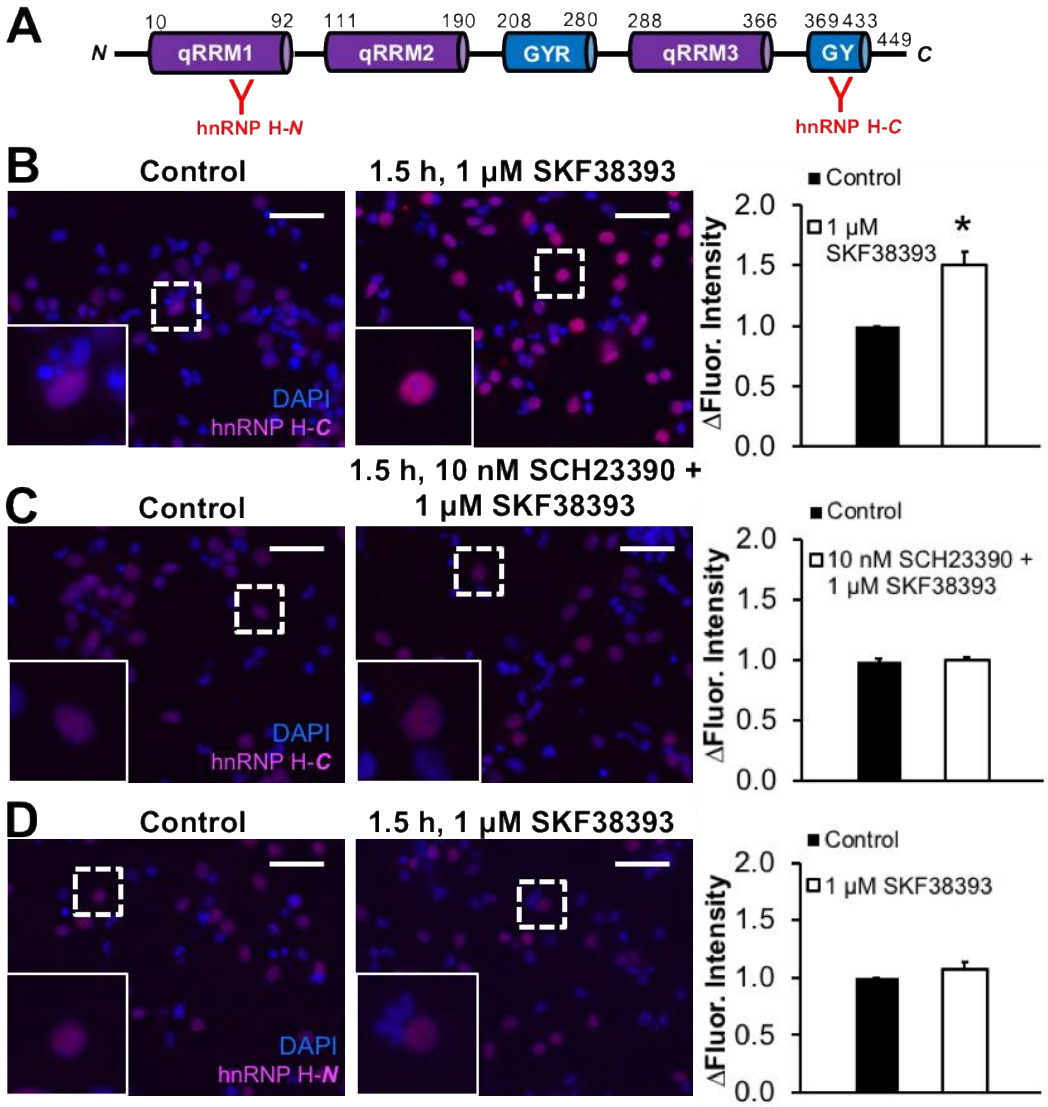

Fig. 1. Increase in hnRNP H nuclear immunofluorescence at the C-terminus but not the N-terminus following D1 dopamine receptor activation. DIV8 primary rat cortical neurons were treated with vehicle (Control), 1 μM of SKF38393 (D1 dopamine receptor agonist) and/or 10 nM of SCH23390 (D1 dopamine receptor antagonist) for 1.5 h. **(A):** Schematic shows hnRNP H and its quasi-RNA recognition motifs (qRRMs) and glycine-rich domains (GYR and GY). The red "Y" markings indicate the relative antibody epitopes in the N-domain or C-domain regions of hnRNP H. **(B,C):** ICC and intensity analysis revealed a significant increase in C-domain staining of nuclear hnRNP H following 1 μM SKF38393 [hnRNP H-C; unpaired Student's t-test, $t_6 = -4.94$, *$p = 0.003$; Control: n = 4, 1 μM SKF38393: n = 4] that was blocked by co-administration of 1 μM SKF38393 and 10 nM SCH23390 [hnRNP H-C; unpaired Student's t-test, $t_7 = -0.34$, $p = 0.74$ Control: n = 4, 1 μM SKF38393+10 nM SCH23390: n = 5]. **(D)** No significant difference was found in N-domain immunoreactivity of nuclear hnRNP H following 1 μM SKF38393 treatment [hnRNP H-N; unpaired Student's t-test, $t_6 = -1.12$ $p = 0.30$; Control: n = 4, 1 μM SKF38393: n = 4]. Scale bars represent 40 μM.

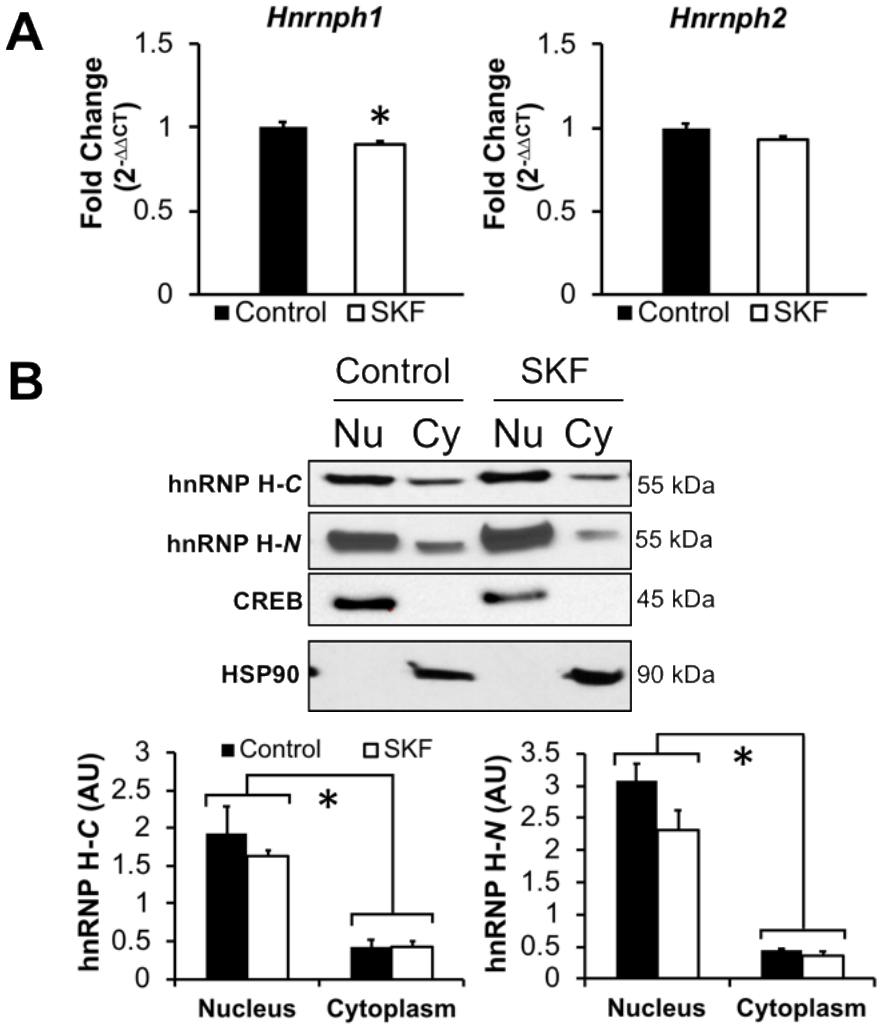

Fig. 2. Changes in *Hnrnph1* and *Hnrnph2* transcript abundance and hnRNP H protein in response to D1 dopamine receptor activation. DIV8 primary rat cortical neurons were treated with vehicle (Control) or 1 μM of SKF38393 (D1 dopamine receptor agonist) for 1.5 h. **(A)** qPCR analysis revealed a significant decrease in *Hnrnph1* mRNA (*top left*) [unpaired Student's t-test, $t_6 = 2.866$, *p = 0.03; Control: n = 4, 1 μM SKF38393: n = 4] and a trend in *Hnrnph2* mRNA reduction (*top right*) [unpaired Student's t-test, $t_6 = 2.432$, p = 0.051; Control: n = 4, 1 μM SKF38393: n = 4]. **(B)** Immunoblot analysis of fractionated samples indicated no significant change in hnRNP H protein levels in the nucleus (Nu) or cytoplasm (Cy), as detected by either C-domain (*left*) or N-domain (*right*) antibodies. Two-way ANOVA with Cellular Compartment and Treatment as factors revealed a significant effect of Compartment [C-domain: $F_{(1,12)} = 51.45$, $p = 1.13 \times 10^{-5}$; N-domain: $F_{(1,12)} = 123.52$, $p = 1.13 \times 10^{-7}$], no significant effect of Treatment [C-domain: $F_{(1,12)} = 1.06$, p = 0.32; N-domain: $F_{(1,12)} = 4.11$, p = 0.07], and no interaction [C-domain: $F_{(1,12)} = 0.26$, p = 0.62; N-domain: $F_{(1,12)} = 2.66$, p = 0.13]. Successful nuclear and cytoplasmic fractionation was confirmed by CREB and HSP90, with CREB expression restricted to the nuclear fractions and HSP90 expression restricted to the cytoplasmic fractions. For either C- and N-domain immunoblot study: Control: n = 4, 1 μM SKF38393: n = 4.

4. Discussion

Neuronal depolarization with KCl ([21] & **Fig. S1**) as well as D1 dopamine receptor activation induced a robust increase in nuclear fluorescence of hnRNP H in primary rat cortical neurons (**Fig. 1**). Previous studies demonstrated that KCl stimulation of neurons induced the translocation and accumulation of RNA-binding proteins (RBPs) such as HuD and hnRNP A2 from the nucleus to dendritic processes [10,23]. RBPs like hnRNP A2 can target and transport mRNAs encoding synaptodendritic proteins, kinases, and cytoskeletal elements to dendritic processes to regulate spine density, morphology, and function [4,8–10]. Although several hnRNPs exhibit activity-dependent neuronal translocation, we did not detect any gross change in hnRNP H localization from the nucleus to the cytoplasm in response to neuronal depolarization or dopamine receptor activation (**Fig. S7 and Fig 2**). Interestingly, we detected a low level of hnRNP H in the cytoplasmic fraction of DIV8 primary rat cortical neurons. Cytoplasmic hnRNP H has previously been reported using myc-tagged hnRNP H in COS7 cells [24]. Here, we have extended this observation to rat cortical neurons. Thus, both nuclear (e.g., splicing) and cytoplasmic functions of hnRNP H (e.g., translational regulation) could contribute to activity-dependent synaptic plasticity.

An important question is whether the SKF38393-mediated increase in nuclear fluorescence of hnRNP H finding is specific to the DIV of primary rat cortical neurons. hnRNP H transcript and protein levels in primary rat cortical neurons have been shown to decrease between DIV 1 through 15, which correlates negatively with neuronal differentiation [25]. The decrease in hnRNP H over time could potentially explain our observation of a less pronounced but still significant effect of SKF38393 treatment on the nuclear fluorescence of hnRNP H in DIV16 neurons (**Fig. S4**). Thus, the nuclear hnRNP H immunofluorescence to SKF38393 and KCl treatments could vary with neuronal age and maturation.

The C-domain of hnRNP H contains glycine-rich domains (GYR and GY; **Fig. 1A**) which mediate protein-protein interactions [7]. Both D1 receptor and KCl stimulation induced an increase in nuclear immunofluorescence of hnRNP H, as detected by an antibody that binds to the GY domain in the C-domain (**Fig. 1B; Fig. S1B**). This increased fluorescence occurred without any change in protein level (**Fig. 2; Fig. S7**). Using BN PAGE, we tested whether a change in hnRNP H-associated protein complexes was associated with the increase in immunofluorescence. We did not observe any visible changes in global hnRNP H-associated protein complexes (**Fig. S9**). It is possible that the denaturing conditions induced by immunoblot transfer could limit our ability to detect changes in hnRNP H protein complexes due to changes in protein conformation. Additionally, the similarly-sized protein complexes that we identified in treated versus untreated neurons could be heterogeneous and differ in weight and composition at a higher resolution of analysis. Future *in vivo* studies with D1 agonists combined with immunoprecipitation under multiple conditions and mass spectrometry will determine whether or not hnRNP H protein complexes change in molecular weight and composition.

It is possible that depolarization or D1 receptor activation disrupts ribonucleoprotein complexes containing hnRNP H, perhaps through a post-translational modification that increases epitope availability in the C-domain. Interestingly, both KCl-mediated neuronal depolarization and D1 receptor stimulation activate adenylate cyclase activity and increase the level of cytoplasmic cyclic adenosine monophosphate (cAMP), activating several kinases, including protein kinase A (PKA) [26,27]. In maintaining energy homeostasis, activation of PKA results in its translocation to the nucleus where it can phosphorylate hnRNP H, leading to increased glucose uptake [28]. There are several predicted phosphorylation sites of hnRNP H [29]. Phosphorylation of hnRNP A1, I, K and L have been shown to alter RBP nucleotide-binding capacity, nucleo-cytoplasmic

shuttling, ribonucleoprotein complexes, and/or alternative splicing [6,30–34]. Notably, modifications such as mutations in the GYR domain nuclear-localization sequence of hnRNP H2 has been hypothesized to cause cytoplasmic retention of this isoform, and perturb neurodevelopment in females [7,35]. Mutations in the glycine-rich or prion domains of other RBPs, such as FUS, TDP-43, hnRNPA1 and A2B1, induced aberrant ribonucleoprotein complexes, contributing to neurodegenerative disease [36]. Future phosphoproteomic studies examining phosphorylation states at the glycine-rich C-terminus of hnRNP H following D1 dopamine receptor activation will identify specific phosphorylation sites that can then be studied experimentally with the development of phospho-specific hnRNP H antibodies. These findings could inform the mechanisms underlying the observed D1-induced increase in C-domain epitope availability and hypothesized changes in protein-protein interactions.

We previously proposed that hnRNP H could regulate mesocorticolimbic dopaminergic neuron development and function [17,21]. Alcohol-binge drinking in mice identified a correlation between alcohol intake and *Hnrnph1*, *Hnrnpm*, and *Hnrnpc* expression in dopaminergic neurons of the ventral tegmental area, implicating a potential role of hnRNP H1 in dopaminergic neuroadaptive changes [37]. In the context of psychostimulant addiction, we recently identified *Hnrnph1* as a quantitative trait gene underlying differential sensitivity to MA [17]. It is unclear whether hnRNP H1 functions at a presynaptic or postsynaptic level to modulate behavioral responses to drugs of abuse. Based on transcriptome analysis of the QTL containing *Hnrnph1*, we suggested a pre-synaptic mechanism based on predicted deficits in the dopaminergic neuron development [17,21]. The cellular observations of the current study suggest the possibility that post-synaptic D1 receptor signaling could initiate acute and chronic downstream cellular adaptations via hnRNP H. Our findings warrant additional proteomic studies to clarify the dynamics of pre-synaptic versus post-synaptic hnRNP H-associated protein complexes *in vivo* during D1 dopamine receptor signaling.

5. Conclusion

D1 dopamine receptor-dependent changes in nuclear immunofluorescence of the C-domain, but not the N-domain, of hnRNP H in the absence of any change in nuclear or cytoplasmic levels of hnRNP H protein. We propose that D1-dependent dopamine signaling could affect the interaction of hnRNP H with other proteins and mRNAs and contribute to long-term neurobehavioral adaptations in response to psychostimulants. Our findings warrant further investigation into potential changes in protein-protein interactions with hnRNP H in response to dopamine receptor stimulation.

Conflict of Interest

The authors have no conflict of interest to declare.

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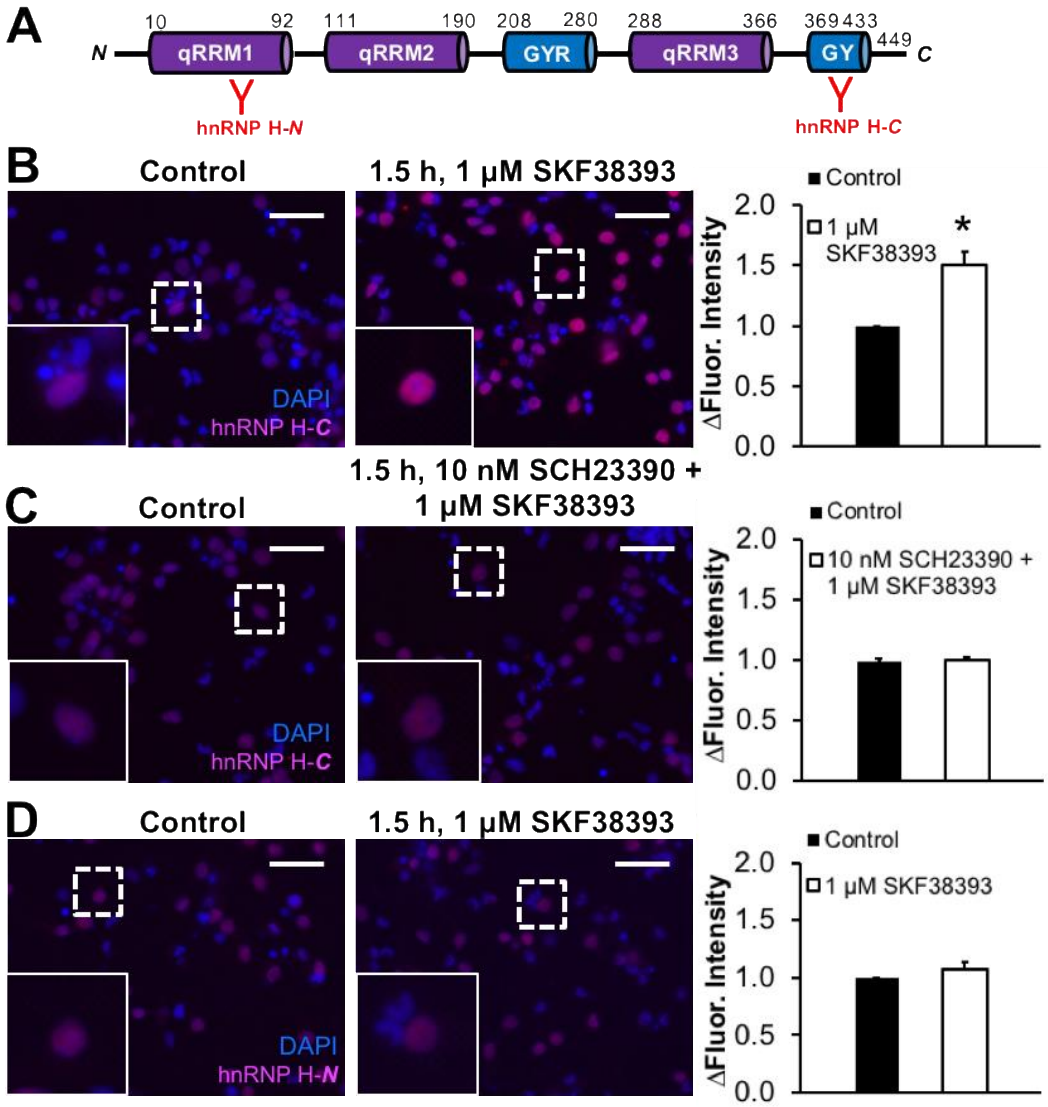

Fig. 1. Increase in hnRNP H nuclear immunofluorescence at the C-terminus but not the N-terminus following D1 dopamine receptor activation. DIV8 primary rat cortical neurons were treated with vehicle (Control), 1 μM of SKF38393 (D1 dopamine receptor agonist) and/or 10 nM of SCH23390 (D1 dopamine receptor antagonist) for 1.5 h. **(A):** Schematic shows hnRNP H and its quasi-RNA recognition motifs (qRRMs) and glycine-rich domains (GYR and GY). The red "Y" markings indicate the relative antibody epitopes in the N-domain or C-domain regions of hnRNP H. **(B,C):** ICC and intensity analysis revealed a significant increase in C-domain staining of nuclear hnRNP H following 1 μM SKF38393 [hnRNP H-C; unpaired Student's t-test, $t_6 = -4.94$, *$p = 0.003$; Control: n = 4, 1 μM SKF38393: n = 4] that was blocked by co-administration of 1 μM SKF38393 and 10 nM SCH23390 [hnRNP H-C; unpaired Student's t-test, $t_7 = -0.34$, $p = 0.74$ Control: n = 4, 1 μM SKF38393+10 nM SCH23390: n = 5]. **(D)** No significant difference was found in N-domain immunoreactivity of nuclear hnRNP H following 1 μM SKF38393 treatment [hnRNP H-N; unpaired Student's t-test, $t_6 = -1.12$ $p = 0.30$; Control: n = 4, 1 μM SKF38393: n = 4]. Scale bars represent 40 μM.

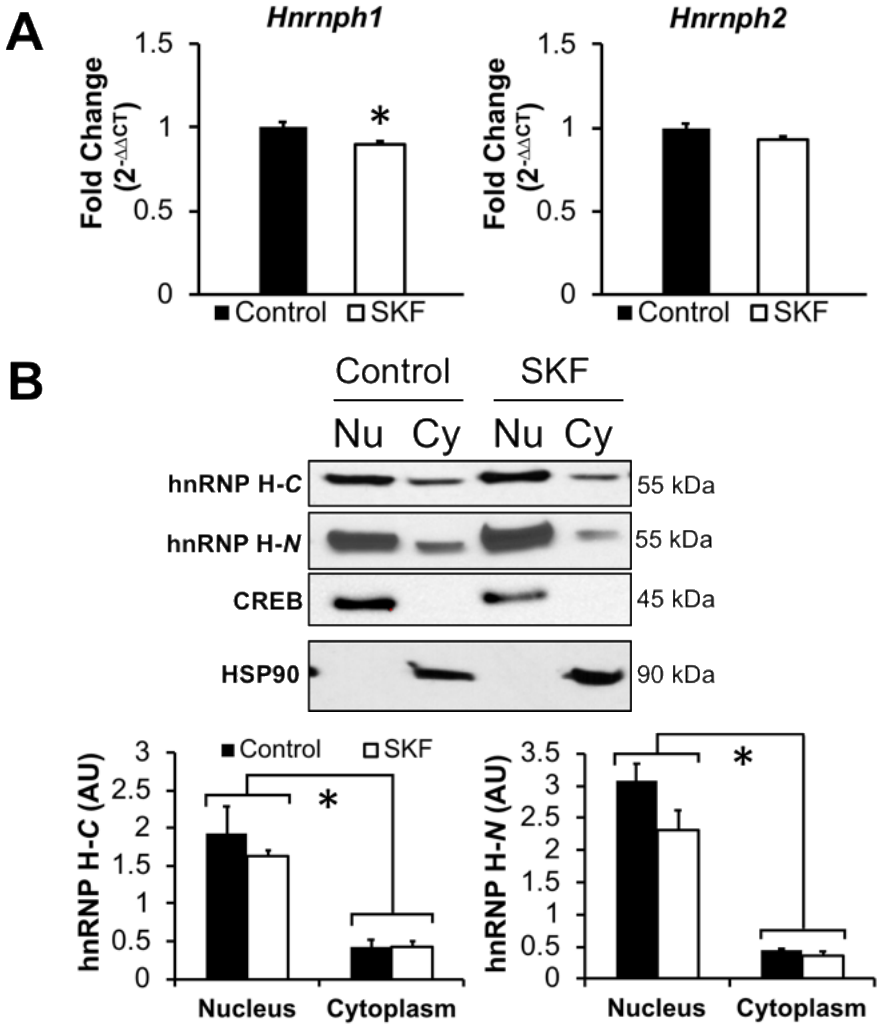

Fig. 2. Changes in *Hnrnph1* and *Hnrnph2* transcript abundance and hnRNP H protein in response to D1 dopamine receptor activation. DIV8 primary rat cortical neurons were treated with vehicle (Control) or 1 μM of SKF38393 (D1 dopamine receptor agonist) for 1.5 h. **(A)** qPCR analysis revealed a significant decrease in *Hnrnph1* mRNA (*top left*) [unpaired Student's t-test, $t_6 = 2.866$, *p = 0.03; Control: n = 4, 1 μM SKF38393: n = 4] and a trend in *Hnrnph2* mRNA reduction (*top right*) [unpaired Student's t-test, $t_6 = 2.432$, p = 0.051; Control: n = 4, 1 μM SKF38393: n = 4]. **(B)** Immunoblot analysis of fractionated samples indicated no significant change in hnRNP H protein levels in the nucleus (Nu) or cytoplasm (Cy), as detected by either C-domain (*left*) or N-domain (*right*) antibodies. Two-way ANOVA with Cellular Compartment and Treatment as factors revealed a significant effect of Compartment [C-domain: $F_{(1,12)} = 51.45$, p = 1.13×10^{-5}; N-domain: $F_{(1,12)} = 123.52$, p = 1.13×10^{-7}], no significant effect of Treatment [C-domain: $F_{(1,12)} = 1.06$, p = 0.32; N-domain: $F_{(1,12)} = 4.11$, p = 0.07], and no interaction [C-domain: $F_{(1,12)} = 0.26$, p = 0.62; N-domain: $F_{(1,12)} = 2.66$, p = 0.13]. Successful nuclear and cytoplasmic fractionation was confirmed by CREB and HSP90, with CREB expression restricted to the nuclear fractions and HSP90 expression restricted to the cytoplasmic fractions. For either C- and N-domain immunoblot study: Control: n = 4, 1 μM SKF38393: n = 4.