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# HSP70 and constitutively active HSF1 mediates protection against CDCrel-1-mediated toxicity

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## Abstract

Defects in cellular quality control mechanisms are thought to contribute to Parkinson's disease (PD) neuropathology. Overexpressing heat shock proteins (HSP) may be a powerful therapeutic strategy for PD as it boosts the ability of the cell to eliminate unwanted proteins. We investigated the neuroprotective potential of HSP70, HSP40 and H-BH, a constitutively active form of heat shock factor 1, in a rat model of PD based on adeno-associated virus (AAV) vector-mediated overexpression of CDCrel-1, a parkin substrate known to be toxic to dopaminergic neurons. AAV vector-mediated overexpression of H-BH and HSP70 afforded similar levels of protection against CDCrel-1 toxicity, with ~20% improvement in survival of dopaminergic neurons compared to controls, as assessed by tyrosine hydroxylase and HuC/D immunohistochemstry, Fluoro-Gold retrograde tracing and preservation of spontaneous and drug-induced motor function. In contrast, HSP40 overexpression exacerbated CDCrel-1-mediated cell death. Real time RT-PCR analysis showed that H-BH upregulated endogenous HSP70 and HSP40 mRNA levels by 10-fold and 4-fold over basal levels, respectively, whereas AAV vector-mediated HSP70 and HSP40 mRNA levels were over 100-fold higher. Our results suggest that a comparatively modest upregulation of multiple HSPs may be an effective approach for achieving signficant neuroprotection in PD.

## Introduction

An impairment in cellular quality control leading to the build-up of proteins prone to misfolding and aggregation may be the key underlying pathogenic mechanism in both sporadic and familial forms of Parkinson's Disease (PD) [1, 2]. The intracellular accumulation of these proteins ultimately renders the dopamine neurons in the substantia nigra pars compacta (SNc) selectively and progressively vulnerable to cell death. Loss of ~55–65% of dopaminergic neurons in this region and the associated dopamine deficit in the striatum leads to the progressive development of movement abnormalities characteristic of the disease.

A crucial player in cellular quality control is the ubiquitin-proteasome system (UPS) which targets misfolded or mutated proteins for degradation by the 26S proteasome [3]. The

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importance of the UPS in PD is underscored by the finding that several familial forms of PD are associated with mutations in genes that directly or indirectly influence key components of this system including ubiquitin C-terminal hydrolase [4] and parkin, an ubiquitin E3 ligase that is involved in tagging client proteins for degradation [5].

Another group of proteins that closely interact with the UPS are the heat shock proteins (HSPs), a family of multifunctional proteins that participate in the folding of newly synthesized proteins, intracellular protein trafficking and cell stress responses [6, 7]. In the mammalian brain, the predominant HSPs are HSP70 and HSP90. These function in a multiprotein complex and are influenced by a variety of co-chaperones, such as HSP40, CHIP and BAG-1 that determine protein fate [8–10]. HSP70 is found at low levels in the central nervous system (CNS) under normal conditions but is upregulated in response to cell stress [11]. The effects of increased expression of various HSP family members on protein aggregation and neuronal survival in the context of neurological diseases including those associated with mutated polyglutamine expansion [12, 13], amyotrophic lateral sclerosis (ALS) [14, 15] and PD [16, 17] have been well documented, with HSP70 and HSP40 being the most effective HSPs in promoting neuronal survival [18]. While these studies have demonstrated neuroprotection following upregulation of individual HSPs, simultaneous upregulation of multiple HSPs could provide an approach that may lead to an enhanced level of protection. This could be achieved by modulating the function of heat shock factor 1 (HSF1) which is involved in the transcriptional regulation of multiple heat shock protein genes [19]. Human HSF-1 exists as a monomer in unstressed cells. The interaction between three hydrophobic leucine zipper repeats (LZ1-LZ3) within the HSF1 molecule plays an important role in stabilizing the monomer and repressing trimerization [20]. Upon exposure to cell stress, HSF1 is induced and forms homotrimers that translocate to the nucleus to bind to the heat shock element in the promoter of HSP genes to regulate gene transcription. Pharmacological activation of HSF1 delays disease progression in the SOD93A transgenic model of ALS and protects against MPTP-induced toxicity in mice supporting this therapeutic approach [15, 21]. However an alternative genetic approach could involve expressing a mutant form of HSF1 produced by deletion of amino acids 187 to 201 encompassing the LZ2 hydrophobic domain [20, 22]. Deletion of this region allows HSF1 trimerization and constitutive gene transcription to occur in the absence of cell stress [20].

In this study, we have investigated the therapeutic potential of H-BH in a rat PD model based on adeno-associated viral (AAV) vector-mediated overexpression of CDCrel-1 (cell division control related-1; also called septin 5). CDCrel-1 is a parkin substrate that accumulates in the brains of autosomal-recessive juvenile PD patients [23, 24] and AAV-mediated overexpression of CDCrel-1 in the SNc leads to significant dopaminergic cell loss [25] accompanied by motor impairment (manuscript submitted).

## Results

### Transcriptional activity of H-BH *in vitro*

We firstly characterized transcriptional activity of the constitutively active HSF1 construct, H-BH (Fig. 1a), using the reporter construct pHSE-luc. pHSE-luc consists of the firefly luciferase (luc) gene under the control of multiple copies of the HSE consensus sequence, the DNA binding site for HSF1. Basal levels of luciferase expression were low in human embryonic kidney 293 (HEK293) cells transiently transfected with pHSE-luc alone, with exposure to heat-shock leading to a 2-fold induction of luciferase expression (Fig. 1b). HEK293 cells transfected with a plasmid expressing wild-type HSF1 showed higher basal levels of luciferase expression in the absence of cell stress, with exposure to heat-shock only producing a minimal induction in expression. This result is consistent with previous reports showing that HSF1 can be constitutively activated under certain experimental conditions

(e.g. significant overexpression due to transfection with large quantities of DNA or prolonged overexpression) [26]. Cells co-transfected with the H-BH and reporter constructs showed 4-fold higher levels of luciferase activity over the maximal induced levels achievable in HSF1-transfected cells subjected to thermal stress ($p < 0.001$). The transcriptional activity of H-BH was mediated by specific binding to the HSE element of the reporter plasmid, as luciferase expression was abolished in cells co-transfected with H-BH and pTAL-luc, a plasmid identical to pHSE-luc but lacking the HSE sequence.

The functionality of H-BH was further confirmed by determining whether H-BH overexpression could induce the upregulation of endogenous HSP protein levels in HEK293 cells. Indeed, HSP27 and HSP70 protein levels as detected by ELISA methods were increased by 70 ± 8% and 210 ± 5% respectively. These results suggest that H-BH has high levels of constitutive transcriptional activity and is effective in upregulating expression of multiple endogenous HSPs.

To confirm that the LZ2 deletion did not affect the ability of a rabbit anti-HSF1 antibody to detect H-BH transgene expression, we conducted immunocytochemistry on HEK293 cells transfected with either the HSF1, H-BH or dYFP plasmid (Fig 1c). A robust and similar pattern of transgene expression was detected in the HSF1 and H-BH transfected cells compared to the control.

## Expression of AAV-mediated transgenes in the SNc

We next determined whether sustained H-BH overexpression by viral vector-mediated gene transfer would have neuroprotective efficacy in a genetic rat model of PD. We have recently characterized a rat model of PD based on AAV-mediated gene transfer of CDCrel-1 to the dopamine neurons in the SNc (manuscript submitted). CDCrel-1 is a putative substrate of parkin and has been associated with autosomal recessive forms of PD [23, 24]. Consistent with previous reports [25], we found robust expression of CDCrel-1 in dopaminergic neurons by 2 weeks following vector infusion. By 4 weeks, progressive degenerative changes were evident, including the formation of intracellular inclusions, swollen soma and dystrophic neurites in CDCrel-1-overexpressing neurons. Loss of dopaminergic neurons progressively occurs from 4 to 8 weeks to 57 ± 7%, with no further reduction in neuronal numbers for up to 20 weeks, which was associated with impaired performance in motor function tests (manuscript submitted). As HSP70 and HSP40 overexpression has been reported to be neuroprotective in various models of PD [16, 17, 27], we also generated groups of animals overexpressing these individual HSPs to compare the neuroprotective efficacy between the H-BH, HSP70 and HSP40 treatments.

AAV vectors expressing CDCrel-1, H-BH, HSP70, HSP40 along with luciferase and dYFP reporter controls were packaged. As a prelude to our neuroprotection study, we firstly assessed transgene expression patterns at 2 and 8 weeks following infusion of these vector stocks into the rat SNc using immunohistochemical methods. Transgene expression typically peaks 2–3 weeks following vector infusion and persists for up to 1.5 years following a single intracerebral injection in the absence of any overt toxicity or immunological response [28]. Robust neuronal expression of the transgenes was detected in the SNc as early as 2 weeks (Figs. 2a–e) and remained stable for at least 8 weeks after vector infusion (not shown). CDCrel-1, HSP70, HSP40, dYFP (Figs. 2a,c–e) and luciferase protein (not shown) was expressed throughout the cell and filled the cell soma, axons, dendrites and axon terminals, whereas H-BH, consistent with HSF-1's role as a transcription factor, was expressed exclusively in the nucleus as detected with an anti-HSF1 antibody (Fig. 2b). No immunoreactivity for the respective transgenes was observed in the contralateral, uninjected hemisphere (not shown). We next confirmed that the predominant population of transduced cells in the SNc were dopaminergic neurons by conducting double immunohistochemical

labeling of the respective transgenes with the dopaminergic neuron marker tyrosine hydroxylase (TH). At 2 weeks post-vector infusion, 85 ± 9.8% of TH-positive neurons in the SNc were co-localized with transgene-expressing cells in a given section (Figs. 2f–h), consistent with the high degree of tropism for dopaminergic neurons as previously demonstrated for AAV vectors [29, 30].

### H-BH and HSP70 protect against dopaminergic cell loss

To evaluate the neuroprotective efficacy of our treatments, AAV-CDCrel-1 vector was co-infused simultaneously with either H-BH, HSP70, HSP40, luciferase or dYFP vectors unilaterally into the SNc of rats. As the effectiveness of the treatments will be dependent on expression of both transgenes in the same set of neurons, we analyzed the brains from subgroups of animals at 2 weeks to determine the proportion of dopaminergic neurons expressing both CDCrel-1 and the respective therapeutic or control transgenes. Double immunohistochemical labeling was performed using antibodies to detect CDCrel-1 and H-BH, HSP70, HSP40, luciferase or dYFP expression and we found that 74 ± 2.9% of the cells expressing CDCrel-1 co-localized with the respective transgenes at the 2 week time point (Fig. 3; luciferase not shown).

To determine whether HSP treatment could prevent CDCrel-1-mediated neuronal degeneration, further subgroups of rats were generated and the brains analyzed by immunohistochemistry at 8 weeks post-vector injection. CDCrel-1 overexpression caused a clear depletion in the number of dopamine neurons as visualized by TH-immunostaining in the injected SNc relative to the uninjected contralateral side, but the extent of cell loss was variable between the treatments (Figs. 4a–e). Unbiased stereological cell counts showed that 49 ± 4% of the dopaminergic neurons in the CDCrel-1/reporter gene treatment groups (luciferase and dYFP results pooled together) remained after 8 weeks (Fig. 4f). This is consistent with the level of cell loss previously observed at this time-point following overexpression of CDCrel-1 alone, where 57 ± 7% of dopamine neurons remain at 8 weeks (manuscript submitted). In contrast, overexpression of H-BH or HSP70 led to significant protection against CDCrel-1-induced TH-positive cell loss, with approximately 70% of neurons (H-BH: 71 ± 6%; HSP70: 72 ± 8%; ANOVA, $p < 0.02$) surviving the insult. Unexpectedly, HSP40 overexpression appeared to exacerbate CDCrel-1-mediated cell death, with only 25 ± 6% cell survival (ANOVA, $p < 0.02$) compared to the control groups (Fig. 4f). We also confirmed this pattern and extent of cell death by conducting immunohistochemistry against HuC/D, a neuronal marker that is highly expressed in the SNc neurons. More HuC/D immunoreactive neurons were found in the SNc when CDCrel-1 was co-expressed with either H-BH or HSP70, compared to the control vectors (not shown). Furthermore, H-BH and HSP70 overexpression protected against loss of striatal dopaminergic innnervation as assessed by density measurements of TH and dopamine transporter (DAT) immunoreactivity on striatal sections. There was a significant reduction in striatal TH and DAT protein levels in the control and HSP40 treatment groups compared to the H-BH and HSP70 groups (Figs. 4g,h). To confirm that the reduction of TH-positive cell numbers in the SNc of the controls was due to actual loss of neurons as opposed to downregulated expression of the cell markers used, a subgroup of CDCrel-1 and HSP/reporter vector-infused rats received bilateral injections of the retrograde tracer Fluoro-Gold into the striatum 1 week prior to euthanasia at 8 weeks. In line with the TH cell count data, more Fluoro-Gold labelled dopaminergic neurons were observed in the CDCrel-1 group co-expressing either H-BH (51.7 ± 1.4% of the contralateral side) or HSP70 (63 ± 8.6%) (Figs. 5a,b) compared to the controls (30.0 ± 4.4%) (ANOVA $p<0.05$) (Figs. 5d,e) whereas there was a trend towards fewer Fluoro-Gold-labelled cells in the CDCrel-1/HSP40 treatment group (21.9 ± 4.9%) compared to the controls (Fig. 5c).

Similarly, Fluoro-Jade B staining, a marker for degenerating neurons, was observed most intensely in the SNc of the CDCrel-1/dYFP-luciferase treatment groups but was reduced when CDCrel-1 was co-expressed with either H-BH or HSP70 (Figs. 5f,g,i,j). CDCrel-1/HSP40-injected animals appeared to have greater numbers of Fluoro-Jade B-positive cells that based on morphological characteristics, appeared to resemble residual cell debris, thus potentially reflecting an accelerated cell death process (Fig. 5h).

### Endogenous upregulation of HSP70 and HSP40 by H-BH overexpression

Our results suggested that H-BH and HSP70 overexpression was able to confer a similar level of protection of dopaminergic neurons. We firstly determined at the protein level by immunohistochemistry which endogenous HSPs were upregulated following H-BH overexpression and found that HSP27, HSP70 and HSP40 expression were elevated but not HSP90 or the co-chaperone CHIP, when compared to the dYFP vector-injected hemispheres (Figs. 6a–d; HSP27 not shown). We quantified this at the mRNA level using quantitative real-time PCR and found that overexpression of H-BH upregulated endogenous HSP70 by ten-fold and HSP40 by two-fold over basal levels in the SN (Fig. 6e,f). In comparison, AAV-mediated expression of HSP70 and HSP40 resulted in mRNA levels 300 and 100-fold higher than basal level, respectively. These results suggest that a comparatively modest and physiological upregulation of multiple HSPs (via AAV-mediated H-BH expression) is sufficient in providing a similar neuroprotective benefit as that of strong overexpression of HSP70 alone.

### Prevention of motor function impairment in the H-BH and HSP70 treatment animals

Unilateral dopamine neuron loss and depletion of striatal dopamine levels to 50–60% of basal levels result in impairments in contralateral limb use that can be readily assessed by drug-induced and spontaneous behavioral tests. To determine whether HSP overexpression could prevent impaired motor function, amphetamine-induced rotation testing and the forepaw adjusting steps test were conducted prior to vector infusion in naïve rats and at 8 weeks post-vector infusion. There was no significant difference in amphetamine-induced rotational bias in the CDCrel-1/H-BH and CDCrel-1/HSP70 treatment groups at 8 weeks compared to baseline levels (Fig. 7a). In contrast, the number of rotations was significantly increased in the CDCrel-1/HSP40 ($p < 0.05$) and in the CDCrel-1/reporter vector-injected animals ($p < 0.01$) at 8 weeks when compared to pre-surgery values, suggestive that TH-positive nigral cell loss had exceeded a critical threshold of ~50%.

The maintenance of motor function of the H-BH and HSP70-treated animals was further investigated by assessing spontaneous motor behaviors in the forepaw adjusting steps test. No significant difference in baseline forepaw usage was observed prior to the vector infusion (Fig. 7b). However by 8 weeks there was a significant impairment in contralateral forepaw usage in the forehand direction in the animals co-injected with CDCrel-1/reporter gene vectors (ANOVA, $p < 0.001$). In contrast, H-BH and HSP70-treated animals showed no impairment in contralateral forepaw movement compared to the CDCrel-1/reporter vector groups (ANOVA, $p < 0.001$; Fig. 7c). Forepaw usage was impaired to a similar level in the HSP40 treatment group when compared to the control reporter injection group.

## Discussion

We have investigated the effects of overexpressing H-BH, a constitutively active form of HSF1, and HSP70 or HSP40 as a strategy to protect against CDCrel-1-induced toxicity in the SNc. Our results show that H-BH and HSP70 afforded a similar degree of neuronal protection against CDCrel-1-mediated toxicity in dopaminergic neurons and to our

knowledge, this is the first demonstration that direct overexpression of H-BH is neuroprotective in a PD model.

Consistent with our previous findings, AAV-mediated CDCrel-1 overexpression led to a progressive and rapid decline in numbers of nigral dopaminergic neurons by 8 weeks post-vector injection. CDCrel-1 is a putative substrate of parkin, which ubiquitinates and targets CDCrel-1 for degradation through the ubiquitin-proteasome pathway [31]. The intracellular accumulation and failure to degrade CDCrel-1 could decrease exocytosis of several neurotransmitters, including dopamine [25], and consequently chronic inhibition of dopamine release may cause dopamine-mediated neuronal death [32]. Furthermore, CDCrel-1 overexpression may overload parkin activity, which may result in accumulation of other parkin substrates leading to neurodegeneration [25, 31, 32].

Various HSPs have previously been found to show protection in several neurodegenerative disease models [16, 27, 34, 35]. Chemical agents that increase HSF1 activity, such as celastrol protect against MPTP-induced toxicity in mice [21] and similarly, arimoclomol is neuroprotective in a transgenic model of ALS [15]. We have investigated an alternative approach to increasing HSF1 activity by overexpressing H-BH, which upregulated various HSPs, including HSP27, HSP40 and HSP70, and compared the protective effects of H-BH against overexpressing HSP70 or HSP40 alone. Co-injection of AAV-CDCrel-1 with AAV-HSP70 led to significant protection against dopaminergic cell loss and motor deficits compared to controls. Despite AAV-H-BH inducing significantly lower levels of HSP70 expression than that produced by AAV-HSP70, the degree of protection against CDCrel-1-mediated cell death was similar with both H-BH and HSP70 vectors. Previous studies in ALS cell culture models have shown that overexpression of multiple HSPs have greater protective effect than overexpressing a single HSP [36, 37]. While specific HSPs are protective in many neurodegenerative diseases, it is possible that H-BH-mediated induction of multiple HSPs at lower levels may provide a similar or greater level of protection.

The neuroprotective effects of HSP70 demonstrated in our study are consistent with that found in previous studies. Co-expression of HSP70 with wild-type or mutant human α-syn in *Drosophila* models of PD can completely ameliorate dopaminergic cell loss [16]. AAV-mediated overexpression of HSP70 reduced MPTP toxicity in the SN and the striatum [27]. Elevating HSP70 levels by chemical stressors, such as with geldanamycin, also protects against MPTP toxicity in mice [38] and α-syn toxicity in *Drosophila* [39].

HSF1 has also been found to have some protective effects against neurodegenerative disorders. HSF1 is the main transcription factor in the mammalian system that induces HSPs in response to cellular stress [40, 41]. In a *Drosophila* α-syn model, abolishing HSF activity decreased induction of HSP70 and geldanamycin was no longer able to protect against α-syn toxicity [39].

Interestingly, we found that HSP40 overexpression potentiated CDCrel-1-mediated toxicity, resulting in a greater extent of cell loss in the SNc and as a consequence, more pronounced motor impairment when compared with the CDCrel-1/reporter gene-treated rats. This is consistent with findings that HSP70 appears to be the predominant molecular chaperone involved in mediating cell protection, while HSP40 overexpression alone increased the sensitivity of cultured hamster fibroblasts to heat shock, or it had no effect on providing protection against a truncated androgen receptor with expanded polyglutamine tract in cultured neurons [42, 43]. Elevated levels of HSP40 could bind and trap misfolded proteins and prevent them from binding to endogenous HSP70, which could lead to additional toxicity [42]. Similarly, although HSP40 enhances the ATPase activity of HSP70 leading to more stable binding to its substrates [44], high (imbalanced) ratios of HSP40/HSP70 might

disturb the normal HSP70-HSP40 bound state as well as prevent other co-chaperones from binding to the HSP70-HSP40 complex [42, 45]. However, HSP40 alone did provide significant protection against toxicity produced by a truncated polyglutamine-expanded huntingtin in a neuronal cell line [46].

In summary, our study has shown that AAV-mediated overexpression of H-BH and HSP70 lead to significant protection against CDC-re1-1-mediated toxicity and thus may be useful as a therapeutic strategy for PD.

## Materials and Methods

### Plasmid construction

The human CDCrel-1 sequence was PCR amplified from an image clone (Image Clone CDCrel-1: accession number BC025261; Invitrogen, Carlsbad, CA) with specific CDCrel-1 primers which contained C-terminal hemaglutinin (HA) tag. The HSF1 open reading frame was PCR amplified from human brain cDNA using appropriate primers and cloned into pBSIISK- plasmid. To make H-BH, pBSIISK- was digested with BamHI (blunted with Mung Bean Nuclease) and HincII then religated, as previously described in [20]. Following sequence verification, the CDCrel-1 and H-BH PCR products were subcloned into an AAV backbone containing the 1.1 kb CMV enhancer/chicken β-actin (CBA) promoter, the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), and the bovine growth hormone polyA signal flanked by AAV2 inverted terminal repeats. Human HSP70 and HSP40 (Hdj-1 isoform) cDNA (generously provided by C O'Brien) were also subcloned into the AAV/CBA-WPRE-bGHpA vector. Luciferase or destabilized YFP (dYFP) were cloned into the same AAV/CBA-WPRE-bGHpA vector backbones and were used as control vectors.

### Generation of AAV virus

High-titer AAV serotype AAV1 and chimeric AAV1/2 (virons containing a 1:1 mixture of AAV1 and AAV2 capsid proteins) vectors were generated for the study. AAV1 serotype vectors were packaged by calcium phosphate transfection into HEK293 with AAV1 helper plasmid (pNLrep, with AAV2 rep and AAV1 cap genes), the adenovirus helper plasmid (pFΔ6) and either AAV/CBA-luciferase-WPRE-bGHpA or AAV/CBA-H-BH-WPRE-bGHpA. Seventy-two hours post-transfection, cells were harvested and purified on an iodixanol gradient, concentrated and dialyzed against 1 x PBS and sterilized through a 0.2 μm filter. To generate AAV1/2 vectors HEK293 cells were transfected with the AAV1 (pH21) and AAV2 (pNLrep) helper plasmids, pFΔ6 and either AAV/CBA-HSP70-WPRE-bGHpA, AAV/CBA-HSP40-WPRE-bGHpA or AAV/CBA-dYFP-WPRE-bGHpA by calcium phosphate transfection. Sixty hours after transfection, cells were harvested and purified using heparin column purification [30]. Genomic titers of vectors were determined using the Applied Biosystem 7700 real time PCR cycler (Applied Biosystems, Foster City, CA) with primers designed to WPRE.

### AAV vector administration

Male Wistar rats (250–300 g) were used and experiments were conducted in accordance with animal ethics guidelines of the University of Auckland. Three μl of AAV1/2-CDCrel-1 ($6 \times 10^{11}$ genomes/ml), and 2μl of either AAV1-H-BH ($2 \times 10^{11}$), AAV1/2-HSP70 ($2 \times 10^{11}$), AAV1/2-HSP40 ($4 \times 10^{11}$), AAV1-luciferase ($2 \times 10^{11}$) or AAV1/2-dYFP ($9 \times 10^{11}$) were mixed and stereotactically injected unilaterally into the SNc using the following coordinates from bregma: anterior-posterior (AP) −5.1 mm, medial-lateral (ML) +2.0 mm and dorsal-ventral (DV) +8.5 mm.

## Behavioral tests

Behavioral tests were conducted prior to and at 8 weeks after surgery for all animals (n=8 for each vector at each time point). All tests were conducted by an investigator blinded to the treatments. Stress to the animals was minimized by daily handling for 1 week prior to testing.

**Amphetamine-induced rotational testing—**Amphetamine-induced rotations were performed with an automated counting system, Rota Count v2.03 (Columbus Instruments, Columbus, OH,). Rats were injected with amphetamine (3 mg/kg, i.p.; dexamphetamine sulphate, PSM Healthcare, Auckland, NZ). Thirty min later, full body turns were recorded for 60 min. Data was expressed as net ipsilateral rotations by subtracting the total number of contralateral from the total number of ipsilateral turns.

**Forepaw adjusting steps test—**While the rat's hindlimbs and one forelimb were lightly restrained by the investigator, the rat was moved laterally through a distance of 90 cm over 10 sec in both the forehand and backhand directions, with the unrestrained forelimb remaining in contact with the surface of the bench. The number of adjusting movements the animal made with the paw was recorded for both paws. The test was repeated six times on each animal and data averaged. Forehand and backhand steps were treated as separate data sets.

## Quantitative polymerase chain reaction

Real-time quantitative RT-PCR was used to measure H-BH, HSP70 and HSP40 mRNA levels. AAV-H-BH, AAV-HSP70, AAV-HSP40 vectors were injected into the SNc (n=4 per vector) and at 4 weeks postinjection, the SN was dissected out for RNA preparation. RNA was isolated using the RNeasy Midi kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Primer design, DNase treatment, cDNA synthesis and SYBR Green I RT-PCR were carried out as described previously [47]. In brief, all primers were designed with PrimerExpress 1.0 software (Applied Biosystems) to maximize amplification efficiency while minimizing non-specific amplification. 500 ng of each RNA sample was treated with the RNase-Free DNase (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized using random hexamers and Superscript II reverse transcriptase (Invitrogen) according to manufacture's instructions. RT-PCR amplification was conducted with 10 ng template cDNA, 2 x SYBR Green I Master Mix buffer (Applied Biosystem) and 3 μM forward and reverse primers. RT-PCR reactions were run on an Applied Biosystem 7700 real time PCR cycler (Applied Biosystems), which measured gene expression levels of H-BH, HSP70, HSP40 as well as housekeeping genes (18sRNA, RPLPO, PPIA, GAPDH). Expression levels of the transgenes were normalized against the most stable housekeeping genes determined by geNorm VBA (version 3.4) application (PrimerDesign, Southampton, UK).

## Immunohistochemistry

Animals were euthanized at 2 and 8 weeks after vector infusion (n=8 for each vector per time point) and perfused transcardially with saline (0.9% (w/v) NaCl) followed by 10% (w/v) buffered neutral formalin (Sigma, St. Louis, MO). Brains were cryoprotected in 30% sucrose in phosphate-buffered saline before cryosectioning. Immunohistochemistry was carried out on 40 μm coronal sections as described previously [48]. Transgene expression was assessed using the respective primary antibodies; monoclonal mouse anti-HA (1:3000; Covance, Princeton, NJ) and monoclonal rabbit anti-HA (1:2000; Abcam, Cambridgeshire, UK) to detect CDCrel-1 expression, rabbit anti-HSF1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) to detect H-BH, mouse anti-HSP70 (1:1000, Stressgen), rabbit anti-HSP40

(1:1000, Stressgen), rabbit anti-HSP27 (1:1000, Santa Cruz Biotechnology), rabbit anti-luciferase (1:1000, Cortex Biochem, San Leandro, CA), and rabbit anti-GFP (1:10000; Abcam) were also used. Anti-rabbit tyrosine hydroxylase (TH; 1:2500; Chemicon), anti-mouse HuC/D (1:500; Molecular Probes, Eugene, OR), rat anti-DAT (1:2500, Chemicon) immunohistochemistry was conducted to assess dopamine neuron viability. Following an overnight incubation with primary antibodies, sections were incubated with the appropriate biotinylated secondary antibodies (Sigma; 1:250). After the secondary antibody incubation, sections were treated with ExtrAvidin peroxidase (Sigma; 1:250) and immunoreactivity visualized with diaminobenzidine. Negative controls were included where sections were treated in the same manner except that the primary antibody was omitted.

For double-labelling, representative brain sections covering the region with the most transgene staining were selected from each treatment group. The HA antibody was incubated concurrently with HSF1, HSP70, HSP40, luciferase antibodies or dYFP fluorescence to examine the degree of co-localization of CDCrel-1 with the treatment transgenes. Antibodies to the transgenes were also incubated with the TH antibody to detect the degree of transduction of dopaminergic cells. After primary antibody incubation, appropriate secondary fluorescent antibodies (1:250; Alexa 488 and Alexa 594, Molecular Probes) were applied. Immunostaining was visualized using a Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) and individual counts of TH or CDCrel-1 or HSP-positive cells and the total numbers of co-localised cells in (n=6 sections) were quantified.

### Quantification of TH-positive cell numbers in the SN

For each animal, ten sections throughout the rostral-caudal extent of the SN (every $4^{th}$ 40 μm section) were selected for TH immunohistochemistry and TH-immunoreactive cells were quantified by unbiased stereological cell counting techniques using the optical fractionator method on a Stereo Investigator system (MicroBrightField, Williston, VT). Cell counts were performed under a 40 x objective lens by an observer blinded to the treatment groups.

### Statistical analysis

The stereological cell count data and behavioral data were analyzed by Student’s t-test and one-way ANOVA with post-hoc Tukey and Scheffé test with significance level set at $P < 0.05$.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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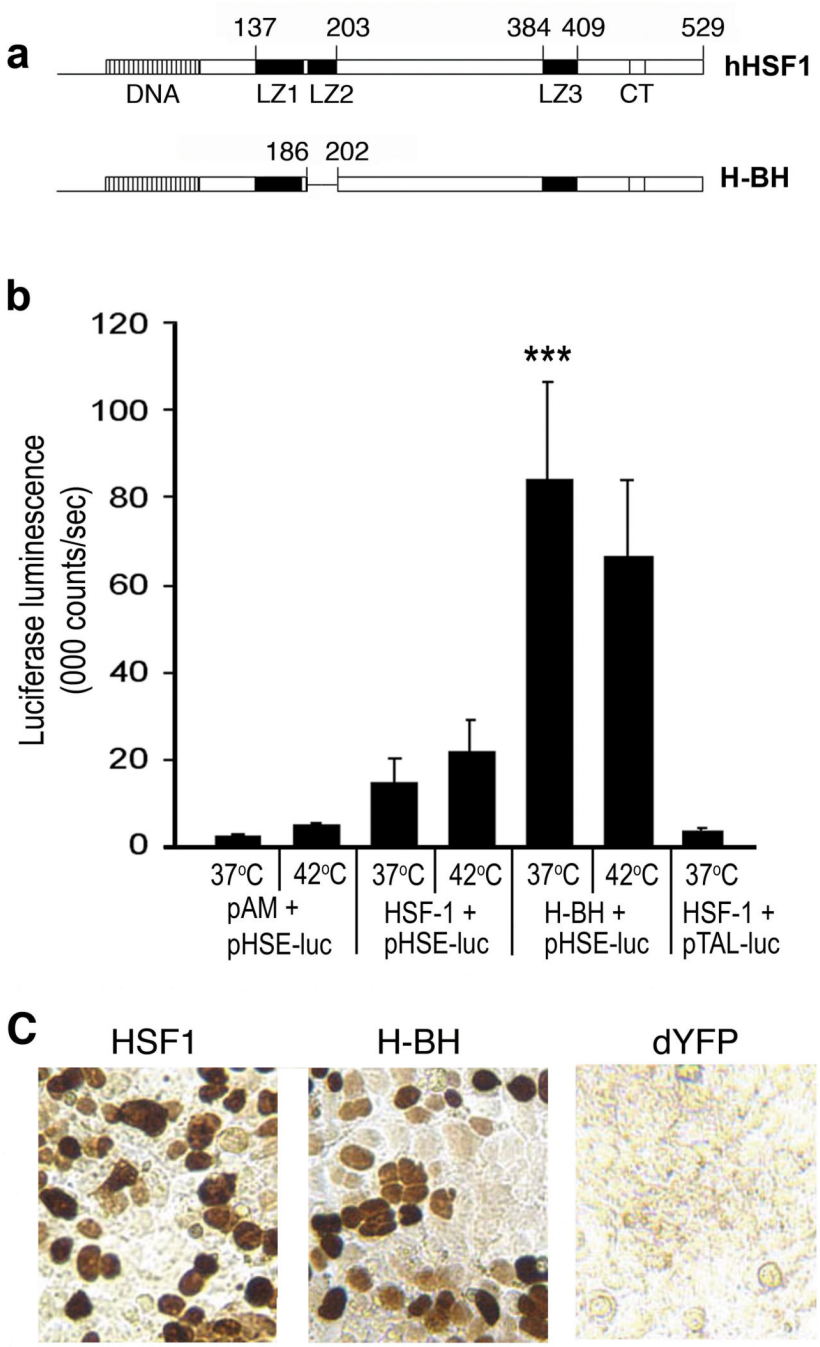


**Figure 1. Transcriptional activity of HSF1 and H-BH**

(**a**) Schematic diagram of human HSF1 (hHSF1) and H-BH created by deletion of residues 187–201 within the LZ2 region. Numbers indicate amino acid positions. DNA, DNA-binding domain; LZ, leucine zipper repeat; CT, carboxy-terminal mammalian homology region. (**b**) HEK293 cells were co-transfected with pHSE-luc in the presence of plasmids expressing HSF1, H-BH or the parent plasmid pAM and were maintained at 37°C. Cells co-transfected with the pTAL-luc plasmid that lacks the HSE sequence was included as a negative control. Cells were exposed to heat shock for 1 hour at 42°C and allowed 3 hours of recovery at 37°C prior to luciferase assay. (ANOVA, *** $p<0.001$). Each bar represents

the mean ± S.E.M for n = 6. (**c**) Immunocytochemistry using the rabbit anti-HSF1 antibody showed specific detection of both HSF1 and H-BH transgenes in HEK-293 cells transfected with the HSF1 and HBH plasmids compared to a control plasmid expressing dYFP.

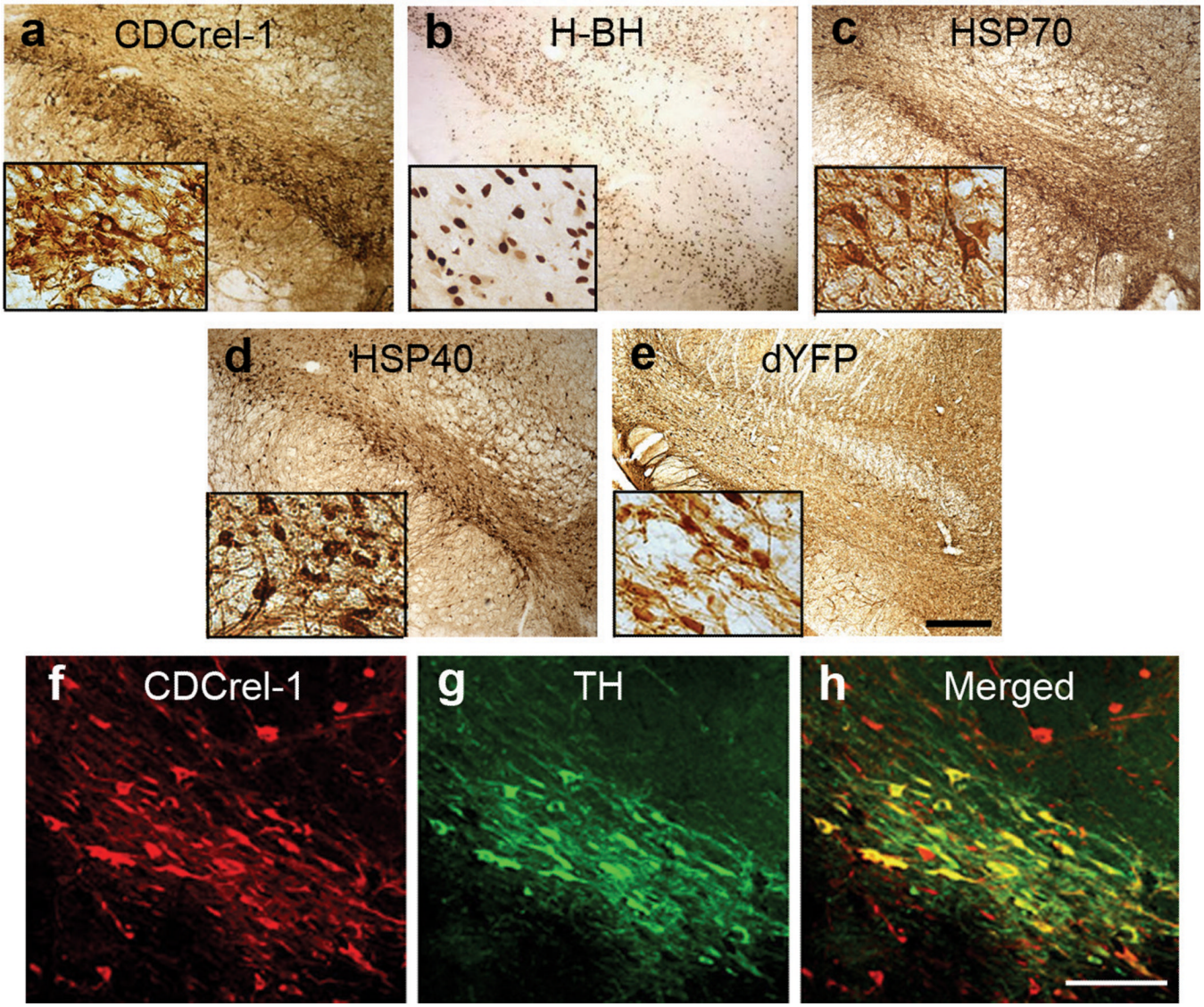


**Figure 2. Robust transgene expression in the SNc following AAV vector-mediated gene transfer**

(**a–e**) Robust expression of transgenes as assessed by immunohistochemistry at 2 weeks post-vector infusion in the SNc. Inner panels: magnified images. (**f–h**) Double immunohistochemical labeling of CDCrel-1 and tyrosine hydroxylase (TH). Co-localized cells are shown in yellow. Scale bars = 250 μm (a–e); 400 μm (f–h).

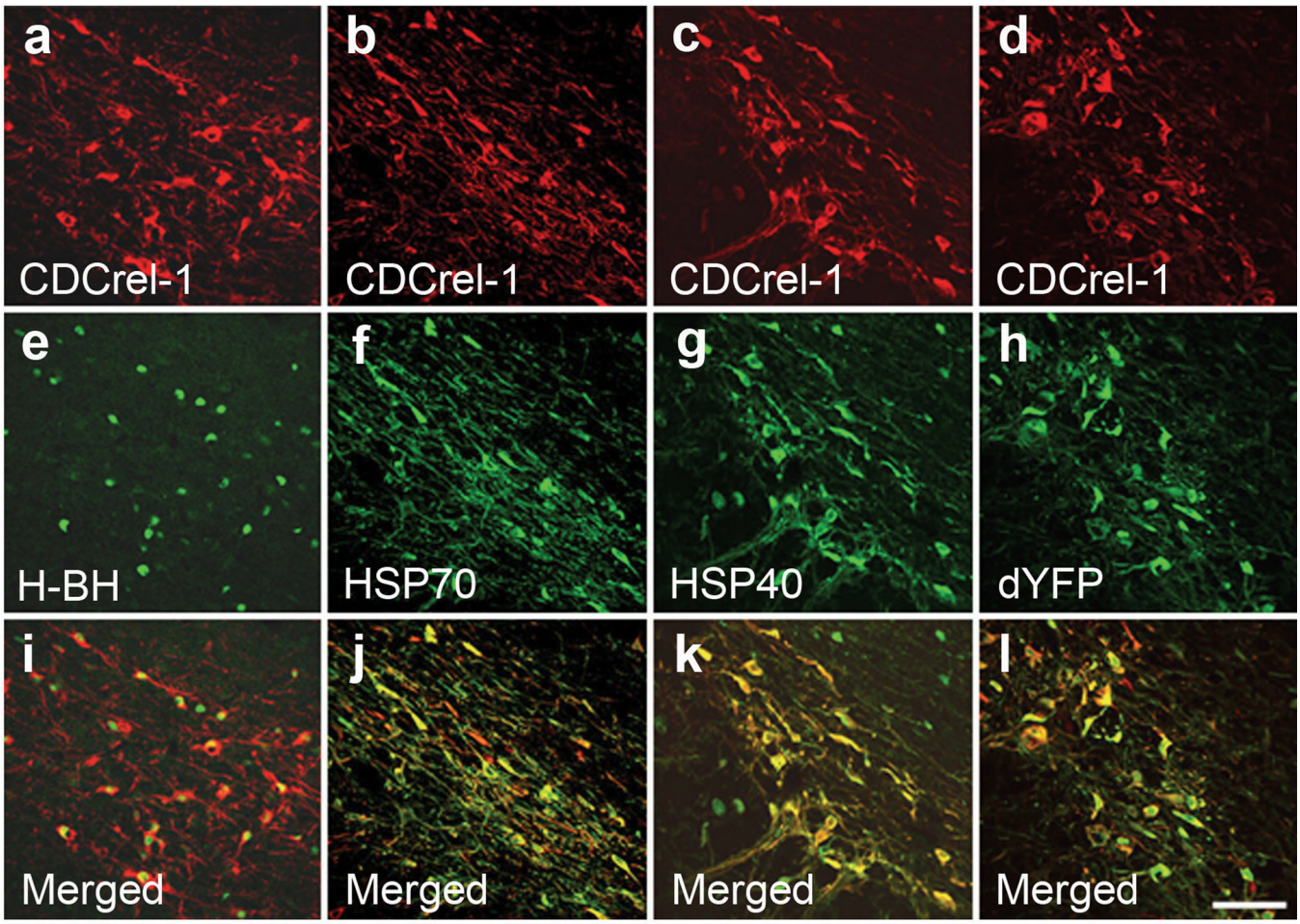


**Figure 3. Double immunohistochemical labeling to show co-localization of transgenes**

High number of (**e**) H-BH, (**f**) HSP70, (**g**) HSP40, and (**h**) dYFP transduced cells co-localized with cells overexpressing (**a–d**) CDCrel-1 in the SNc. (**i–l**) Co-localization of the transgenes are shown in yellow. Scale bar = 400 μm.

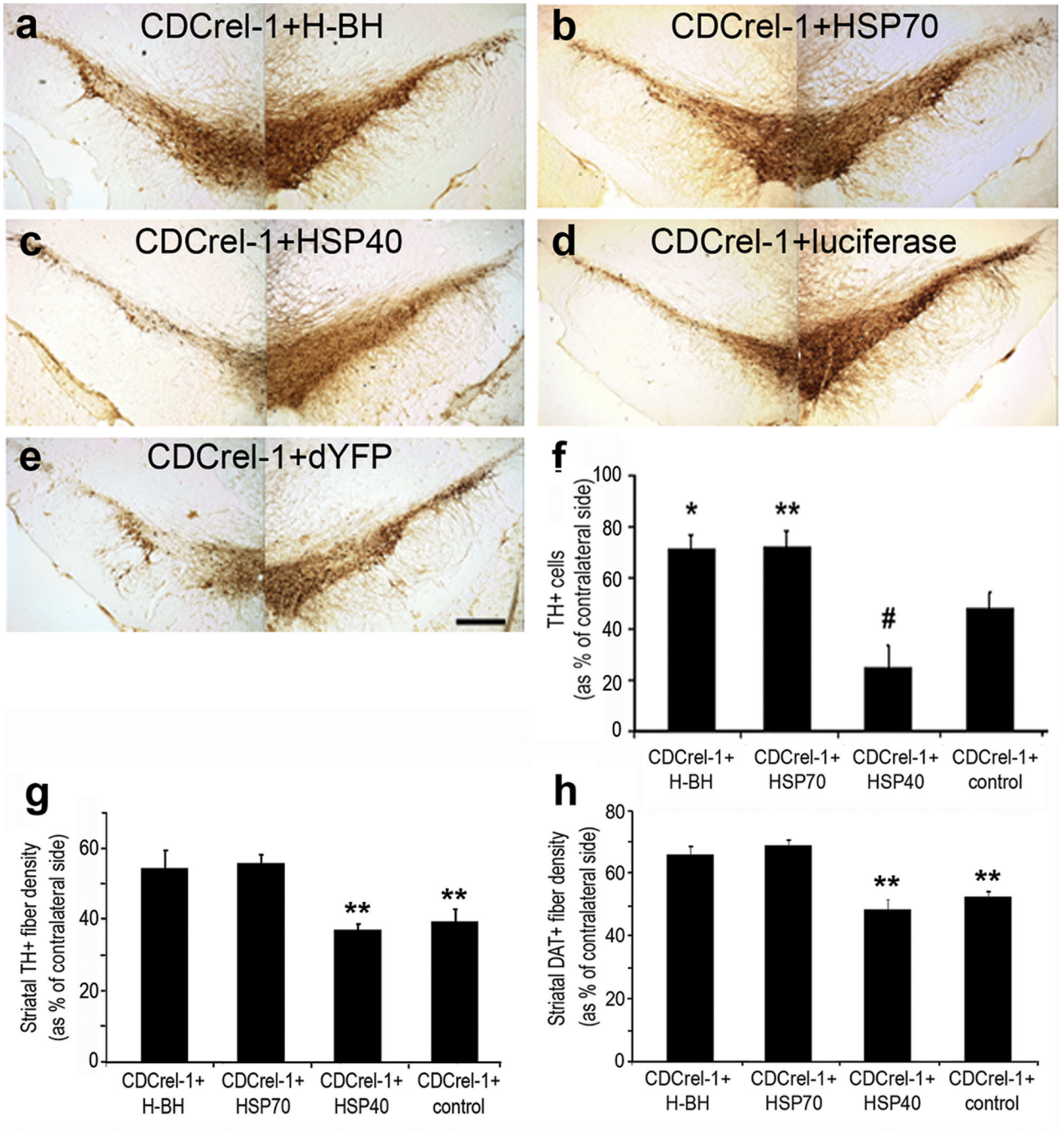


**Figure 4. H-BH and HSP70 protects TH cells against CDCrel-1 overexpression**

Dopamine neuron numbers in the SN in rats co-injected with CDCrel-1 and (**a**) H-BH, (**b**) HSP70, (**c**) HSP40, (**d**) luciferase or (**e**) dYFP. (**f**) Unbiased stereological cell counts in the SN expressed as a percentage of TH-positive cell numbers in the ipsilateral compared to the contralateral hemispheres. Striatal TH (**g**) and DAT (**h**) fiber density at 8 weeks expressed as a percentage of the contralateral hemisphere. Scale bar = 250 μm. ANOVA, *$p = 0.02$, # $p = 0.015$ and ** $p < 0.01$. Each bar represents mean +/− SEM, n=8.

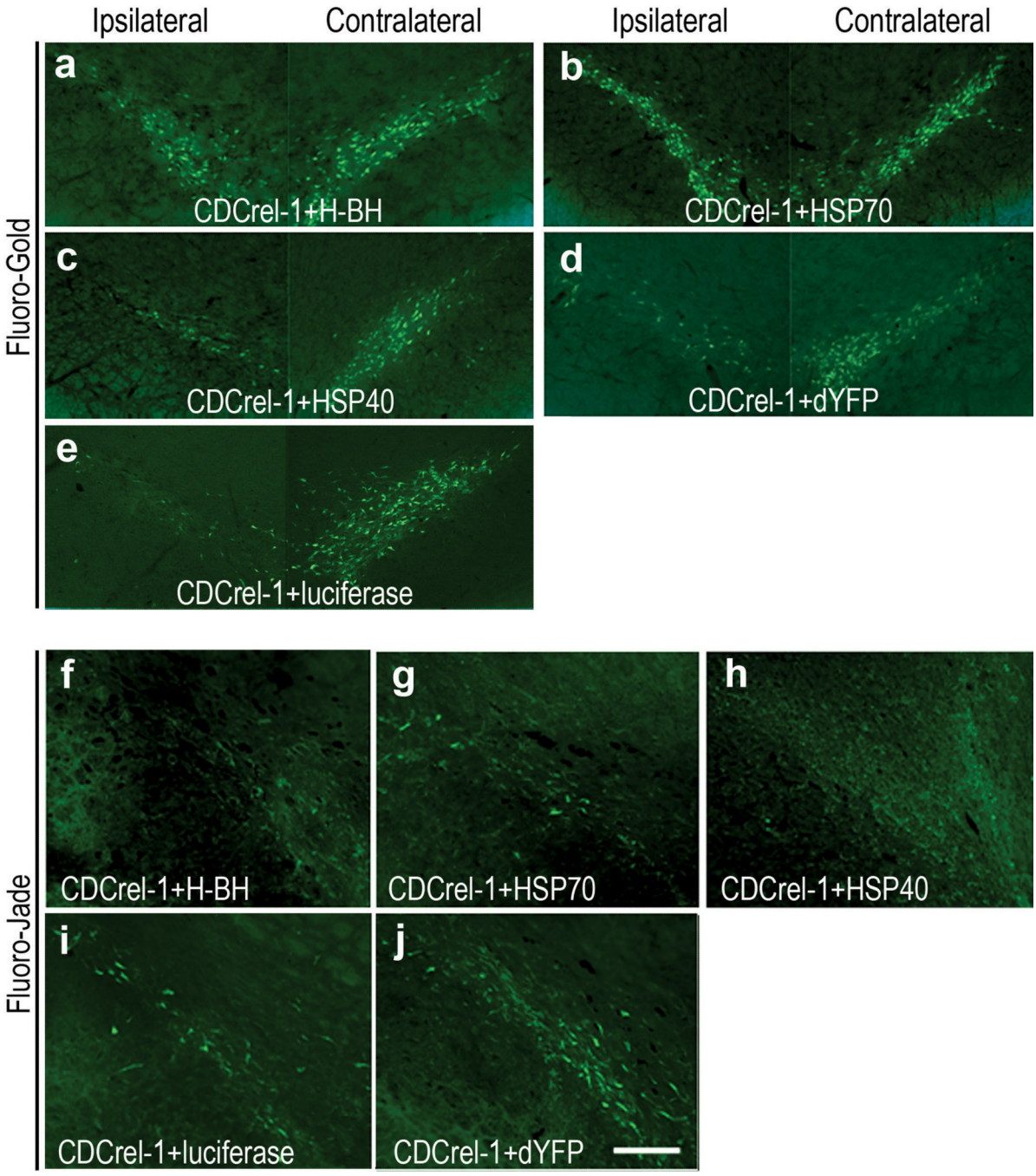


**Figure 5. Fluoro-Gold and Fluoro-Jade B staining in the SNc**

(**a–e**) Fluoro-Gold was injected bilaterally into the striatum of CDCrel-1/treatment vector rats 1 week prior to euthanasia at 8 weeks to label an intact nigrostriatal pathway. (**f–j**) Fluoro-Jade B staining on sections from CDCrel-1/treatment vector rats to show degenerating cells in the SNc. Scale bars = 250 μm (a–e), 200 μm (f–j).

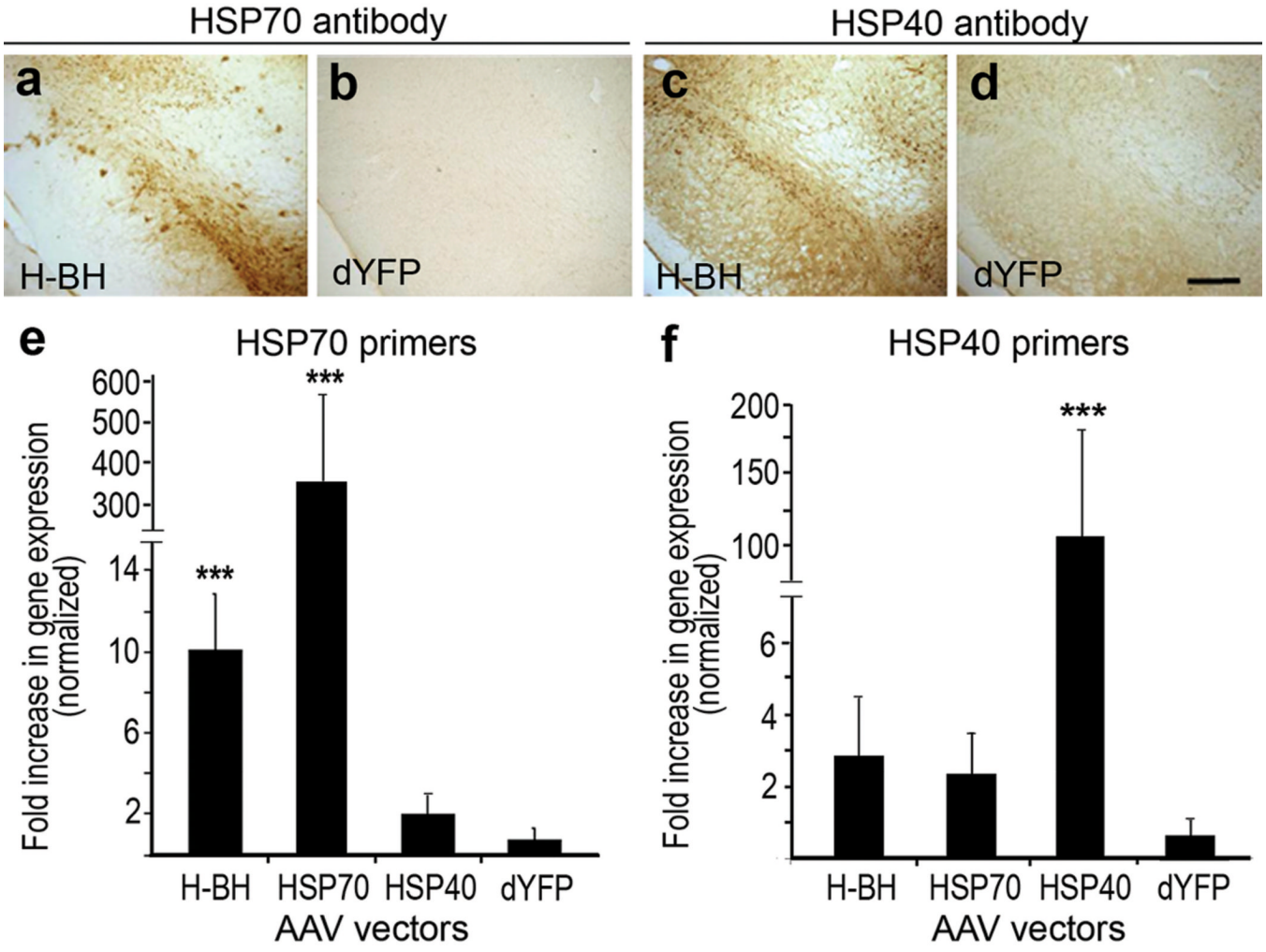


**Figure 6. AAV-mediated H-BH overexpression upregulated various endogenous HSPs**

(**a–d**) Overexpression of H-BH upregulated endogenous (**a**) HSP70 and (**c**) HSP40 in the SNc compared to the (**b,d**) dYFP vector injected hemispheres as detected by immunohistochemistry. Normalized qPCR results showing amount of (**e**) HSP70 expression, detected with HSP70 primers, or (**f**) HSP40 expression, detected with HSP40 primers, after AAV vector injection at 4 week time point. Scale bar = 250 μm. ANOVA, ***$p < 0.001$ compared with dYFP vector injected tissue. Each bar represents mean +/− SEM, n=4.

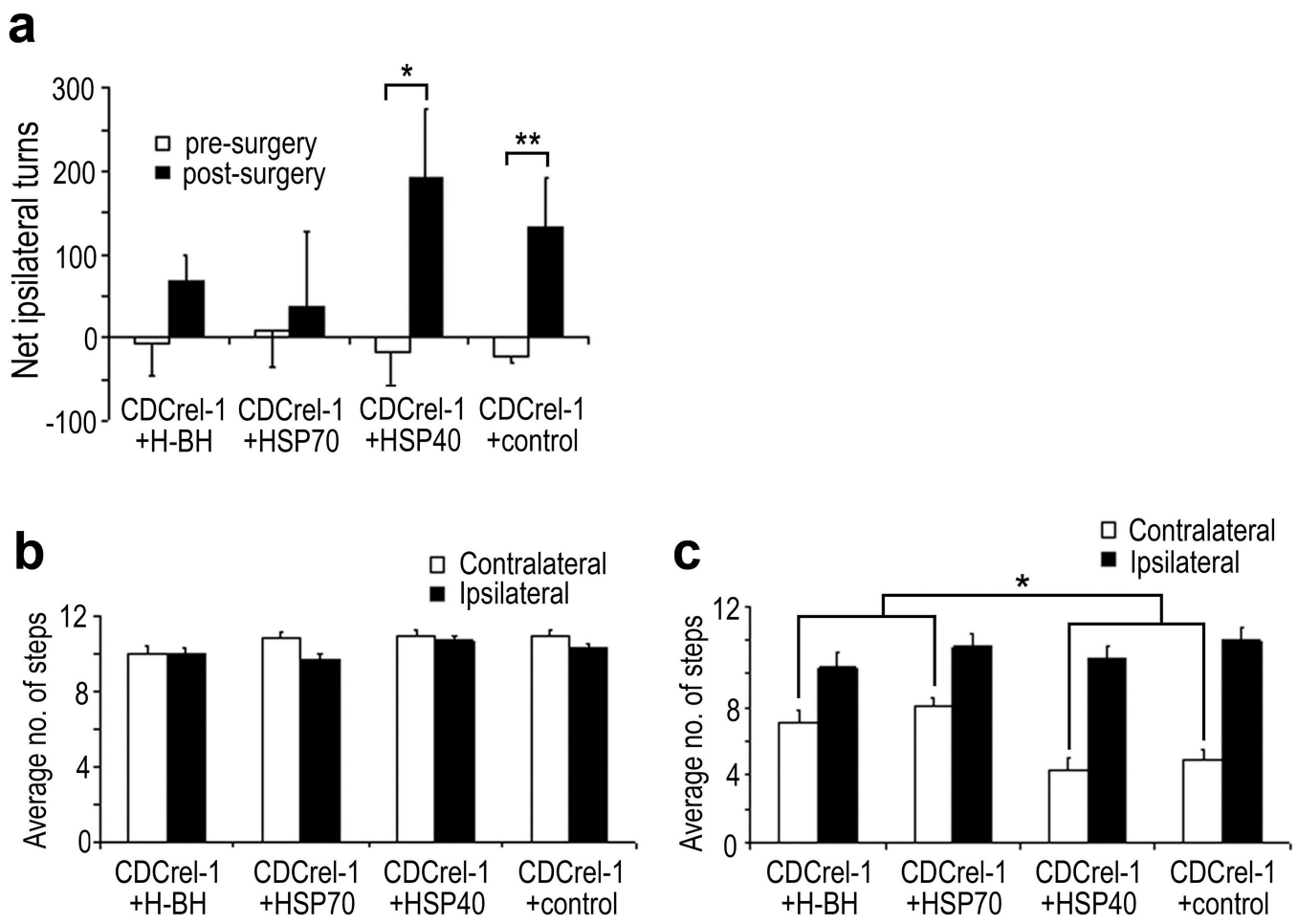


**Figure 7. H-BH and HSP70 prevents motor impairments**

(**a**) Baseline (pre-surgery) amphetamine-induced rotational testing and at 8 weeks postinjection (post-surgery) of H-BH, HSP70, HSP40 and control vectors. Paired t-test, *$p$ = 0.041, **$p$ = 0.006. (**b,c**) The forepaw adjusting steps test conducted before surgery (**b**) and at (**c**) 8 weeks postinjection. ANOVA, *$p$ < 0.001. Each bar represents mean +/− SEM, n=8.