Transcriptional dysregulation in striatal projection- and interneurons in a mouse model of Huntington’s disease: neuronal selectivity and potential neuroprotective role of HAP1


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Transcriptional dysregulation has been described as a central mechanism in the pathogenesis of Huntington’s disease (HD), in which medium spiny projection neurons (MSN) selectively degenerate whereas neuronal nitric-oxide-synthase-positive interneurons (nNOS-IN) survive. In order to begin to understand this differential vulnerability we compared mRNA levels of selected genes involved in N-methyl-D-aspartate (NMDA) glutamate receptor and calcium (Ca2+) signaling pathways in MSN and nNOS-IN from 12-week-old R6/2 mice, a transgenic mouse model of HD and wild-type littermates. We undertook a laser capture microdissection (LCM) study to examine the contribution of transcriptional dysregulation in candidate genes involved in these two signaling pathways in discrete populations of striatal neurons. The use of LCM in combination with quantitative real-time polymerase chain reaction (Q-PCR) allowed us to quantify the neuronal abundance of candidate mRNAs. We found different transcriptional alterations in R6/2 neurons for both MSN and nNOS-IN, indicating that global transcriptional dysregulation alone does not account for selective vulnerability. Further, we observed a striking enrichment of several mRNAs in the nNOS-IN population, including that for the NMDA receptor subunit NR2D, the postsynaptic density protein 95 (PSD-95) and the huntingtin-associated protein 1 (HAP1) as well as nitric-oxide-synthase (nNOS) mRNA itself. The higher expression levels of these molecules in nNOS-IN when compared with MSN together with an association of nNOS, NR2D and HAP1 in a protein complex with PSD-95 suggest that these proteins may be involved in protective pathways that contribute to the resistance of this interneuron population to neurodegeneration in HD.

INTRODUCTION

Huntington’s disease (HD) is one of the nine inherited neurodegenerative diseases caused by a CAG/polyglutamine (polyQ) expansion (1). In HD, the mutant huntingtin (mt Htt) is expressed throughout the brain and peripheral organs. In brain, it accumulates forming neuronal intranuclear and cytoplasmic inclusions that are associated with neuronal dysfunction. Postmortem brains show severe atrophy of both the striatum as well as the globus pallidus and cortex (2–4).

The striatum, the primary afferent structure of the basal ganglia, comprises ~90% medium spiny GABAergic projection neurons (MSN) and 10% aspiny interneurons. Four subpopulations of interneurons are well characterized: cholinergic interneurons, GABAergic interneurons expressing parvalbumin or calretinin and interneurons expressing nitric-oxide-synthase (nNOS), somatostatin and neuropeptide Y (NPY) (5–7).

In HD, the MSN degenerate (8); however, sparing of striatal interneurons occurs (9–11). In particular, there is preferential sparing of the small population of interneurons co-expressing nNOS, somatostatin and NPY (12–14). The molecular basis of this preferential neuronal vulnerability is unknown although animal models that recapitulate the neuronal dysfunction of HD are under investigation. One of the most extensively studied
animal models is the R6/2 mouse line developed by Mangiarini et al. (15). It expresses the N-terminal portion of human huntingtin with ~150 CAG repeats, driven by the human huntingtin promoter. This model has a severe disease phenotype including learning and motor deficits and a lifespan limited to 12–15 weeks. Pathologically, the brains of these mice show inclusions from age 3.5 weeks (16). At least at the molecular level, these animals appear to recapitulate the striatal neuronal dysfunction evident in postmortem human HD tissue. For example, a marked reduction in the cellular expression of several neuropeptides and components of neurotransmitter receptor signaling pathways has been reported in human HD brain (17,18) as well as in the R6/2 mice starting from 6 weeks of age and reaching a maximum at age 12 weeks (19–22). However, at the time of our previous studies it was not possible to examine the cell type specificity of these gene expression changes. Now, with the advent of laser capture microdissection (LCM) these cell type specific questions can be readdressed.

In the present study, we have used LCM and quantitative real-time polymerase chain reaction (Q-PCR) to test the hypothesis that cell type specific transcriptional dysregulation underlies the difference in vulnerability between MSN and interneurons (IN) in HD. Therefore, we selected candidate genes based on our previous findings. Further, as N-methyl-D-aspartate (NMDA) receptor activation mimicks HD striatal pathology, in particular the loss of MSN and the preservation of nNOS-IN, and as NMDA receptor subunits may impact on selective neurodegeneration (23–25), we focused our studies on mRNAs of the NMDA receptor family as well as on known candidate genes that are important in the calcium signaling cascade. The functional significance of our Q-PCR findings was then tested further at the protein level in tissue homogenates to examine protein–protein interactions that may contribute to the selective protection of striatal nNOS-IN.

**RESULTS**

**Optimization of Q-PCR for LCM**

To establish conditions for quantification of gene expression levels in neuronal LCM samples by Q-PCR, we first examined samples obtained from coronal brain sections stained with methylene blue, visualizing all neuronal perikarya. We laser-microdissected 4000 methylene blue-stained striatal neurons per animal (Fig. 1A–D). In order to estimate the amount of RNA per laser-microdissected neuron we compared the Q-PCR signal for the 32 kDa dopamine and adenosine 3′,5′-monophosphate-regulated phosphoprotein (DARPP-32), a solely neuronally expressed gene (26), in striatal homogenates and laser-captured neuronal samples. This lead to an estimated cDNA yield of ~1 pg of total RNA per captured neuronal profile. In order to determine the limits of sensitivity of the Q-PCR assay, we studied stepwise dilutions of dissected neuronal RNA samples (200, 100, 50, 25, 12.5 neuronal profiles = ‘neuronal equivalents’). We found that our Q-PCR technique would generate reproducible signals that were proportional to the input using as little as 50 neuronal equivalents. Thus, all subsequent studies were performed with 50 neuronal equivalents of cDNA. All samples were normalized to β-actin mRNA, which was detected in parallel Q-PCR reactions.

Every target and normalizing gene specific primer set was tested on four known concentrations of mouse striatal cDNA (1 ng, 250 pg, 62.5 pg, 15.6 pg) and each assay was found to be linear over this concentration range. The resulting standard curve allowed the calculation of the individual primer efficiency as considered in our data analysis (see Materials and Methods). We also confirmed that the abundance of the target mRNAs in our LCM samples comprising 50 neurons fell into the linear range of the dilution series of known cDNA concentrations.

**Neuronal enrichment of laser-dissected striatal samples**

LCM of our striatal neuron samples involved the dissection of a round profile (diameter 7–9 μm) on the tissue section containing primarily the neuronal perikaryon, but may also

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**Figure 1.** Visualization of MSN and nNOS-IN for LCM. (A–H) Staining of 8 μm coronal cryosections from 12-week-old R6/2 mice and controls. (A–D) Methylene blue stain, neurons chosen for dissection (A), section after dissection (B), harvested neurons attached to a thermoplastic film (C); (E–H), immunofluorescent stain using an nNOS-antibody and a Cy3-conjugated secondary antibody. Three nNOS-IN are chosen for dissection (E), holes in tissue after dissection (F), harvested neurons (G); (D and H), comparison of abundance of MSN and nNOS-IN in tissue section. While 1000 MSN can be harvested from a coronal section of one hemisphere (D), only 30 nNOS-IN can be laser-microdissected (H). Scale bar (A–C; E–G) 20 μm. Scale bar (D, H) 250 μm.
include a small component of closely adherent glial elements. In order to assess the extent to which our LCM technique effectively separated neuronal and glial tissue, we compared levels of neuronal and glial markers in wild-type (WT) homogenate (neurons and glia) with WT LCM samples (neurons). After normalizing the data to neurofilament light chain (NF-L), we found 5.7 times less myelin basic protein (MBP, oligodendrocytic marker) in LCM samples than homogenates (P < 0.005) and 6.0 times less glial fibrillary acidic protein (GFAP, astrocytic marker) in LCM samples than homogenates (P < 0.05). We did the same comparisons for R6/2 transgenic tissue and found a 6.8-fold reduction for MBP (P < 0.0001) and a 10.7-fold reduction for GFAP (P < 0.01) in LCM samples. These data demonstrate a significant enrichment of neuronal elements in our LCM samples compared with tissue homogenates.

Transcriptional alterations in laser-microdissected neurons

Previous studies of striatal tissue homogenate from the R6/2 mouse have determined that the magnitude and number of gene expression changes is maximal around the age of 12 weeks (19–22). We thus chose this time point to ask whether those changes occur in striatal neurons.

We first studied those genes reported to have robust changes in R6/2 striatal homogenates in microarray studies, northern blots and in situ hybridization histochemistry (ISHH): dopamine D1 and D2 receptor, DARPP-32 and preproenkephalin (PPE), an endogenous opioid precursor peptide, all of which are known to be enriched in striato-pallidal or striato-nigral MSN (5,26,27). We also compared hippocalcin mRNA levels, a member of the neuronal calcium sensor family of calcium binding proteins (28). Figure 2 illustrates how the data were analyzed: the graphs show overlaying curves representing PCR products of WT and R6/2 cDNA derived from RNA isolated from laser-dissected neurons. These data demonstrate a significant enrichment of neuronal elements in our LCM samples compared with tissue homogenates.

Transcriptional dysregulation in MSN and nNOS-IN in R6/2 mice

We hypothesized that gene expression changes would differ in the MSN and nNOS-IN populations of striatal neurons, mirroring their selective vulnerability in HD.

Since MSN comprise the predominant cell type in the striatum (90%), the methylene blue-stained neurons primarily represent MSN. We identified nNOS-IN using an nNOS immunohistochemical stain (31) on adjacent sections (Fig. 1E–H), which enabled us to visualize and harvest ~30 nNOS-IN per striatum from a coronal mouse brain section (Fig. 1H).

We began by quantitating levels of nNOS itself. As expected, we found an enrichment of nNOS in nNOS-IN compared with methylene blue-stained neurons in both mouse strains (91-fold in WT and 55-fold in R6/2; Table 2). In R6/2 mice, nNOS mRNA levels were significantly decreased in nNOS-IN (to 31.3% of WT) (Fig. 3A). The nNOS signal
Table 1. mRNA expression changes in laser-dissected striatal neurons

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Q-PCR-WT normalized value</th>
<th>Q-PCR-R6/2 normalized value</th>
<th>P-Value</th>
<th>Q-PCR (%)</th>
<th>ISHH (%)</th>
<th>NB (%)</th>
<th>Microarray (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 dopamine receptor</td>
<td>0.1569 ± 0.0246</td>
<td>0.0865 ± 0.0235</td>
<td>&lt;0.05</td>
<td>55.1</td>
<td>37.7a</td>
<td></td>
<td>38.5b</td>
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<tr>
<td>D2 dopamine receptor</td>
<td>0.0123 ± 0.0009</td>
<td>0.0049 ± 0.0006</td>
<td>&lt;0.001</td>
<td>39.8</td>
<td>39a</td>
<td>40c</td>
<td>24.4e</td>
</tr>
<tr>
<td>DARPP-32</td>
<td>0.0033 ± 0.0009</td>
<td>0.0011 ± 0.0003</td>
<td>&lt;0.002</td>
<td>33.3</td>
<td>33b</td>
<td>40c</td>
<td>24.4e</td>
</tr>
<tr>
<td>Hippocalcin</td>
<td>0.0049 ± 0.0031</td>
<td>0.0019 ± 0.0002</td>
<td>&lt;0.05</td>
<td>38.8</td>
<td>43.6e</td>
<td></td>
<td>24.4e</td>
</tr>
<tr>
<td>PPE</td>
<td>0.0974 ± 0.0416</td>
<td>0.0301 ± 0.0046</td>
<td>&lt;0.01</td>
<td>30.9</td>
<td>21b</td>
<td>30.6e</td>
<td>24.4e</td>
</tr>
<tr>
<td>NF-L</td>
<td>0.2934 ± 0.0440</td>
<td>0.3867 ± 0.0731</td>
<td>n.s.</td>
<td>131.8</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NMDA receptor subunit NR1</td>
<td>0.0124 ± 0.0124</td>
<td>0.0107 ± 0.0008</td>
<td>n.s.</td>
<td>86.3</td>
<td>96b</td>
<td>89b</td>
<td>76.9b</td>
</tr>
<tr>
<td>g2-2-Microglobulin</td>
<td>0.0641 ± 0.0258</td>
<td>0.1145 ± 0.0336</td>
<td>&lt;0.05</td>
<td>178.6</td>
<td>220a</td>
<td></td>
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<tr>
<td>STAT1</td>
<td>0.0000048 ± 0.0000012</td>
<td>0.0000093 ± 0.0000025</td>
<td>&lt;0.02</td>
<td>193.8</td>
<td></td>
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</tbody>
</table>

In the first column, the assessed genes are listed. The following two columns show means of normalized values from R6/2 mice (n = 4) and controls (n = 4) with standard deviations. The next column lists P-values of a two-tailed Student’s t-test. The fifth column expresses the results as a percentage of WT (forming a ratio between the means of the normalized values). The last three columns present data from previously published mRNA expression studies in R6/2 mice using ISHH, northern blot (NB) and microarray. These are compared with the LCM–Q-PCR data.

aCha et al. (20).
bLuthi-Carter et al. (21).
cLuthi-Carter et al. (22).
dLuthi-Carter unpublished data.

Table 2. Relative abundance of mRNAs in nNOS-IN compared with MSN

<table>
<thead>
<tr>
<th>IN/MSN</th>
<th>nNOS</th>
<th>NR2D</th>
<th>PSD-95</th>
<th>CPLX2</th>
<th>IP3R</th>
<th>HAP1</th>
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<tbody>
<tr>
<td>WT</td>
<td>91x</td>
<td>30x</td>
<td>5x</td>
<td>0.4x</td>
<td>11x</td>
<td></td>
</tr>
<tr>
<td>R6/2</td>
<td>55x</td>
<td>28x</td>
<td>7x</td>
<td>3x</td>
<td>6x</td>
<td></td>
</tr>
</tbody>
</table>

P-Value <0.0001 <0.0001 <0.0001 0.0064 <0.0001

Values express enrichment of target genes in nNOS-IN compared with MSN based on normalized values calculated for the two cell types, analyzed as split-plot ANOVA with Dunn–Sidak post hoc correction. The fold enrichment is listed separately for each genotype.

Table 2 shows that the relative abundance of mRNAs in nNOS-IN compared with MSN was measurable in methylene blue-stained neurons was also reduced in R6/2 tissue compared with WT (Fig. 3A). However, the abundance in this population was much lower than in the nNOS-IN pool and may reflect the small proportion of nNOS-IN expected to be present in the methylene blue-stained sample.

Our laboratory has previously demonstrated that NMDA receptor subunits are differentially distributed in striatal neuron subtypes (32–34). In the present study, we focused on the NR2B and NR2D subunits which are particularly abundant in the adult rodent striatum (35,36). Using samples isolated by LCM, we found that NR2B mRNA was expressed in R6/2 MSN and that its expression did not differ significantly from control MSN levels (Fig. 3B). NR2B mRNA could not be quantified by Q-PCR in our assay using mRNA isolated from 50 nNOS-IN. In contrast, NR2D mRNA was highly enriched in nNOS-IN: 30-fold in WT and 28-fold in R6/2 (Table 2). We did not find a significant change in the abundance of NR2D mRNA in either R6/2 MSN or nNOS-IN compared with their WT counterparts (Fig. 3C).

Both nNOS and NR2 subunits are able to bind to the second PDZ-domain of the postsynaptic density protein 95 (PSD-95) (37,38). In WT animals, we found that PSD-95 mRNA levels in nNOS-IN were 10 times higher than in MSN (Table 2). A comparison of PSD-95 mRNA levels revealed that the effect of the transgene depended on the cell type (interaction P = 0.0256). In R6/2 nNOS-IN, there was a 59% decrease in PSD-95 transcript levels (P = 0.0004) whereas levels in MSN of R6/2 mice did not change (Fig. 3D).

We also analyzed mRNAs encoding proteins involved in calcium signaling, as these have been shown previously to be dysregulated in several mouse models of HD (21,39,40). The mRNA encoding the presynaptically enriched protein CPLX2, a binding partner of the SNARE complex of the synaptic vesicle fusion machinery (41), was more abundant in nNOS-IN than in MSN (Table 2). CPLX2 was downregulated to 44.4% of WT levels in R6/2 nNOS-IN (Fig. 3E) without a change in MSN.

The inositol-1,4,5-triphosphate receptor 1 (IP3R), which can release Ca²⁺ from intracellular stores in the endoplasmic reticulum (42), showed higher mRNA levels in MSN than in nNOS-IN. There was no significant change in IP3R mRNA levels in either MSN or nNOS-IN in R6/2 compared with WT animals (Fig. 3F).

Huntingtin-associated protein 1 (HAP1), an interacting partner of IP3R (43), was very abundant in nNOS-IN (11-fold enriched in WT; Table 2), and showed no change either in R6/2 nNOS-IN or MSN compared with control (Fig. 3G).

PSD-95 coimmunoprecipitates with NR2D, nNOS and HAP1

Finally, we investigated whether some of the genes we have identified as being enriched in nNOS-IN were functionally related to each other. We anticipated that such a relationship might elucidate important aspects of their differential vulnerability to toxicity of mt htt.

Whereas the NR1 and NR2A and NR2B subunits are known to coimmunoprecipitate with PSD-95 (38), this had not been shown for NR2D. We chose thalamic rat tissue for this analysis because of its abundance of NR2D protein (44). Deoxycholate extract of the membraneous fraction (P2) was
immunoprecipitated with a monoclonal PSD-95 antibody or non-specific IgG as a negative control. The input and precipitates (pellet) were separated on a polyacrylamide gel and probed for PSD-95, NR2B and NR2D subunits (Fig. 4). As expected, PSD-95 was found in the input (Fig. 4, lane 1) and was enriched in the anti-PSD-95 pellet (Fig. 4, lane 2) but not in the negative control immunoprecipitate (lane 3). As shown previously, NR2B and nNOS were also detected in the PSD-95 pellet (37,38). The NR2D subunit was abundant in the input and could also be detected in the anti-PSD-95 pellet fraction (lane 2). These results demonstrate for the first time that NR2D is associated with PSD-95-containing complexes.

Given that HAP1 mRNA is also enriched in nNOS-IN, we then asked whether HAP1 and its interactor IP3R are present in a complex with PSD-95 as well. Although the low level of IP3R was beyond the level of detection in our experimental paradigm, we found that both isoforms of HAP1 are indeed associated in a complex containing PSD-95 (Fig. 4).

**DISCUSSION**

**Studying mRNA expression by LCM–Q-PCR**

Selective cellular vulnerability is a common feature of most human neurodegenerative diseases, and LCM proves an important new approach for examining cell type specificity of a disease process and elucidating the underlying mechanisms (45). Indeed, our study supports the utility of this technique, demonstrating the ability to enrich for neuronal elements by 6–10-fold compared with homogenates of the same tissue.

The 50 dissected neuronal perikarya used per assay were too few to allow us to measure the RNA concentrations by spectrophotometry. Rather than amplifying the RNA extracted from our dissected neurons [which would have introduced a 3′ bias (46)], we normalized our samples to an internal control, β-actin. We know from previous studies that β-actin mRNA levels do not change in R6/2 compared with WT (19,20) and that β-actin mRNA is abundant in neuronal perikarya.

In this study, we used staining for nNOS as a marker of striatal interneurons. We observed that nNOS mRNA was decreased in R6/2 nNOS-IN compared to WT, but remained 55 times that observed in MSN, and the staining of the nNOS cells remained robust. Although we cannot rule out the possibility that the decrease in nNOS mRNA might lead to a biased selection of interneurons from R6/2 tissue, it seems unlikely that it had an important effect on the isolation of the stained cells.

**Transcriptional alterations in MSN and nNOS-IN**

Transcriptional dysregulation appears to be an important mechanism in the pathogenesis of HD (47,48). Studying mRNA in two distinct populations of striatal neurons revealed subsets of genes downregulated, upregulated or unchanged in R6/2 laser-dissected neuronal cell bodies compared with WT. Our results are strikingly consistent with previous microarray studies of tissue homogenates (Table 1). Although it is likely that glial gene expression changes occur in HD, especially considering that gliosis is evident during the course of the disease (2), our data indicate that many of the
HD related changes in gene expression arise from transcriptional abnormalities in neurons.

Our initial hypothesis that global transcriptional dysregulation determines the vulnerability of MSN versus IN was not confirmed. Clearly, there are transcriptional alterations not only in MSN but also in nNOS-IN. Moreover, we found changes in nNOS-IN in the R6/2 mice compared with control mice, which did not occur in the vulnerable MSN for nNOS, PSD-95 and CPLX2 (Fig. 3).

A downregulation in nNOS mRNA in R6/2 animals is consistent with a previously described decrease in nNOS protein in these mice (49) and also with the reduction of nNOS in human HD striatum (50). Given that the nNOS promoter has several specificity protein (Sp) 1-binding sites (51,52) and that mt htt interferes with Sp1-dependent transcription (53,54), it is possible that mt htt is directly responsible for the decrease in nNOS expression.

R6/2 nNOS-IN revealed a decrease in PSD-95 whereas PSD-95 levels in MSN did not differ between R6/2 and controls (Fig. 3D) demonstrating differential regulation of the PSD-95 gene in these two striatal cell populations. Our laboratory has previously found no change in PSD-95 protein in 12-week-old R6/2 striatal homogenates (48). The inability to detect a change in PSD-95 protein expression in homogenates could reflect a selective change in nNOS-IN that is not measurable in total PSD-95 levels due to dilution of interneuronal protein in the total sample.

In our study of seven selected genes, we were unable to demonstrate that huntingtin-induced transcriptional dysregulation alone accounts for selective vulnerability of neuronal subtypes. This conclusion implies that other factors must contribute to the resistance of interneurons in HD.

Impact of differential expression of NMDA receptor subunits on selective vulnerability

A variety of evidence has implicated NMDA glutamate receptor systems in the pathophysiology of HD. Intrastriatal injection of the NMDA receptor agonist quinolinic acid selectively destroys MSN with sparing of nNOS-IN (23). Striatal neurons in the R6/2 mice are also more sensitive to NMDA receptor activation (55,56).

In contrast, R6/2 mice are resistant to quinolinic acid-induced cell death from 6 weeks of age (57). Mt htt was reported to interfere with the ability of normal htt to interact with PSD-95 and to cause sensitization of NMDA receptors in a cellular model (58). Another mouse model of HD, the YAC72 mice, showed increased sensitivity to quinolinic acid. In order to attenuate the activity of the NMDA receptor, mainly composed of the NR2B subunit in MSN, an NR2B receptor antagonist was able to abolish NMDA-mediated toxicity (59). This is in line with our observation that vulnerable MSN contain NR2B very abundantly whereas NR2B is not detectable in nNOS-IN (Fig. 3B).

Previous studies have shown that in rats, striatal interneurons, and not MSN, express the NR2D subunit (34). Our data confirm that this is also the case in mouse, and demonstrate that this selectivity is not altered in the R6/2 model of the disease state (Fig. 3C).

Recordings of activated NMDA receptors containing the NR2D subunit show low conductance with slow deactivation kinetics which could slow down other synaptic channels (60,61) and thereby attenuate excessive excitability. Thus, the composition of interneuron NMDA receptor complexes may be an important determinant of susceptibility to excitotoxic injury.

Possible neuroprotective roles of HAP1 and PSD-95

HAP1, identified as the first interactor of htt (62), also protects neurons against polyQ toxicity when coexpressed with mt htt (63). One of the possible mechanisms of this protection is HAP1’s maintenance of epidermal growth factor (EGF) signaling (63–65). Thus, high HAP1 levels in nNOS-IN (Fig. 3G), previously described as a colocalization (66), might enhance cell survival and be important for their resistance against neurodegeneration.

HAP1 forms a complex with PSD-95 (Fig. 4), whose mRNA levels were also found highly enriched in nNOS-IN, and both appear to have roles as scaffolding proteins. Spatiotemporal signals mediated by glutamate are controlled by such scaffolding proteins by virtue of PSD-95’s binding to and clustering of NMDA receptors (67,68). Thus, differential expression of HAP1 and PSD-95 in nNOS-IN versus MSN may modulate htt and postsynaptic complexes of NMDA glutamate receptors and be responsible for the relative polyQ insensitivity of NR2D-containing channels in nNOS-IN (Fig. 5).

Besides its role in promoting survival by maintaining EGF signaling and serving as a scaffolding protein, HAP1 has been shown to interact with the IP3R (43). In our experiments, IP3R does not coimmunoprecipitate with PSD-95 (Fig. 4), presumably because the IP3R resides in the membrane of the endoplasmic reticulum and most of the immunoprecipitated PSD-95 is in the postsynaptic density (Fig. 5). Nonetheless, it is also possible that HAP1 protects nNOS-IN from htt toxicity through its ability to regulate IP3R signaling and Ca2+ dynamics.

In conclusion, we show that a discrete set of mRNAs, isolated from two distinct striatal subtypes, undergo different mRNA expression changes in a transgenic mouse model of HD. These results indicate that transcriptional dysregulation occurs in both MSN and nNOS-IN. We also postulate that
selective enrichment of nNOS, NR2D, HAP1 and PSD-95 mRNA, as measured in WT nNOS-IN, and seen in R6/2 nNOS-IN compared with MSN, contributes to the protection of nNOS-IN in HD. In addition, we have provided new evidence that the four corresponding proteins are co-associated in vivo. Our data suggest that prosurvival effects of nitric oxide-, NMDA- and HAP1-mediated signaling cascades may be important mitigators of htt toxicity and provide motivation for future studies to further explore the possible interrelatedness of these three biochemical pathways.

**MATERIALS AND METHODS**

**Animals**

Female R6/2 mice (15) and WT littermates were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All animals were age-matched and sacrificed at 12 weeks. Brains for LCM studies were snap frozen in methylbutane and stored at −80°C. For coimmunoprecipitation experiments, thalamic tissue from adult male Sprague-Dawley rats was utilized.

**Antibodies and reagents**

Rabbit anti-nNOS (Sigma N7155, St Louis, MO, USA); Cy3-conjugated goat anti-rabbit IgG (111-165-144; Jackson ImmunoResearch Laboratories, West Grove, PA, USA); rabbit NR2B (69); rabbit NR2D (44); rabbit IP3R1 (Chemicon AB5882; Chemicon, Temecula, CA, USA); rabbit nNOS (Chemicon AB5380); mouse PSD-95 (Sigma P-246); mouse HAP1 (BD Transduction Laboratories, Chicago, IL, USA); goat anti-mouse and anti-rabbit HRP conjugate IgG (Bio-Rad 170-5047 and 170-5046, Bio-Rad Hercules, CA, USA); non-immune purified rabbit IgG (Sigma).

**LCM**

Coronal 8 μm sections from four R6/2 and four WT mouse brains were cut on a cryostat (Shandon, Cheshire, UK) and thaw mounted on non-coated glass slides (Gold Seal® RITE-ON® micro slides). Sections were stained using the following protocols: (1) methylene blue: 70% ethanol 40 s, 50% ethanol 20 dips, 0.1% methylene blue 40 s, processing through graded ethanol 50/70/95/100% and xylene; (2) nNOS-immunostaining

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Figure 5. Schematic model for selective vulnerability. Striatal MSN and nNOS-IN receive glutamatergic (Glu) input from cortex (CTX), activating NMDA receptors, followed by Ca2+ influx activating the IP3R. nNOS-activation through Ca2+/calmodulin releases nitric oxide (NO*) that can nitrosylate the NMDA receptor and render it less sensitive to activation (75–77). NO* can be scavenged by the superoxide dismutase (SOD) in nNOS-IN (78). NO* easily diffuses to the neighboring MSN where it can react with the superoxide anion (O2•−) and exert toxicity. The high abundance of HAP1 and PSD-95 in nNOS-IN as opposed to MSN might modulate postsynaptic complexes of NMDA glutamate receptors containing NR2D and mediate polyglutamine insensitivity. The open arrows indicate up- or downregulation in R6/2 mice versus WT. The size and color intensity of the molecules express abundance of mRNAs comparing MSN and nNOS-IN.
for interneurons (31) (discussed subsequently). After visualization, striatal neurons were dissected using the PixCell II LCM instrument (Arcturus, Mountain View, CA, USA), diameter 7.5 μm, onto CapSure™ HS caps covered with a thermoplastic film. The harvested cells were solubilized from the film in extraction buffer provided in the Arcturus Pico Pure™ RNA isolation kit for 30 min at 42°C and stored at −80°C.

**Immunohistochemistry**

For visualization of nNOS-IN, coronal sections were stained as follows (31): 70% ethanol 40 s, 50% ethanol 20 dips, 1× phosphate buffer (PB) (p. 7.4) 20 dips, 1× PB 20 dips again, incubation with primary antibody (rabbit anti-nNOS 1:250) for 5 min at room temperature, 1× PB 20 dips twice, incubation with secondary antibody (Cy3™ 3-conjugated IgG, 1:50) for 5 min at room temperature, 1× PB 20 dips twice, processing slides through graded ethanols 50/70/95/100%/xylene.

**RNA extraction and reverse transcription**

Methylene blue- and nNOS-immunostained neurons were harvested for each animal in parallel and underwent RNA extraction according to the Arcturus Pico Pure™ RNA isolation kit instructions including a DNase treatment (Qiagen, Valencia, CA, USA). Samples were eluted in a volume of 30 μl.

Reverse transcription of RNA was conducted with a SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) using random hexamer primers according to manufacturer’s instructions.

**Q-PCR**

Q-PCR studies were performed with a Bio-Rad iCycler by using SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) through 50 PCR cycles (95°C for 30 s, 57°C for 60 s, 72°C for 90 s). Each cDNA sample, equivalent to RNA from 50 laser-dissected neurons, was run in triplicate for the target and the normalizing gene (β-actin) in the same 96-well plate for all eight animals. Specificity of amplicons was determined by melt curve analysis, gel electrophoresis and DNA sequencing. Primer pair sequences were as follows: β-actin (GenBank accession no. X03672) AGGTATCCCTGACCCCTGAAG forward primer and GCTCATTTGAGAAGGTGGTGG reverse primer, complexin 2 (NM_009946) TGGCCTGGAAGAAGAGGAA and CCGGAGGAGGGTTTA, dopamine D1 receptor (XM_283136) GACGGACGACGACGACGAGAG and TCGAGACCCCGTATGGTGAGA and AGGGTCGGGCTCATAGGTG were designed using Oligo 6.7 software (Molecular Biology Insights, Cascade, CO, USA).

**Immunoprecipitation and western blot**

Non-denaturing conditions of protein solubilization. Rat thalami were homogenized with a polystyrene (Brinkmann Instruments, Westbury, NY, USA) in ice-cold Tris–EDTA buffer (10 mM Tris–HCl, 5 mM EDTA, pH 7.4, 320 mM sucrose) and centrifuged at 700g for 10 min at 4°C. The pellet containing mainly nuclei and large debris was discarded (P1). The supernatant was centrifuged at 10 000g at 4°C for 16 min, and the resulting pellet with membrane enriched proteins (P2 fraction) was resuspended in ice-cold Tris–EDTA buffer and sonicated. After addition of one-tenth volume of 10% sodium deoxycholate in 500 mM Tris–HCl, pH 9.0, samples were incubated in a 36°C waterbath for 30 min and another tenth volume of a buffer containing 1% deoxycholate, 1% Triton X-100, 500 mM Tris–HCl, pH 9.0 was added. The samples were dialyzed overnight in a dialysis tubing system (Pierce, Rockford, IL, USA) at 4°C. After centrifugation at 37 000g for 40 min at 4°C, the supernatants were used for immunoprecipitation after determination of protein concentration.

Precoupling antibodies to protein A—Sepharose. Mouse PSD-95 and non-immune rabbit IgG were incubated with protein–Sepharose A beads at a concentration of 20 μg of antibody per 50 μl of hydrated protein A—Sepharose beads with gentle rotation at 4°C overnight in 10 mM sodium borate, pH 8.0. The beads were washed once with sodium borate buffer, followed by three washes in immunoprecipitation buffer (150 mM NaCl, 50 mM Na2SO4, pH 7.2, 1% sodium deoxycholate, 2 mM EDTA, 1% Triton X-100) and used for precipitation.

Immunoprecipitation. Immunoprecipitation was performed as previously described (70). Solubilized protein samples were diluted 20-fold with immunoprecipitation buffer and incubated with the 50 μl of the protein A—Sepharose/antibody-coupled beads for 3 h at 4°C with gentle rotation. After washing with ice-cold immunoprecipitation buffer followed by brief centrifugation, the pellets were boiled in 2×
loading buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 50 mM DTT, 7.5% glycerol) for 10 min with rocking and centrifuged for 5 min at room temperature.

**Gel electrophoresis.** SDS–polyacrylamide gel electrophoresis (PAGE) and transfer of separated proteins to polyvinylidene difluoride were performed. 7.5% polyacrylamide gels were used for protein separation. The concentration of antibodies for immunoblotting was 1–2 μg/ml. Protein concentrations were determined with the Bio-Rad protein assay kit and bands were visualized on film by enhanced chemiluminescence (BioRad Immun-Star HRP Chemiluminescent Kit). Stripping of the blots was performed using the Western Blot Stripping Kit (Pierce).

**Calculations and statistical analysis**

Expression of the mRNA of interest for each sample was calculated for Q-PCR by normalization of $C_t$ values to β-actin using the equation:

$$V = \frac{(1 + E_{\text{reference}})C_{\text{actin}}}{(1 + E_{\text{target}})C_{\text{target}}}$$

in order to correct for potential differences in PCR primer efficiencies (71,72). Here $V$ = relative value of target gene normalized to reference (β-actin), $E$ = primer efficiency and $C_t$ = threshold crossing cycle number. Differences between genotypes for MSN (Table 1) and differences between LCM samples and homogenates were assessed using an unpaired, two-tailed Student’s $t$-test. Data comparing transcript levels in MSN and nNOS-IN from R6/2 and WT mice (Fig. 3; Table 2) were analyzed as a split-plot ANOVA to detect significant effects of genotype, cell type and interactions using the mixed procedure in SAS statistical software version 8.0 (73). A significance level of 0.05 was used for main effects and interactions. The significance level was adjusted for planned post hoc comparisons to reduce type I error probability using the Dunn–Sidak method (74) and determined to be 0.01274.

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