Decreased VIP and $VPAC_2$ receptor expression in the biological clock of the R6/2 Huntington disease mouse.

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Running head: VIP loss in R6/2 Huntington mice

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Abstract

Huntington's disease (HD) is a fatal genetic neurodegenerative disorder caused by a CAG triplet repeat expansion in the gene encoding the protein huntingtin. The most studied model of HD, the R6/2 transgenic mouse, replicates many features of the disease. In addition to motor, cognitive and endocrine dysfunctions, these mice exhibit a progressive disruption of circadian rhythms. This is accompanied by an altered expression of the circadian clock genes in the suprachiasmatic nucleus, the principal circadian pacemaker in the brain. The neuropeptide vasoactive intestinal polypeptide (VIP) and its receptor $VPAC_2$ are highly expressed in the suprachiasmatic nucleus and VIPergic signalling plays an essential role in maintenance of ongoing circadian rhythmicity. We found a marked reduction in both VIP mRNA and $VPAC_2$ receptor mRNA, quantified by RT-PCR, as well as a decrease in VIP immunostaining in the suprachiasmatic nucleus of R6/2 mice. These changes were coupled to a disruption of circadian rhythm. We observed no loss of neurons in the suprachiasmatic nucleus and therefore suggest that the changes in VIP and $VPAC_2$ receptor are due to their decreased expression. In conclusion, we propose that the impaired VIPergic signalling is an additional candidate mechanism for disruption of circadian rhythms in R6/2 mice.

Introduction

Huntington’s disease (HD) is a fatal, genetic neurodegenerative disorder caused by an unstable CAG expansion on the gene (*htt*) encoding the protein huntingtin. This expansion results in the formation of excessive polyglutamine repeats (≥36) at the N-terminus of the protein (Huntington's disease collaborative research group, 1993). It causes a cascade of alterations in cell function leading ultimately to neuronal degeneration, particularly in the cerebral cortex, striatum and lateral hypothalamus (Maat-Schieman *et al.*, 1999; Petersén *et al.*, 2005; VonSattel and DiFiglia, 1998).

The most studied animal model of HD is the R6/2 transgenic mouse which expresses *exon* 1 of the human mutant HD gene containing approximately 150 CAG repeats (Mangiarini *et al.*, 1996). R6/2 mice display cognitive deficits and motor hyperactivity starting at 4-5 weeks of age, show an overt behavioural phenotype and endocrine changes around 8 weeks of age, and in advanced stages of disease progression these mice display several clinical features reminiscent of HD (Carter *et al.*, 1999; Mangiarini *et al.*, 1996). The R6/2 mice were recently reported to show a progressive disruption of circadian rhythm with increased daytime activity. This behavioural disturbance was accompanied by an impaired expression of the circadian clock genes, *Per2* and *Bmal1*, in the suprachiasmatic nucleus (SCN), the principal circadian pacemaker in the brain (Morton *et al.*, 2005). The neuropeptide vasoactive intestinal polypeptide (VIP) is expressed in the core region of the SCN, and VIP-containing neurons project to cells within the entire SCN and other brain areas outside the nucleus (Abrahamson and Moore, 2001). Studies using exogenous application of VIP and experiments in VIP- and VIP receptor ($VPAC_2$)-deficient mice indicate that VIPergic signalling plays an essential role in maintenance of ongoing circadian rhythmicity, probably by synchronizing the SCN cells (Aton *et al.*, 2005; Colwell

et al., 2003; Cutler *et al*., 2003; Harmar *et al*., 2002; Hughes *et al*., 2004; Maywood *et al*., 2006; Reed *et al*., 2003). Considering the similarity in the previously reported disturbances in circadian activity patterns in R6/2 mice and in VIP/$VPAC_2$ receptor-deficient mice, we hypothesized that the VIPergic system in the SCN of R6/2 mice is perturbed.

In the present study, we first monitored the circadian cycles in R6/2 mice.

We then examined both VIP and $VPAC_2$ receptor mRNA contents, quantified by RT-PCR, as well as VIP immunostaining in the SCN of R6/2 mice.

Material and methods

Animals: Ten male R6/2 mice, code B6CBA-TgN (HDexon1) 62 Gpb ((Mangiarini *et al*., 1996) and ten age-matched male controls, code B6CBAF 1/1 100011 were purchased from Jackson Laboratory, Bar Harbor, ME, USA. The mice were housed under a 12 h/12 h light/dark cycle with free access to food and water. The animals were treated according to the principles of Laboratory Animal Care (Law on Animal Experiments in Denmark, publication 382, June 10, 1987).

Analysis of circadian behaviour: To confirm the previously reported disturbances of circadian activity in R6/2 mice running-wheel activity was continuously monitored in six of the ten animals in each group from seven until 11 weeks of age. R6/2 mice and controls were housed in individual cages equipped with a running-wheel in ventilated, light-tight chambers with controlled lightning. Wheel-running activity, collected continuously in 10 min bins, was monitored by an on-line personal computer connected via a magnetic switch to the MiniMitter Running Wheel activity system (consisting of QA-4 activity input modules, DP-24 data ports and Vital View data acquisition system, version 2.19; MiniMitter Company, Sunriver, OR, USA). Animals were entrained to a 12 h/12 h light/dark cycle for 12 d followed by 10 d in constant darkness after which they were re-entrained to the 12 h/12 h light/dark schedule and included in the groups of animals used for VIP/$VPAC_2$ receptor mRNA quantification or VIP immunohistochemistry. The locomotor activity of mice kept in 10 d of constant darkness were analysed by periodogram analysis combined with the χ2-test with a 0.001 significance level on the raw data (ClockLab, ActiMetrics software, Coulbourn Instruments, Allentown, PA, USA). The periodogram shows the amplitude (power) of the periodicities in the time series for the period of interest.

Quantification of VIP and $VPAC_2$ receptor mRNAs in the SCN by real-time RT-PCR: The SCN from seven R6/2 mice and seven wild type (WT) control mice at 11 weeks of age was dissected out as previously reported (Fahrenkrug *et al.*, 2005). All animals were entrained to a 12 h/12 h light/dark cycle and decapitated at midday.

From individual animals total SCN RNA and cDNA were made as described previously (Hannibal *et al.*, 2005). Mouse cerebral cortex and hypothalamus RNA was used to make two large batches of cDNA, applied as standards for the VIP and VPAC2 receptor assay, respectively. Each curve consisted of five serial five-fold dilutions frozen in aliquots. The most concentrated sample contained: cDNA from 50 ng total RNA/µl and the least concentrated one held: cDNA from 80 pg total RNA/µl. The highest point of the standard curve was arbitrarily set to 12,500 and the lowest to 20. Real-time PCR was performed on an ABI7000 instrument using Taqman based chemistry (Applied Biosystems, CA, USA). For both VIP and VPAC2 receptor expression Assay-on-Demand (Mm00669234_m1 and Mm00437316_m1, Applied Biosystems, CA, USA) was used. The primers and TaqMan probe for the β2-microglobulin (β2MG) assay used as internal control were designed employing Primer Express software (Applied Biosystems, CA, USA). Probe of 200 nM having a 5'-VIC reporter and a 3'-TAMRA-quencher (VIC-CCTCAAATTCAAGTATACTCACGCCACCCA-TAMRA), and 600 nM of both the forward: CGGCTTGTATGCTATCCAGAAAA, and reverse primer, AGTATGTTCGGCTTCCCATTCTC, were used. All reactions were done in 25 µl containing cDNA from 25 ng total RNA or 2.5 µl standard and by TaqMan Universal PCR Master Mix containing AmpErase7UNG (Applied Biosystems, CA, USA). The VIP, $VPAC_2$ receptor and the β2MG assays were run in separate wells on the same plate and all samples, standards, and the non-template negative controls were made in duplicate. The ABI prism

7000 SDS software program (Applied Biosystems, CA, USA) was used to calculate the concentrations (in arbitrary units) of VIP/$VPAC_2$ receptor and β2MG mRNA. The amount of VIP/$VPAC_2$ receptor mRNA was normalised with the amount of β2MG obtained from the same run leading to a normalized VIP/$VPAC_2$ concentration.

Immunohistochemistry: Three R6/2 mice and three WT mice entrained to a 12 h/12 h light/dark cycle were anaesthetized at midday with 3-bromoethanol (250 μg/kg, ip) and transcardially perfused with Stefanini's fixative (2% paraformaldehyde, and 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.2). The brains were removed and postfixed in the same fixative overnight, cryoprotected in 30% sucrose and frozen. The brains were cut throughout the entire SCN in a cryostat as 12 μm thick sections and processed for VIP immunohistochemistry as previously described (Fahrenkrug and Hannibal, 1998; Hannibal and Fahrenkrug, 2004) using VIP antiserum, code no. 291E-3, raised and characterized in our own laboratory. To evaluate if there was a loss of neurons in the SCN of R6/2 mice as compared to controls we counted the neuronal nuclei on 3 sections from each animal representing the rostral, middle and caudal SCN using an antibody against NeuN (code no. MAB 377, Chemicon, CA, USA). VIP fluorescence images were obtained with an Olympus IX70 confocal microscope equipped with Fluoroview version 2.1.3.9 (Olympus, Tokyo, Japan) and appropriate filter setting for detection of Cy2. Images were edited for contrast and brightness by Adobe Photoshop and combined into plates using an Adobe Illustrator.

Statistical analysis: Data were presented as means ± SEM. Mann-Whitney U-test (using GraphPad Prism version 3.0 software) was performed to determine significant differences between R6/2 mice and controls. $p < 0.05$ was considered statistically significant.

Results

Disturbance of circadian rhythm in R6/2 mice

Patterns of running wheel activity were monitored in R6/2 and WT mice. From 7 weeks of age the animals were entrained to a 12 h/12 h light/dark cycle for 12 d followed by 10 d in constant darkness after which they were re-entrained to the 12 h/12 h light/dark schedule. All mice were able to synchronize to a 12 h/12 h light/dark cycle, but some of the R6/2 mice had higher daytime activity than the WT controls (Fig. 1). When transferred to continuous darkness WT mice exhibited robust circadian rhythms of activity. In contrast the R6/2 mice displayed only weak rhythmic activity with a less clear time of onset (Fig. 1), and the activity occurred both during the subjective night and day phase. The periodograms obtained during constant darkness are data segments of 24 h length, averaged as the activity profile (calculated using X^2-test) and plotted with the X^2-line (99.9% confidence level) showed weak circadian behaviour in the R6/2 mice as compared to WT mice (Fig. 1).

Reduction of VIP and $VPAC_2$ receptors in the SCN of R6/2 mice

As observed by real-time RT-PCR quantification, 11-week-old R6/2 mice displayed a marked and significant ($p = 0.002$) reduction of VIP mRNA level in the SCN, compared with age-matched controls (Fig. 2A). The mRNA for $VPAC_2$ receptor was also significantly ($p = 0.007$) reduced in R6/2 mice compared to WT controls (Fig. 2B). The amounts of β2MG mRNA used as internal control did not differ between R6/2 mice and controls.

The staining intensity of VIP immunoreactive cell bodies and nerve fibres was markedly reduced in the SCN of R6/2 mice as compared to controls, both at the age of 11 weeks (Fig. 3). The decreased VIP immunostaining in R6/2 mice was observed at all levels of the

SCN. However, counting of neuronal nuclei in sections of the SCN showed no significant difference between R6/2 mice (178 ± 8 per section) and WT mice (182 ± 15 per section).

Discussion

Patients with HD exhibit disturbances in circadian rhythm (Morton *et al.*, 2005). In mammals, the principal pacemaker is situated in the SCN of the hypothalamus and coordinates circadian rhythms of physiology and behaviour (Reppert and Weaver, 2002). The SCN integrates information from the retina and from the periphery and imposes circadian patterning across the organisms via neural and neuroendocrine pathways (Buijs and Kalsbeek, 2001). Rhythmic circadian activity within the SCN is dependant on cyclic expression of a family of “clock genes” (Okamura, 2004; Reppert and Weaver, 2001). There is evidence that the individual SCN neurons are autonomous circadian pacemakers (Herzog *et al*., 1998; Honma *et al*., 1998; Welsh *et al*., 1995), which in the intact SCN synchronise to one another with defined phase relationships (Herzog *et al*., 1997; Quintero *et al*., 2006; Schaap *et al*., 2003; Yamaguchi *et al*., 2003). How synchrony is maintained is slowly being unravelled, and at present the peptide neurotransmitter VIP is a strong regulatory candidate. VIP is synthesised in a subset of SCN neurons (Abrahamson and Moore, 2001; Moore *et al*., 2002) in the ventrolateral region of the SCN, which is innervated by the retina. The VIPergic neurons provide an extensive intrinsic innervation of the SCN. A receptor for VIP, the $VPAC_2$ receptor, is highly expressed in the SCN (Kalamatianos *et al*., 2004). Approximately 60% of SCN neurons respond to VIP with changes in firing rate (Cutler *et al*., 2003; Reed *et al*., 2002). Mice lacking VIP or $VPAC_2$ receptor show diminished or abolished behavioural rhythms in constant darkness (Colwell *et al*., 2003; Itri *et al*., 2004). Both types of mutant mice exhibit markedly reduced percentages of rhythmic SCN neurons and the synchrony between the neurons is eliminated. Rhythmicity and synchrony can be restored in SCN neurons from VIP-deficient mice by daily application of a $VPAC_2$ agonist (Aton *et al*., 2005). Recently Maywood *et al.*

(2006) used real-time imaging of cellular circadian gene expression to show that VIPergic signalling in addition to cell-to-cell synchronization also plays a role in maintaining molecular timekeeping within individual SCN neurons.

In the present study we confirmed that circadian rhythms are disturbed in R6/2 mice at the age when neurodegenerative symptoms typically begin to appear. Importantly, we reveal that VIP/VPAC$_2$ receptor mRNAs and VIP immunostaining are markedly reduced in the SCN of the R6/2 mice. Since the circadian behavioural changes in the R6/2 mice are similar to those described in VIP- and VPAC$_2$ receptor-deficient mice it is tempting to suggest that impaired VIPergic signalling is responsible for the altered circadian behaviour. Only minor to moderate cell loss has been reported in the neostriatum and cerebral cortex of R6/2 mice (Iannicola *et al.*, 2000; Stack *et al.*, 2005; Turmaine *et al.*, 2000). Our analysis of sections immunostained for NeuN did not disclose a significant loss of SCN neurons. Thus the reductions in VIP and VPAC$_2$ receptor mRNA level are likely to be due to decreased gene expression rather than cell death.

Circadian sleep disturbances are a pathological feature of HD and the behavioural rhythm in R6/2 mice resembles the disrupted night/day activity pattern in HD patients (Morton *et al.*, 2005). Whether HD patients have altered VIP/VPAC$_2$ receptor expression and disrupted molecular oscillation and synchronization within the SCN remains to be determined. The mechanism behind the impaired VIP and VPAC$_2$ receptor gene expression in the SCN is unknown, but it is possible that progressive formation of neuronal intranuclear and cytoplasmic polyglutamine aggregates causes transcriptional down-regulation. Transcriptional alterations have definitely been implicated in the pathology of HD (Luthi-Carter *et al.*, 2000, 2002; Zucker *et al.*, 2005), but the involvement of huntingtin aggregates in these processes is not fully elucidated. While some studies indicated that

huntingtin aggregates may sequester polyglutamine-containing transcription factors, such as p53 (Steffan *et al*., 2000), TATA-binding protein (TBP) (Huang *et al*., 1998) and the transcriptional coactivator CREB-binding protein (CBP) (Kazantsev *et al*., 1999; Nucifora *et al*., 2001), and deplete their concentration in the nucleus causing altered gene expressions, other reports claim that inclusions are not necessary and suggest that soluble mutant huntingtin interacts with nuclear transcriptional factors and thereby induces transcriptional repression (Arrasate *et al*., 2004; Li *et al*., 2002; Steffan *et al*., 2000). Pathogenesis of disease in transgenic mice models of HD indicates the association of the severity of functional impairments with huntingtin inclusion accumulation. However, recently Sadri-Vakili *et al.* (2006) reported no differences in the transcript levels of specific genes (for preproenkephalin, presomatostatin and dopamine D1 receptor) between striatal neurons with intranuclear inclusions and without them in R6/2 mice. Therefore, their study suggests that the intranuclear inclusions are not the crucial component that triggers transcriptional dysregulation, and that free mutant huntingtin as well as cytoplasmic inclusions may both play a role in transcriptional dysregulation. A study from Kotliarova *et al*. (2005), on the other hand, shows co-localization of mutant huntingtin intranuclear and cytoplasmic inclusions with down-regulation of genes for neuropeptides (dopamine and cAMP-regulated phosphoprotein, oxytocin, vasopressin, cocaine-amphetamine-regulated transcript) in specific population of hypothalamic neurons. Taken together, there is no clear evidence of causal relation between transcriptional dysregulation with neither intranuclear nor cytoplasmic huntingtin aggregates.

In the SCN of R6/2 mice the mRNA levels of the clock gene *Bmal1* are attenuated and failed to exhibit circadian oscillation (Morton *et al*., 2005). Furthermore, SCN expression of *prokinecitin 2*, a transcriptional target of *Bmal1* encoding a neuropeptide, is reduced. It is

possible that the observed reduction in VIP mRNA and $VPAC_2$ mRNA in the SCN of R6/2 mice could be secondary to the decrease in *Bmal1*. Recently, we described that cells containing gonadotrophin releasing hormone (Papalexi *et al.*, 2005) and neurons expressing orexin are reduced in the hypothalamus of R6/2 mice (Petersén *et al.*, 2005), and clinical studies suggest that cells producing these peptides may be altered in HD (Markianos *et al.*, 2005, Petersén *et al.*, 2005). The neuropeptide orexin is a highly important regulator of sleep/wakefulness states and deficiency of orexin signalling causes narcolepsy in animal models as well as humans (Taheri *et al.*, 2002). Interestingly, the orexin-containing neurons are probably innervated by nerve fibres from VIPergic SCN neurons (Abrahamson *et al.*, 2001). This raises the possibility that impaired VIPergic input could play a role in the reduction of orexin neurons. VIP levels have been reported to be normal or increased in the caudate nucleus and frontal cortex of HD patients (Emson *et al.*, 1979; Mazurek *et al.*, 1997), but there are no published studies examining levels of VIP or $VPAC_2$ receptor in the HD hypothalamus. Future studies will hopefully clarify if HD patients have pathological changes within the SCN that lead to sleep disturbances.

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Legends to figures

Fig. 1: Disruption of circadian activity patterns in R6/2 mice.

Double-plotted actograms showing wheel-running activity of three representative wild type (WT) controls (first panel) and three R6/2 mice (third panel). After a 12 h/12 h light/dark schedule animals were transferred to constant darkness (transition indicated by DD) followed by re-entrainment to a 12 h/12h light/dark schedule (transition indicated by LD). The corresponding periodograms for each animal (second and fourth panels) show the dominant period during constant darkness. The amplitude of the peaks above the diagonal line (indicating the 99.9% confidence level) can be used as a rough guideline for significant periods.

Fig. 2: Reduction of VIP and $VPAC_2$ receptor mRNA in SCN of R6/2 mice. **A**: VIP mRNA concentration determined by real-time RT-PCR in SCN of wild type (WT) controls (white bar) and R6/2 mice (black bar) at 11 weeks of age. The VIP mRNA amount (arbitrary units) was normalized with the β2-microglobulin (β2MG) mRNA amount. Values are given as mean ± SEM (n = 7). ** p = 0.002. **B**: $VPAC_2$ receptor mRNA concentration determined by real-time RT-PCR in SCN of wild type controls (WT) (white bar) and R6/2 mice (black bar) at 11 weeks of age. The $VPAC_2$ receptor mRNA amount (arbitrary units) was normalized with the β2-microglobulin (β2MG) mRNA amount. Values are given as mean ± SEM (n = 7). ** p = 0.007.

Fig. 3: Loss of VIP immunoreactivity in cell bodies and nerve fibres in SCN of R6/2 mice. Coronal sections of the rostral (A, B), rostro-/middle (C, D), middle (E, F) and caudal (GH) SCN processed for VIP immunohistochemistry in wildtype (WT) (A, C, E, G) and R6/2 mice (B, D, F, H) at 11 weeks of age. 3 v: third ventricle, oc: optic chiasma. Scale bar: 100 µm.

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