

# Suprachiasmatic Nucleus Neuropeptide Expression in Patients with Huntington's Disease

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**Study Objective:** To study whether sleep and circadian rhythm disturbances in patients with Huntington's disease (HD) arise from dysfunction of the body's master clock, the hypothalamic suprachiasmatic nucleus.

**Design:** Postmortem cohort study.

**Patients:** Eight patients with HD and eight control subjects matched for sex, age, clock time and month of death, postmortem delay, and fixation time of paraffin-embedded hypothalamic tissue.

**Measurements and Results:** Using postmortem paraffin-embedded tissue, we assessed the functional integrity of the suprachiasmatic nucleus in patients with HD and control subjects by determining the expression of two major regulatory neuropeptides, vasoactive intestinal polypeptide and arginine vasopressin. Additionally, we studied melatonin 1 and 2 receptor expression. Compared with control subjects, the suprachiasmatic nucleus contained 85% fewer neurons immunoreactive for vasoactive intestinal polypeptide and 33% fewer neurons for arginine vasopressin in patients with HD ( $P = 0.002$  and  $P = 0.027$ ). The total amount of vasoactive intestinal polypeptide and arginine vasopressin messenger RNA was unchanged. No change was observed in the number of melatonin 1 or 2 receptor immunoreactive neurons.

**Conclusions:** These findings indicate posttranscriptional neuropeptide changes in the suprachiasmatic nucleus of patients with HD, and suggest that sleep and circadian rhythm disorders in these patients may at least partly arise from suprachiasmatic nucleus dysfunction.

**Keywords:** Arginine vasopressin, Huntington's disease, hypothalamus, melatonin receptor, suprachiasmatic nucleus

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## INTRODUCTION

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a CAG repeat expansion in the gene encoding the protein huntingtin. Patients characteristically develop motor, cognitive, and behavioral deficits during the course of the disease.<sup>1</sup> Other features include sleep and circadian rhythm disorders<sup>2-4</sup> and symptoms of cognitive decline,<sup>5,6</sup> as well as disturbances in peripheral circadian pathways such as the liver's metabolic cycle.<sup>7</sup> These disturbances in intrinsic circadian rhythmicity and a delayed phase position of hormones such as melatonin and cortisol<sup>3,8</sup> suggest a disorder of the endogenous biological masterclock, the suprachiasmatic nucleus (SCN), in patients with HD similar to SCN pathology in the R6/2 transgenic mouse model of HD.<sup>8-11</sup> One of the principal circadian hormones released under the influence of the SCN is melatonin, which in turn has a feedback mechanism on the SCN during which its actions are mediated by the melatonin 1 and 2 receptors (MT<sub>1</sub> and MT<sub>2</sub>).<sup>12,13</sup>

Elucidation of the mechanisms underlying sleep and circadian rhythm disturbances in patients with HD is of great importance because restoration of sleep-wake cycles may not only

improve quality of life, but might also ameliorate cognitive and motor dysfunction.<sup>10,11</sup> To test the hypothesis that the biological clock is affected in patients with HD, we directly assessed the functional integrity of the SCN in HD and control brains by determining the expression of two major regulatory neuropeptides, vasoactive intestinal polypeptide (VIP) and arginine vasopressin (AVP), as well as MT<sub>1</sub> and MT<sub>2</sub>, which are crucial for SCN entrainment.

## METHODS

### Postmortem Material

All brain material for patients with HD and control subjects was obtained through the Netherlands Brain Bank (NBB) and consisted of formalin-fixed, paraffin-embedded hypothalamic material (patients with HD  $n = 8$ , control subjects  $n = 8$ ). Control subjects were matched to patients with HD for sex, age, clock time and month of death, postmortem delay, and fixation time (Table 1). Different control subjects were used for VIP/AVP and MT<sub>1</sub>/MT<sub>2</sub> analysis when insufficient hypothalamic sections were available from the same control subject while maintaining adequate matching for the putative confounding factors mentioned previously. Written informed consent for brain autopsy as well as for the use of brain material and medical records for research purposes was acquired by the NBB from patients or their next of kin. This study was approved by the institutional review board of the NBB.

The diagnosis of HD was clinically and neuropathologically confirmed in all patients. Additionally, the diagnosis was genetically confirmed (CAG repeat  $\geq 39$ ) in all but one patient (NBB

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**Table 1**—Clinicopathologic characteristics of patients with Huntington's disease and control subjects

NBB	Sex	Age	Onset	CTD	MD	PMD	Fix	CAG	Cause of death
HD patients									
99-108	M	49	40	9:15	8	5:45	49	51	Cachexia
03-047	F	50	35	18:25	6	5:40	55	47	Pneumonia
92-105	M	54	41	9:55	12	3:50	80	ND	Sudden death
95-060	M	57	42	3:30	6	7:30	53	46	Cachexia
08-044	M	59	50	18:10	5	5:05	52	44	Legal euthanasia
01-128	M	61	39	10:55	11	10:25	48	43	Pneumonia
98-047	F	67	56	10:10	4	6:05	41	45	Legal euthanasia
99-120	M	79	54	19:00	10	6:15	34	44	Pneumonia
Mean		60	45	12:25	8	6:19	52	46	
SEM		3	3	1:57	1	0:41	5	1	
Control subjects									
98-006 (VIP, AVP)	M	50	—	11:00	1	8:30	41	—	Septic shock
00-090 (MT <sub>1</sub> , MT <sub>2</sub> )	M	70	—	8:00	7	7:45	34	—	Bladder carcinoma
97-127	F	49	—	3:30	4	13:30	165	—	Cervix carcinoma
98-027	M	54	—	9:00	12	8:00	59	—	Hepatocellular carcinoma
99-141	M	44	—	10:00	7	7:00	149	—	Cardiac infarction
98-127	M	56	—	15:45	8	5:25	35	—	Cardiac infarction
92-042 (VIP, AVP)	M	61	—	21:00	4	13:50	52	—	Esophageal carcinoma
97-143 (MT <sub>1</sub> , MT <sub>2</sub> )	M	79	—	6:10	10	6:00	31	—	Prostate carcinoma
98-036	F	69	—	6:15	3	9:25	31	—	Cardiogenic shock
93-060	M	79	—	14:00	2	3:00	53	—	Leaking aorta prosthesis
Mean (VIP, AVP)		58	—	11:18	5	8:35	73	—	
Mean (MT <sub>1</sub> , MT <sub>2</sub> )		63	—	8:30	7	7:22	44	—	
SEM (VIP, AVP)		4	—	1:57	1	1:18	19	—	
SEM (MT <sub>1</sub> , MT <sub>2</sub> )		5	—	1:27	1	1:05	19	—	
P value (VIP, AVP)		0.63 <sup>1</sup> 0.42 <sup>3</sup>	—	0.91 <sup>2</sup>	0.18 <sup>2</sup>	0.20 <sup>1</sup> 0.09 <sup>3</sup>	0.72 <sup>1</sup> 0.67 <sup>3</sup>	—	
P value (MT <sub>1</sub> , MT <sub>2</sub> )		0.75 <sup>1</sup> 0.60 <sup>3</sup>	—	0.52 <sup>2</sup>	0.89 <sup>2</sup>	0.37 <sup>1</sup> 0.40 <sup>3</sup>	0.83 <sup>1</sup> 0.78 <sup>3</sup>	—	

<sup>a</sup>For the respective analyses different control patients had to be used (see (VIP, AVP), (MT<sub>1</sub>, MT<sub>2</sub>)) because of a lack of hypothalamic material. Unmarked control patients were used in all analyses. AVP, arginine vasopressin; CAG, mutant Huntingtin CAG repeat length; CTD, clock time of death; F, female; FIX, fixation time (in days); HD, Huntington's disease; M, male; MD, month of death; MT, melatonin receptor; NA, not assessed; NBB, Netherlands Brain Bank number; PMD, postmortem delay (in hr); SEM, standard error of the mean; VIP, vasoactive intestinal polypeptide; Vonsattel, HD grading according to Vonsattel et al.<sup>14</sup>; <sup>1</sup>Mann-Whitney-U test; <sup>2</sup>Mardia-Watson test; <sup>3</sup>Wilcoxon signed rank test.

92-105). This patient, however, had a positive family history and the clinical features of HD, as well as a confirmed Vonsattel grade II HD neuropathology. In Vonsattel grade II and higher grades of HD neuropathology, the changes in the caudate nucleus are characterized by atrophy, neuronal loss, and fibrillary astrocytosis, whereas the globus pallidus in stage II is still relatively spared.<sup>14</sup> Exclusion criteria for control subjects were primary neurologic and/or psychiatric disorders and glucocorticoid therapy during the 2 months before death because glucocorticoids can influence the activity of the SCN.<sup>15</sup> Neuroleptic drugs were not considered an exclusion criterion for patients with HD because depletion of dopamine has no effect on clock gene expression in the SCN.<sup>16</sup> Unfortunately, no information was available on type or degree of sleep and circadian disorders in the patients with HD.

### Immunocytochemistry

The immunocytochemical procedures have been described previously.<sup>17</sup> In short, every 25<sup>th</sup> section (6 μm) of the SCN was

mounted on Superfrost Plus slides (Menzel, Braunschweig, Germany) in four series, one for each neuropeptide or receptor, and dried for 2 days at 37°C. After deparaffinization and rehydration, sections for AVP and MT<sub>2</sub> were heated by microwave oven in citrate buffer pH 4.0 or Tris buffered saline (0.05M Tris and 0.15 M NaCl; pH 7.6), respectively, at 700 W. Sections were incubated in either rabbit antihuman-VIP (Netherlands Institute for Neuroscience, Amsterdam, the Netherlands) 1:1500, polyclonal rabbit antihuman-AVP (Netherlands Institute for Neuroscience) 1:800, polyclonal rabbit antihuman-MT<sub>1</sub> (provided by R. Jockers) 1:200 or polyclonal rabbit anti-human-MT<sub>2</sub>-antibody (provided by D. Angeloni and F. Fraschini) 1:4,000 in Supermix (0.25% gelatin, 0.5% TritonX-100, 0.05M Tris and 0.15 M NaCl; pH 7.6). After overnight incubation, sections were incubated in biotinylated goat-antirabbit immunoglobulin G (Vector Laboratories Inc, Burlington, Ontario, Canada) 1:400 followed by ABC-complex (Vector Laboratories Inc.) 1:800. Sections for MT<sub>1</sub> and MT<sub>2</sub> analysis were additionally incubated

in thyramide 1:250 and reincubated in ABC-complex 1:800. Visualization of the immunoreaction product was achieved by incubation in 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO) 0.5 mg/ml Tris buffered saline, containing 0.23% (w/v) nickel ammonium sulfate (Merck, Darmstadt, Germany) and 0.01% (v/v) H<sub>2</sub>O<sub>2</sub> (Merck). Specificity of all antibodies is described in the following paragraphs.

### In situ Hybridization

The methods used for the assessment of AVP and VIP messenger RNA (mRNA) levels by *in situ* hybridization (ISH) are identical to those described before.<sup>18</sup> Briefly, a 48-mer oligonucleotide probe complementary to bases 411 to 458 of the human preprovasopressin precursor (GenBank # X03172.1) and a 48-mer oligonucleotide probe complementary to bases 524 to 571 of human VIP (Genbank # NM003381.2) were used. Both probes were 3'-end labeled using terminal deoxynucleotidyl transferase (Roche, Mannheim, Germany) and [<sup>35</sup>S] deoxyadenosine triphosphate (PerkinElmer, Burlingame, CA, Cat. # NEG612H) and purified by ethanol precipitation. Hybridization buffer consisted of 0.5 M NaCl, 1× Denhardt solution, 10 mM Tris-HCl, 1 mM EDTA, 10% dextran sulphate, 0.5 mg/ml yeast transfer RNA, 50% formamide, and 200 mM dithiothreitol.

For analysis, every 25<sup>th</sup> (VIP) or 50<sup>th</sup> (AVP) (6 μm) section along the rostrocaudal axis of the SCN was mounted on 2% amino-alkyl-silane coated slides and dried at 58°C. After deparaffinization and rehydration, sections were microwave-oven pretreated at 700 W in citrate buffer (pH 4). After delipidation in phosphate buffered saline 0.1% Triton X-100, sections were hybridized in hybridization buffer with 1 × 10<sup>6</sup> cpm of labeled AVP or VIP oligoprobe per slide, coverslipped and hybridized overnight at 42°C. After hybridization, sections were washed in sequential series of standard saline citrate and dehydrated in graded mixtures of 300 mM ammonium acetate (pH 5.5) and absolute ethanol. Afterward, slides were dipped in photographic emulsion (NTB2; Carestream Health, Rochester, NY) at 42°C, dried and stored in a light-tight box at 4°C for 17 days. Slides were developed for 2 min in Dektol Developer (Sigma), fixed in Kodak fixer (Sigma) at 15°C, and counterstained with thionin.

### Quantification

For an estimation of the total number of SCN neurons immunoreactive for VIP, AVP, MT<sub>1</sub>, or MT<sub>2</sub> every 25<sup>th</sup> (6 μm thick) section throughout the entire SCN in rostrocaudal direction of each patient was used for analysis. All analyses were performed on the SCN in the left half of the hypothalamus for all subjects. Estimates were made using an image analysis system (Image-Pro version 4.5, Media Cybernetics, Silver Spring, MD) connected to a camera (JVC KY-F553CCD) and plain objective microscope (Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives, Carl Zeiss GmbH, Jena, Germany). Analysis consisted of two steps: area selection of the entire SCN and determining the number of immunoreactive neurons with a visible nucleolus, serving as a unique marker for individual neurons; and determination of the number of neuropeptide-immunoreactive neurons. Area selection and sampling were performed at 2.5× objective and the image was loaded into the image analysis sys-

tem and displayed on the monitor. Position and scanning stage using *x* and *y* coordinates were stored. In this image the area covered by the entire SCN was manually outlined and over this outlined area a grid was superimposed. From the respective grid fields *x* and *y* coordinates were stored and all individual images were retrieved at 40× objective on the image analysis monitor. In these images the manually outlined borders of the SCN area were visible and within these borders each neuropeptide-immunoreactive neuron containing a nucleolus was counted. This method has been described previously.<sup>18,19</sup>

The total number of SCN neurons in one-half of the hypothalamus for each neuropeptide was estimated using a previously described technique based on multiplication of the neuronal counts by sample frequency (interval distance between sections) of the hypothalamus not requiring cross-sectional area measurements of the SCN.<sup>19,20</sup> Briefly, the amount of immunoreactive neurons per section, defined as A<sub>n</sub>, was used for estimating the number of neurons within the non-assessed sections on either side of A<sub>n</sub>, defined as D<sub>n</sub>. The value for D<sub>n</sub> was calculated by adding the section number (d<sub>n</sub>) of A<sub>n</sub> to the section number of the previous section (d<sub>n-1</sub>) and dividing it by two. The same procedure was repeated for the consecutive section (d<sub>n+1</sub>) and both figures were combined, so  $D_n = A_n * ((d_n + d_{n-1}) / 2 + (d_n + d_{n+1}) / 2)$ . Finally, all counts for individual areas were added up ( $N = \sum D_n$ ) for an estimation of the total number of immunoreactive neurons per SCN. The mean (± standard deviation [SD]) number of sections quantified for each neuropeptide and receptor were as follows (patients with HD and control subjects, respectively): VIP 8.0 ± 2.6 and 11.6 ± 4.3 (P = 0.09); AVP 10.8 ± 3.1 and 12.0 ± 3.7 (P = 0.37); MT<sub>1</sub> 9.4 ± 4.0 and 8.0 ± 2.1 (P = 0.46); MT<sub>2</sub> 9.4 ± 4.0 and 8.0 ± 2.1 (P = 0.32). Neuronal cell counts or cross-sectional area analysis or neuronal density of the SCN on thionin-stained sections were not performed due to the inability to recognize SCN neurons with certainty in thionin staining.<sup>13</sup> We have tried to perform immunohistochemical staining for VPAC (VIP) receptor 2 without success on the paraffin embedded hypothalamic sections.

Quantitative analysis of the VIP and AVP mRNA signal in the SCN was performed in the same way as described previously and has also been described before.<sup>18,21</sup> After retrieval of individual grid fields on the image analysis monitor a mask was superimposed on the silver grains. All profiles identified as neurons were manually outlined. After correcting for background silver grains, the total mask area covered by the silver grains in these profiles was calculated. Finally, the total number of profiles expressing VIP and AVP mRNA in the SCN, total mask area of the silver grains in the profiles per SCN, and mean silver grains per profile were calculated as an estimate for the total amount of VIP and AVP mRNA in the SCN and the amount of VIP and AVP mRNA per SCN neuron. The mean (± SD) number of sections quantified were as follows (patients with HD and control subjects, respectively): VIP 5.5 ± 2.5 and 7.0 ± 1.6 (P = 0.11); AVP 5.0 ± 1.1 and 6.3 ± 1.6 (P = 0.27).

All analyses for all neuropeptides were conducted by two observers independently of each other and blinded for the groups. For interrater reliability Cronbach alpha was used as a measure and was 0.960 – 0.962 for all analyses. The results in this study represent the mean of their observations.

### Antibody and Oligonucleotide Probe Specificity

Specificity of the VIP and AVP antibodies (Netherlands Institute for Neuroscience), as well as the AVP oligonucleotide probe, has been described before.<sup>22-24</sup> In addition, neither antibody showed reactivity with spot-blot of the following peptides (20 pmol), formalin-fixed onto 0.2% gelatin-coated nitrocellulose paper,<sup>25</sup> using an identical staining procedure as used for the hypothalamic sections: porcine neuropeptide Y(1–36), agouti-related peptide (83–132),  $\alpha$ -melanocyte stimulating hormone (MSH),  $\beta$ -MSH,  $\gamma$ -MSH, galanin, adrenocorticotrophic hormone (1–39), luteinizing hormone releasing hormone, somatostatin-14 and -28, cocaine- and amphetamine-regulated transcript CART (106–129), oxytocin, corticotropin-releasing hormone, and melanin concentrating hormone. Moreover, the VIP antibody showed no reactivity with AVP peptide and the AVP antibody no reactivity with VIP peptide. VIP and AVP ISH probes were checked for cross homology with other known sequences using BLAST,<sup>26</sup> which did not identify interfering sequences in the databases at the National Center for Biotechnology Information and National Library of Medicine. VIP and AVP ISH sense probes were used alongside the ISH antisense probes serving as negative control samples, producing no autoradiographic signal.

Specificity of the MT<sub>1</sub> and MT<sub>2</sub> antibody has been described before.<sup>27-29</sup> Additionally, spot-blot testing confirmed that the anti-MT<sub>2</sub> antibody bound to synthetic human MT<sub>2</sub> and not to spots containing vasopressin or oxytocin. Moreover, in human hypothalamic sections no immunoreactivity could be seen when the MT<sub>2</sub> antibody was preadsorbed with MT<sub>2</sub> immunogenic peptide (Y-H Wu et al., Netherlands Institute for Neuroscience, 2009, unpublished data).

### Statistical Analysis

All data are presented as median  $\pm$  standard error of the mean unless otherwise specified. Differences between groups were statistically evaluated by the nonparametric Mann-Whitney U test (two-tailed). Intergroup differences in clock time and mo of death were evaluated using the Mardia-Watson test, whereas intergroup differences for dichotomous variables were evaluated by chi-square. Spearman  $\rho$  was used to assess all correlations.  $P < 0.05$  was considered to be significant. All statistical analyses were performed using SPSS Statistics 17.0 (SPSS Inc, Chicago, IL).

## RESULTS

Control subjects did not differ from patients with HD for any of the putative confounders. Groupwise and pairwise comparison yielded similar results, reflecting adequate matching (Table 1). The number of patients with HD and control subjects in each analysis (VIP/AVP and MT<sub>1</sub>/MT<sub>2</sub>) did not differ with respect to season or circadian time of death (i.e., either at day or at night) ( $P \geq 0.14$ ).

### VIP and AVP Immunocytochemistry

Figure 1, A-D provides representative immunocytochemical stainings for VIP and AVP in HD patients and control subjects, showing reductions in both VIP-immunoreactive and AVP-immunoreactive neurons in patients with HD. The number of VIP-immunoreactive neurons in the SCN of patients with HD

( $256 \pm 122$ ) was found to be 85% lower than that in control subjects ( $1,679 \pm 331$ ) ( $P = 0.002$ ). The number of AVP-immunoreactive neurons in the SCN of patients with HD ( $3,455 \pm 588$ ) was 33% lower than that in control subjects ( $5,154 \pm 546$ ,  $P = 0.027$ ) (Figure 3).

### MT<sub>1</sub> and MT<sub>2</sub> Immunocytochemistry

Figure 1, E-H provides representative immunocytochemical stainings for MT<sub>1</sub> and MT<sub>2</sub>, showing no changes in MT<sub>1</sub>- or MT<sub>2</sub>-immunoreactive neurons between HD patients and control subjects. There was no significant difference between the total number of MT<sub>1</sub>- or MT<sub>2</sub>-immunoreactive neurons in HD patients ( $13,717 \pm 1,523$  and  $6,605 \pm 403$ , respectively) and control subjects ( $12,901 \pm 2,203$  and  $4,161 \pm 843$ , respectively) ( $P = 0.88$  and  $0.13$ , respectively) (Figure 4).

### VIP and AVP mRNA Levels

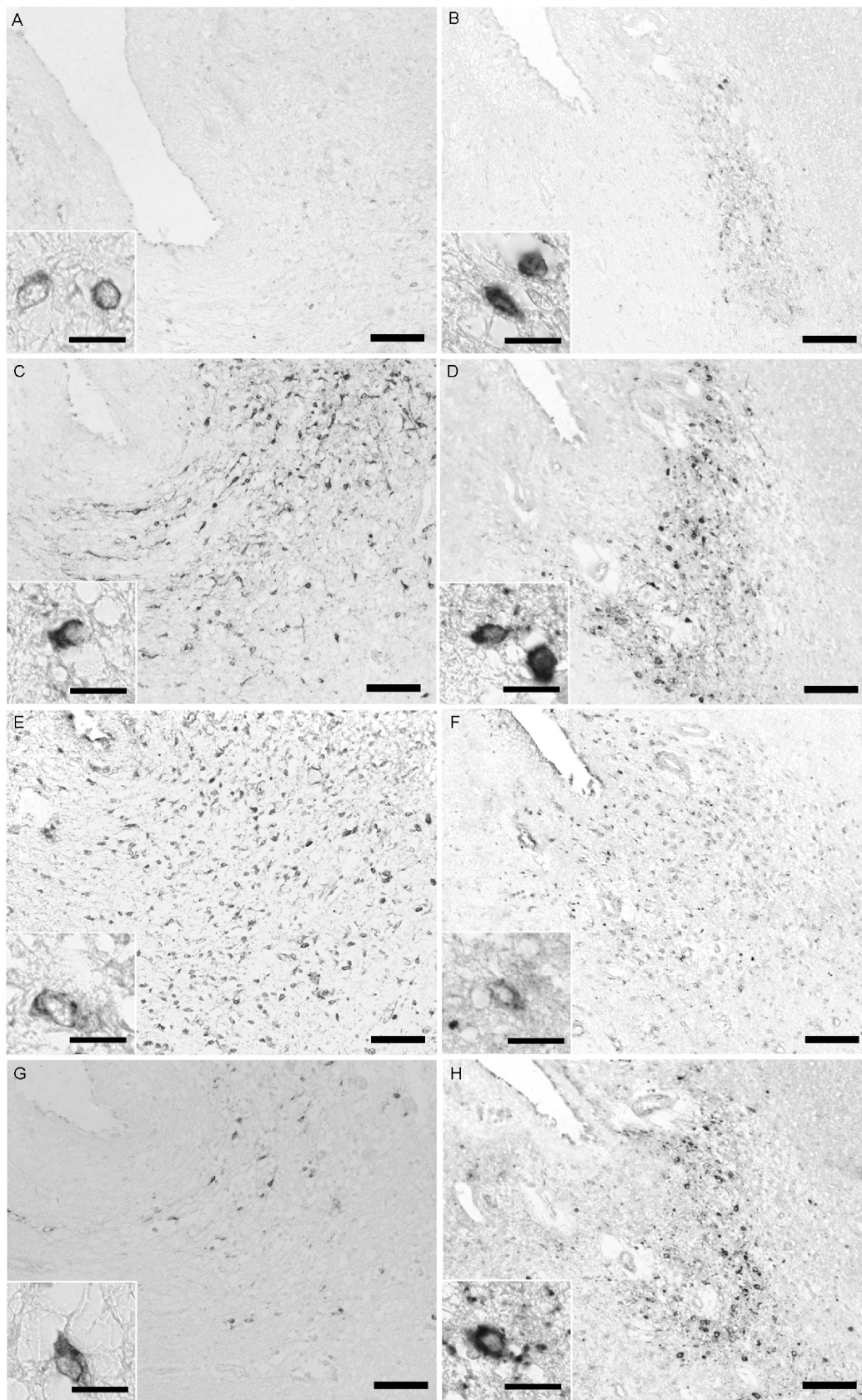
Figure 2, A-D provides representative autoradiographs for VIP and AVP, showing no obvious differences between patients with HD and controls. Compared with control subjects, the total masking area of silver grains for VIP-mRNA (patients with HD  $7.23 \pm 5.27$ , control subjects  $16.19 \pm 1.11$ ), as well as for AVP-mRNA (patients with HD  $13.45 \pm 6.71$ , control subjects  $17.75 \pm 6.86$ ) per SCN was unchanged ( $P = 0.14$  and  $P = 0.58$ , respectively) (Figure 5). Furthermore, masked silver grains per cell profile was unchanged in patients with HD, both for VIP and AVP ( $P = 0.88$  and  $P = 0.55$ , respectively). In addition, the number of cell profiles expressing VIP-mRNA or AVP-mRNA was similar in patients with HD ( $1,549 \pm 600$  and  $6,076 \pm 1,419$ , respectively) and control subjects ( $4,351 \pm 1,939$  and  $6,265 \pm 1,407$ , respectively) ( $P = 0.23$  and  $P = 0.80$ , respectively). The discrepancy between estimated neuronal numbers based on immunocytochemistry and ISH is due to the fact that for immunocytochemistry neurons with a visible nucleolus were counted, whereas in the ISH analyses all SCN neurons on individual sections were counted regardless of a nucleolus.

A positive correlation between the number of VIP-immunoreactive neurons and total masking area for VIP-mRNA was observed in control subjects ( $\rho = 0.833$ ,  $P = 0.010$ ), but was absent in patients with HD ( $\rho = 0.43$ ,  $P = 0.29$ ). No significant correlations were observed between the number of AVP-immunoreactive neurons and total masking area of AVP-mRNA ( $P = 0.57$  for both patients with HD and control subjects).

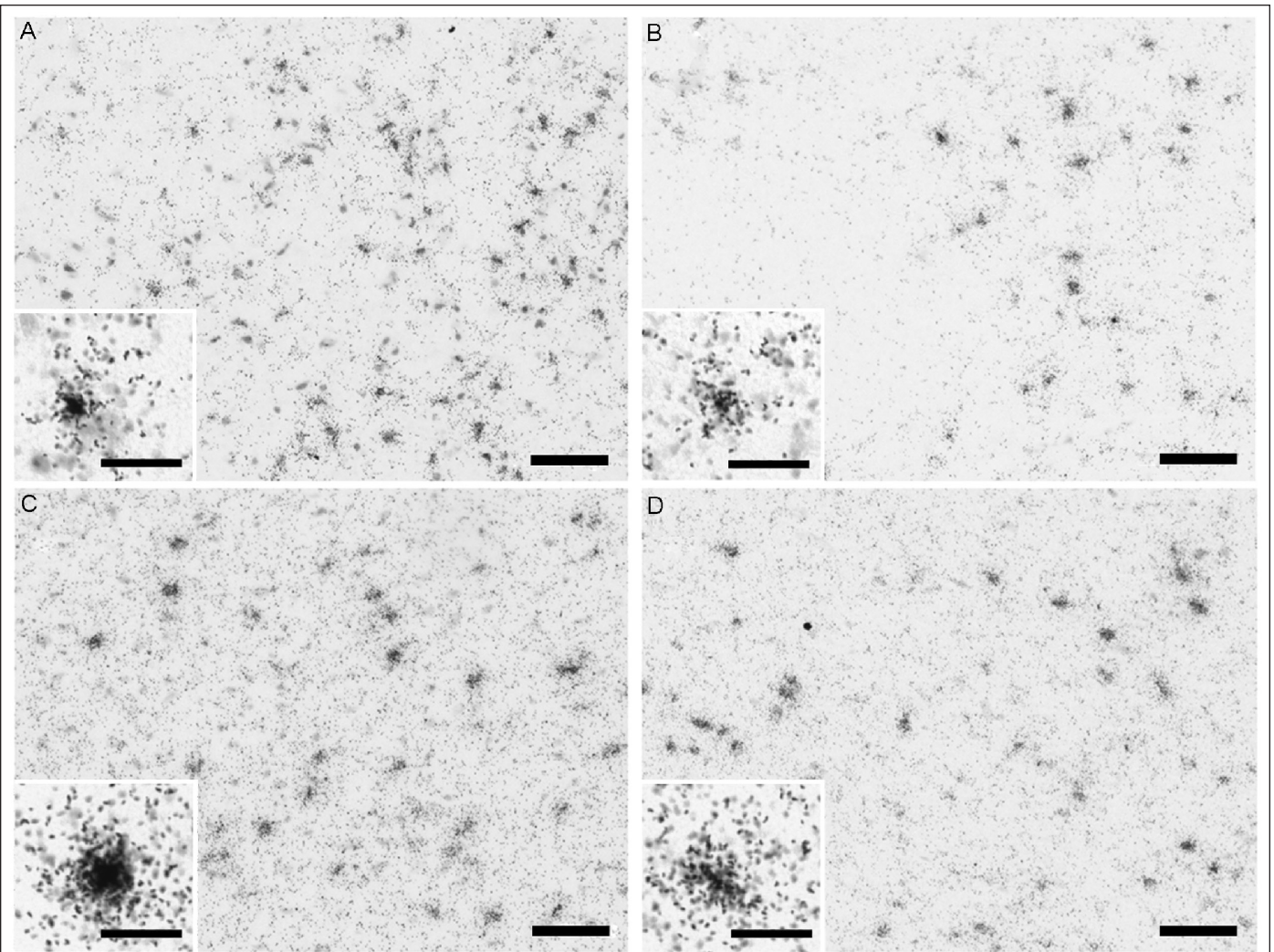
## DISCUSSION

To our knowledge, the current study is the first to report altered morphology of the SCN in patients with HD. The numbers of both AVP and VIP neurons were significantly decreased in patients with HD, whereas the expression levels of AVP- and VIP-mRNA as well as the number of MT<sub>1</sub> and MT<sub>2</sub> neurons were unchanged. These findings suggest that alterations in the levels of two major regulatory neuropeptides in the SCN may contribute to sleep and circadian rhythm disturbances in patients with HD.

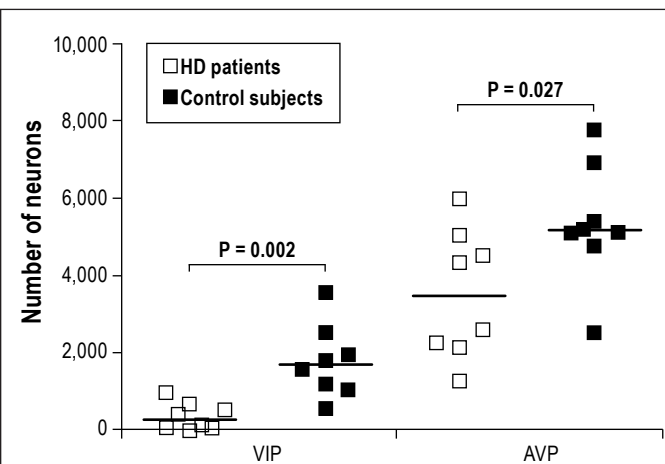
Circadian sleep disorders are frequently found in patients with HD<sup>2</sup> and are partly mirrored in the R6/2 transgenic mouse model of HD, which shows a complete disintegration of circadian behavior.<sup>4</sup> These mice show reduced levels of VIP and its receptor VPAC<sub>2</sub> in the SCN,<sup>9</sup> along with a marked disrupt-



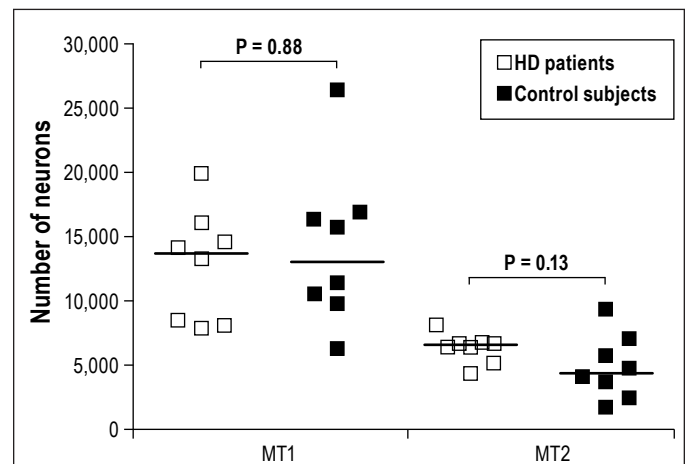
**Figure 1**—Representative immunocytochemical staining microscopic images for arginine vasopressin, vasoactive intestinal polypeptide and melatonin 1 and 2 receptor in the suprachiasmatic nucleus. **A**, In patients with Huntington's disease (HD), the number of vasoactive intestinal polypeptide-immunoreactive neurons was considerably lower than that in matched controls (shown in **B**) ( $P = 0.002$ ). In addition, the number of arginine vasopressin-ir neurons in patients with HD (**C**) was lower than that in controls (shown in **D**) ( $P = 0.027$ ). Melatonin 1 and 2 receptor-immunoreactive neurons were unchanged in patients with HD (**E** and **G**, respectively) compared with control subjects (**F** and **H**, respectively). HD patient NBB 01-128 and control subject NBB 98-127. Bars represent 250  $\mu\text{m}$ ; bars in the inserts represent 100  $\mu\text{m}$ .



**Figure 2**—Representative *in situ* hybridization radiographs for vasoactive intestinal polypeptide and arginine vasopressin in the suprachiasmatic nucleus. mRNA levels of vasoactive intestinal polypeptide (A, patient with Huntington's disease (HD); B, control subject) and arginine vasopressin (C, patient with HD; D, control subject) were unchanged in patients with HD ( $P = 0.14$  and  $P = 0.58$ , respectively). HD patient NBB 01-128 and control subject NBB 98-127. Bars represent 250  $\mu\text{m}$ ; bars in the inserts represent 100  $\mu\text{m}$ .



**Figure 3**—Total numbers of immunoreactive neurons for vasoactive intestinal polypeptide (VIP) and arginine vasopressin (AVP). Both the number of VIP and AVP immunoreactive neurons were significantly decreased in the suprachiasmatic nucleus of patients with Huntington's disease (HD) ( $P = 0.002$  and  $P = 0.027$  respectively). Central bars represent the median.

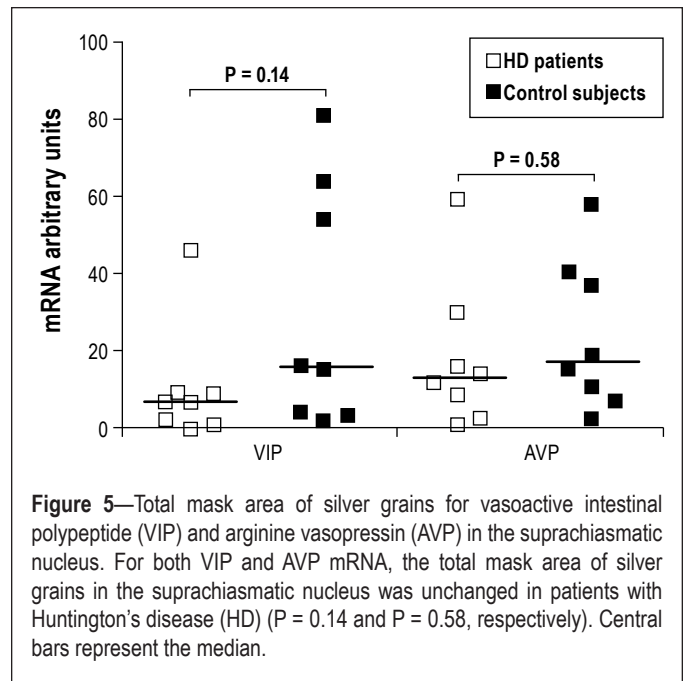


**Figure 4**—Total numbers of immunoreactive neurons for melatonin 1 and 2 receptors. The number of melatonin 1 and 2 receptor ( $\text{MT}_1$  and  $\text{MT}_2$ , respectively) immunoreactive neurons was unchanged in the suprachiasmatic nucleus in patients with Huntington's disease (HD) ( $P = 0.88$  and  $0.13$ , respectively). Central bars represent the median.

tion of two clock genes, *mBmal1* and *mPer2*, both failing to exhibit circadian oscillation.<sup>4</sup> Furthermore, circadian rhythm disturbances are also suggested by an onset delay in nocturnal rise in melatonin,<sup>3</sup> as well as disrupted patterns of cortisol secretion in patients with early-stage HD.<sup>8</sup> The decrease in the numbers of VIP and AVP neurons seems to confirm that circadian rhythm disturbances arise from pathology of the SCN. More specifically, our data suggest a posttranscriptional defect in neuropeptide production or a disturbed balance between production and breakdown/release in SCN neurons of patients with HD. A discrepancy between AVP protein and mRNA levels has also been described in the SCN of patients with major depressive disorder, where an increase in AVP protein levels was found together with a decrease in AVP mRNA,<sup>17</sup> stressing that mRNA and protein levels do not necessarily have to go hand in hand. The difference between mRNA results in our cohort of HD patients and the R6/2 transgenic mice<sup>4</sup> may potentially be explained by the large differences in CAG repeat lengths. In the patients with HD, the average CAG repeat length was approximately 46, whereas in the R6/2 rodent model of HD CAG repeat size was approximately 150.<sup>4</sup> One may reason that the very long CAG repeats in rodents with HD may cause more severe changes in hypothalamic neuropeptide expression. On the other hand, the precise effect of mutant huntingtin in neurons remains unknown. In a previous study it has been shown that the SCN is among the hypothalamic nuclei in patients with HD that contain the least amount of mutant huntingtin inclusions.<sup>8</sup>

Our findings suggest that sleeping disorders in patients with HD may partly stem directly from SCN pathology. Due to lack of polysomnographic data in our cohort of patients with HD it remains unknown, however, to what degree these SCN changes could contribute to sleep and circadian disorders in patients with HD. Other contributing factors may include a reduction in the expression of the hypothalamic neuropeptide orexin and alterations in the histaminergic system in patients with HD, both of which are of considerable importance for the regulation of sleep/wakefulness states.<sup>30,31</sup> Part of the orexin reduction may be induced by a decrease in VIP in the SCN because orexin-containing neurons in the lateral hypothalamus are innervated by VIP nerve fibers from the SCN, at least in rodents.<sup>32</sup> However, although in R6/2 mice orexin levels are markedly decreased,<sup>33</sup> in a previous study including largely the same patients as in the current study, the number of orexin-producing neurons in the lateral hypothalamus was found to be reduced by approximately 30%.<sup>8</sup> It is therefore uncertain whether this modest reduction in orexin levels could contribute to a major degree to sleeping disturbances in patients with HD,<sup>34</sup> although additional (clinical) studies directly assessing the association between orexin levels in the cerebrospinal fluid and sleep disturbances in patients with HD are needed to delineate the precise clinical relevance of dysfunctional orexin signaling in patients with HD.

The changes that were observed in the SCN of patients with HD seem to be specific to the SCN because AVP is unchanged in other areas of the brain affected by HD, such as the hypothalamic paraventricular nucleus (in the same patients with HD as in the current study),<sup>35</sup> locus coeruleus, and substantia nigra.<sup>36</sup> Similarly, the decrease in VIP appears to be specific to the SCN because no change was observed in VIP concentrations in several other brain areas in patients with HD.<sup>37</sup> The changes in the



**Figure 5**—Total mask area of silver grains for vasoactive intestinal polypeptide (VIP) and arginine vasopressin (AVP) in the suprachiasmatic nucleus. For both VIP and AVP mRNA, the total mask area of silver grains in the suprachiasmatic nucleus was unchanged in patients with Huntington's disease (HD) ( $P = 0.14$  and  $P = 0.58$ , respectively). Central bars represent the median.

SCN are similar to changes in the SCN concerning VIP and AVP in patients with Alzheimer disease<sup>38-40</sup> and indicate that bright-light therapy may prove effective in improving circadian rhythmicity and cognitive functioning in patients with HD.<sup>41</sup> Similarly, melatonin (agonist) administration may prove effective in resetting circadian rhythmicity in the SCN through the  $MT_1$  and  $MT_2$  receptors,<sup>13,42</sup> both of which were preserved in the SCN of our cohort of patients with HD. The unchanged expression of the  $MT_1$  and  $MT_2$  receptor seems to suggest that melatonin levels are not decreased in HD patients and that merely the circadian timing of melatonin secretion is altered due to the altered circadian output from the SCN.

Potential limitations of our study include the use of hypothalamic material from patients with end-stage HD and the lack of data on sleep and circadian disorders in patients with HD. This lack of data results in the inability to directly link the observed changes in the SCN to circadian abnormalities in patients with HD. We have, however, found in the same patients as in the current cohort, a disorder in the diurnal fluctuation of the expression of histidine decarboxylase (HDC). HDC is the key enzyme for histamine production, and the histaminergic system is crucial for the regulation of circadian rhythm.<sup>43</sup> Furthermore, a potential limitation is the assessment of neuronal numbers by counting of immunoreactive neurons. Using immunocytochemical markers it is impossible to distinguish between an actual loss of neurons in the SCN or a decrease in neurotransmitter levels without neuronal loss. On the mRNA level, however, we did not observe a significant change in the number of neurons expressing VIP or AVP. These latter findings suggest a posttranscriptional defect rather than a loss of SCN neurons in patients with HD, which is further corroborated by the unchanged number of neurons expressing  $MT_1$  and  $MT_2$ . In addition, it has been shown that markers such as NeuN do not necessarily reflect the total number of neurons<sup>44</sup> and that SCN borders can only be established on the basis of staining with immunocytochemical markers. These borders established by SCN markers such as AVP and VIP are activity-dependent, and SCN

volume and thus the total cell numbers seem, therefore, to fluctuate with activity changes.<sup>39</sup> In the R6/2 transgenic HD mice, the number of SCN neurons is unchanged.<sup>9</sup> This potential post-translational defect may be due to a decrease in prohormone convertases (PC), which are abundantly expressed in the rodent SCN and are involved in converting proVIP and proAVP into VIP and AVP, respectively.<sup>45</sup> This finding seems to be supported by the observation that the number of PC 1/3 and PC2 expressing neurons are significantly reduced in the paraventricular and infundibular nucleus of the current patients with HD where mRNA levels of neuropeptides such as corticotrophin-releasing hormone and neuropeptide Y are unchanged or increased with decreased peptide levels.<sup>35</sup>

In conclusion, our findings provide evidence for severe SCN pathology in patients with HD, reflected by a substantial loss of VIP and AVP immunoreactivity, as well as an imbalance between protein and mRNA levels. No change was observed in MT<sub>1</sub> and MT<sub>2</sub> expression. Future studies should aim to discern potential phenotype differences in circadian and sleep disorders in HD patients and delineate the degree in which such differences would relate to the severity of SCN dysfunction.

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## DISCLOSURE STATEMENT

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