Increased plasma melatonin in presymptomatic Huntington disease sheep (*Ovis aries*): Compensatory neuroprotection in a neurodegenerative disease?

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Abstract

Melatonin is a pleiotrophic hormone, synthesised primarily by the pineal gland under the control of the suprachiasmatic nuclei (SCN). It not only provides a hormonal signal of darkness but also has neuroprotective properties. Huntington's disease (HD) is a progressive neurodegenerative disorder characterised by abnormal motor, cognitive and psychiatric symptoms. There is growing evidence, particularly from animal models, that circadian rhythms may also be disturbed in HD. We measured two circadian-regulated hormones, melatonin and cortisol, in plasma samples collected around-the-clock from normal and presymptomatic transgenic HD sheep (*Ovis aries*) at 5 and 7 years of age, to assess SCN-driven rhythms and the effect of genotype, sex and age. Melatonin-related precursors and metabolites (tryptophan, serotonin, kynurenine) were also measured by liquid chromatography (LC)-mass spectrometry (MS). At 5 years of age in both rams and ewes, plasma melatonin levels were significantly elevated in HD sheep. In ewes measured 2 years later, there was still a significant elevation of nocturnal melatonin. Furthermore, the daytime baseline levels of melatonin were significantly higher in HD sheep. Since increased melatonin could have global beneficial effects on brain function, we suggest that the increased melatonin measured in presymptomatic HD sheep is part of an autoprotective response to mutant huntingtin toxicity that may account, at least in part, for the late onset of disease that characterises HD.

KEYWORDS

cognitive function, Huntington's disease, kynurenine, melatonin biosynthesis, metabolomics, neurodegenerative disease, serotonin, tryptophan metabolites

1 | INTRODUCTION

Melatonin is a pleiotropic hormone secreted primarily from the pineal gland at night.^{1,2} As well as playing an important role in circadian biology, melatonin has an emerging role in neuroprotection.^{3–6} Because patients with neurodegenerative diseases such as Alzheimer's and Parkinson's disease show disturbed

circadian rest-activity rhythms, melatonin has been considered as a potential treatment in both diseases.^{7–11} Huntington's disease (HD) patients also have significantly disrupted circadian rest-activity rhythms^{12,13} and sleep,^{14–17} with deterioration of sleep and rest-activity rhythms correlating with depression and cognitive impairment.¹⁸ Although HD patients show disturbed rest-activity patterns, whether the central pacemaker

in the hypothalamic SCN is disrupted or not remains an open question. Melatonin and cortisol are circadian hormones driven directly by the SCN clock, and assessment of their rhythms is considered a reliable proxy of SCN clock timing. Circulating melatonin levels have been measured in HD patients in a number of studies (see below). Whilst robust day-night rhythms of circulating melatonin were seen in these studies, melatonin levels in HD patients were typically decreased compared to those of normal subjects.

Huntington's disease is a late onset slowly progressing disorder, with symptoms typically appearing in the fourth decade of life. It has long been suspected that excitotoxic mechanisms may contribute to neurodegeneration and that energetic deficits exacerbate neurotoxicity.¹⁹ In particular, mitochondrial dysfunction and oxidative stress have been widely implicated as key factors for the neurodegeneration observed in HD.^{19–21} *In vitro*, melatonin effectively scavenges free radicals and enhances activity of antioxidant enzymes to reduce oxidative damage.^{22–24} As an antioxidant that protects mitochondrial bioenergetic function,²⁴ exogenous melatonin has been used in HD models and found to be neuroprotective both *in vitro*^{25–27} and *in vivo*.^{27–30} The melatonin receptor MT1 but not MT2 are depleted in brains of HD patients and R6/2 mice, and mRNA MT1 levels in mice are restored by melatonin treatment.²⁷ Thus, a decrease in melatonin signalling would potentially affect both circadian function and neurodegeneration in HD.³¹

We (Maywood et al¹³; Morton et al³²) and others (Kudo et al³³; Wang et al³⁴; Whittaker et al³⁵) showed that circadian rest-activity rhythms and SCN clock gene expression is severely disrupted in HD mouse models. However, the knock-in HD mice lines we are currently using to study circadian dysregulation in HD³⁶ are bred on a pure C57/Bl6 background that does not synthesise melatonin,³⁷ so we do not have direct evidence that there is disturbed timing of hormone production by the SCN. More recently, we showed that HD sheep exhibit circadian behavioural abnormalities.³⁸ Because they synthesise melatonin and are large enough to collect the multiple blood samples necessary for an around-the-clock 24 hour study, sheep are ideal for studying circadian SCN-driven melatonin rhythms.

In this study, we aimed to see if there were alterations in the timing of the SCN-driven circadian rhythms in HD sheep. Melatonin and cortisol, as well as the melatonin precursors, tryptophan, serotonin and kynurenine were measured in the plasma of 5 year old HD and control rams, and 5 and 7 year old HD and control ewes in a controlled “around-the-clock” study where samples were taken hourly for 26 or 27 hours. We found that whereas serotonin and cortisol levels were normal or lower than normal, melatonin levels in HD sheep were significantly elevated compared to control sheep, as were tryptophan and kynurenine. This raises the possibility that endogenous melatonin is part of an orchestrated

auto-neuroprotective mechanism that may in part explain the late timing of onset of HD.

2 | MATERIALS AND METHODS

2.1 | Animals

We investigated age- and sex-matched HD and control sheep from the OVT73 line that carries a full-length human transgene with a mutation (a CAG repeat of 73) that would cause juvenile-onset HD. Sheep were aged 5 years (14 HD, 10 control rams; 16 HD, 16 control ewes), or 7 years (N = 16 HD, N = 16 control sheep). The 7 year old group included 10 control and 12 HD ewes that were previously sampled at 5 years. Samples for melatonin and cortisol analysis from rams were taken in parallel with those used for a previous metabolomics study.³⁹

2.2 | Animal husbandry

Sheep were obtained from a mixed HD/control ram flock maintained outdoors at South Australian Research and Development Institute (SARDI) in accordance with OGTR approval NLRD-1037/2003 and NLRD-7574/2015. For circadian blood sampling, sheep were transported from SARDI to the Preclinical Imaging and Research Laboratory (PIRL) at Gilles Plains, South Australia. Animal husbandry and blood sampling was performed in accordance with AEC approvals SAM93 (SAHMRI) and 07/14 (PIRSA). To ensure minimal dietary change throughout the period prior to blood sampling, for at least 4 weeks prior to transport, sheep were fed a defined ration of food that was continued once the sheep had been relocated to PIRL. At PIRL, rams were transferred into undercover pens for at least 1 week acclimatisation before being transferred to indoor holding on the day prior to cannula insertion. Ewes were transferred directly into indoor holding pens for a week of acclimatisation.

Indoors, sheep were housed in individual metabolic crates. During indoor acclimatisation, the light cycle matched the outdoor light/dark cycle. For all groups, on the day of cannulation, the lights were turned off in the evening (at sunset), and remained off until the end of the blood collection period ~45 hours later (see below). Samples were collected in dim light using red head torches.

2.3 | Around-the-clock plasma sample collection

Blood collection commenced at 14:00 the day following placement of the cannulae, as previously described.³⁹ Blood

samples were collected on the hour (± 5 minutes) for 25 hours (rams) or 26 hours (ewes). An appropriate dead volume of blood was discarded, and then, 10 mL samples of blood were withdrawn into a fresh syringe and transferred to lithium heparin tubes. Samples were centrifuged immediately at 1620 g for 10 minutes at 4°C. Plasma was aliquoted and stored at -20°C (melatonin/cortisol) and -80°C (metabolomics), prior to being shipped on dry ice to the UK for analysis.

2.4 | Melatonin and cortisol measurements

Hormone concentrations were measured by radioimmunoassay as previously described.⁴⁰ The limit of detection for the plasma melatonin assay was 3.9 ± 0.8 pg/mL (rams), 4.4 ± 2.2 pg/mL (5 year old ewes) and 4.6 ± 2.0 pg/mL (7 year old ewes). The limit of detection for the plasma cortisol assay was 1.6 ± 0.8 nmol/L (rams); 1.3 ± 0.7 nmol/L (5 year old ewes) and 1.0 ± 0.5 nmol/L (7 year old ewes). For both assays, the inter-assay coefficients of variation of low, medium, high and very high QCs were $<15\%$ (range 6.1%–14.7%). One HD ram and one 7 year old control ewe had undetectable and abnormally high (2–3 fold) melatonin levels, respectively, and were excluded from all the analysis.

To control for inter-animal differences in circadian phase, all the hormone and metabolite data were replotted with respect to each animal's dim light melatonin onset time (DLMO), that is considered a more reliable marker of circadian SCN-phase, than clock time of day. The onset of melatonin (DLMO) was calculated using the 25% threshold method as described in Sletten et al.⁴¹

2.5 | Metabolite measurements

A range of plasma metabolites was measured using targeted liquid chromatography/mass spectroscopy metabolomics as previously described.^{39,42} Plasma tryptophan, serotonin and kynurenine concentrations were measured across the 24-hour day. Two sheep that were outliers in a PCA plot of the metabolite data were confirmed to be ill during sampling and were thus excluded from any subsequent analysis.

2.6 | Statistical analysis

Unless otherwise stated, all data are presented as mean $\pm$ SEM. Parameters derived from analysis of the melatonin rhythm (DLMO, DLMoff, duration, AUC) were compared between genotypes using either unpaired or paired Student's *t* test, as appropriate. Individual melatonin, cortisol, and metabolite levels were analysed in R version 3.1.2 using the linear models and ANOVA methods.³⁹ Linear models were fitted to

the genotype and time of day, with the animal as covariate. Significant differences for time of day, genotype and their interaction were determined using 2-way ANOVA. *P*-values were corrected for multiple comparisons according to the Benjamini-Hochberg False Discovery Rate (FDR). Analytes were considered as significant at a cut-off of FDR-adjusted *P* value $<.05$. To assess 24-hour rhythmicity, cosinor analysis was performed on the analyte profiles of each sheep as well as on the mean *z*-score values for each genotype and group.⁴² Peak time (acrophase, amplitude and significance of a cosine fit or cosine + linear fit ($P < .05$) were determined in each case (MATLAB and Bioinformatics Toolbox Release 2016a, The MathWorks, Inc., Natick, MA, USA).

3 | RESULTS

3.1 | Plasma melatonin is elevated in HD sheep

There was a clear nocturnal rise in melatonin in all sheep (Figure 1), with levels returning to baseline during the day-time. Notably, melatonin levels were significantly higher in HD sheep of both sexes and both ages compared to control sheep (Figures 1 and 2, Tables 1 and 2).

3.2 | Increased melatonin production with age in HD sheep

Baseline melatonin concentrations for 5 year old rams and ewes of both genotypes were comparable, being on or below the detection limit of the assay.

Melatonin levels were higher in 7 than 5 year old ewes irrespective of genotypes (Figures 1C,D and 2C,D; Tables 1 and 2). At 7 years, however, the baseline levels of melatonin were further elevated in HD (23.1 ± 6.6 pg/mL, mean $\pm$ SEM, $n = 16$) compared to control ewes (5.9 ± 0.9 pg/mL, $n = 14$). Of the 7 year old HD ewes, 7/16 had high baseline concentrations (46.4 ± 9.6 pg/mL). Of these, 4/7 had been sampled previously but showed no evidence of high baseline values at 5 years.

3.3 | Timing of melatonin rhythms

When melatonin levels were aligned to each animal's biological time using their DLMO, elevated melatonin levels in HD sheep remained significant (Figure 1 right panels). At 5 years, neither DLMO nor dim light melatonin offset (DLMoff) was different in any group. Likewise, at 7 years there was no significant change in the timing of the melatonin rhythm between genotypes although there was a trend ($P = .055$) to a delay in

FIGURE 1 Plasma melatonin levels in control and Huntington's disease (HD) sheep collected across 24 h. Plasma melatonin levels in control (black symbols) and HD (red symbols) rams (A, B) or ewes at 5 y (C, D) or 7 y (E, F). Data are shown as mean $\pm$ SEM and are plotted against clock (A, C, E) or DLMO-corrected (B, D, F) time. Where error bars are not visible they are occluded by the symbol

FIGURE 2 Plasma melatonin levels in repeated control and Huntington's disease (HD) sheep. Plasma melatonin levels measured in samples taken from control (black symbols) and HD (red symbols) "repeated" ewes at 5 y (A, B) and 7 y (C, D). Data are shown as mean $\pm$ SEM and are plotted against clock (A, C) or DLMO-corrected (B, D) time. Where error bars are not visible they are occluded by the symbol

TABLE 1 Plasma melatonin areas under the curve from all groups of sheep

Group	Age (y)	AUC (pg/mL.h $\pm$ SEM)		AUC (pg/mL.h $\pm$ SEM)	
		Control	n	Huntington's disease	n
Rams	5	483 $\pm$ 81	12	701 $\pm$ 66*	12
Ewes	5	633 $\pm$ 77	16	903 $\pm$ 95*	16
Repeated	5	646 $\pm$ 77	10	961 $\pm$ 117*	12
Ewes	7	1109 $\pm$ 117 [†]	16	1589 $\pm$ 190* [†]	16
Repeated	7	1122 $\pm$ 154 [†]	10	1604 $\pm$ 250 [†]	12

* $P < .05$ compared to controls; [†] $P < .05$ compared to 5 y.

TABLE 2 ANOVA analyses (genotype and time of day) and 24 h mean concentrations ($\pm$ SEM) of the measured analytes in controls and Huntington's disease (HD) sheep in all study groups

Sex	Age (y)	Metabolite	FDR_genotype	FDR_time	FDR_interaction	Control		HD	
						Mean $\pm$ SEM	n	Mean $\pm$ SEM	n
Rams	5	Tryptophan (μ mol/L)	1.7E-03	1.6E-32	4.3E-01	36.49 $\pm$ 0.84	10	38.55 $\pm$ 0.71	12
		Kynurenine (μ mol/L)	2.1E-06	2.1E-23	8.2E-01	2.36 $\pm$ 0.11		2.64 $\pm$ 0.08	
		Serotonin (μ mol/L)	2.9E-01	4.0E-02	1.4E-01	6.59 $\pm$ 0.32		6.11 $\pm$ 0.27	
		Melatonin (pmol/L)	1.2E-04	1.2E-25	3.2E-02	122.11 $\pm$ 8.10		175.60 $\pm$ 9.74	
		Cortisol (nmol/L)	4.6E-01	4.9E-01	9.7E-01	10.19 $\pm$ 0.43		10.06 $\pm$ 0.39	
Ewes	5	Tryptophan (μ mol/L)	2.1E-45	7.5E-99	8.1E-01	38.53 $\pm$ 0.50	15	43.46 $\pm$ 0.42	16
		Kynurenine (μ mol/L)	1.1E-06	9.0E-39	8.7E-01	1.79 $\pm$ 0.04		1.90 $\pm$ 0.03	
		Serotonin (μ mol/L)	2.2E-11	2.4E-01	8.7E-01	7.13 $\pm$ 0.16		6.09 $\pm$ 0.12	
		Melatonin (pmol/L)	3.5E-11	4E-149	9.7E-05	123.84 $\pm$ 5.95		162.37 $\pm$ 7.89	
		Cortisol (nmol/L)	1.9E-02	4.9E-03	1.3E-01	14.75 $\pm$ 0.78		12.70 $\pm$ 0.52	
Ewes Repeated	5	Tryptophan (μ mol/L)	1.5E-47	7.7E-64	6.4E-01	38.84 $\pm$ 0.68	10	45.40 $\pm$ 0.48	12
		Kynurenine (μ mol/L)	1.2E-09	8.4E-20	6.9E-01	1.71 $\pm$ 0.05		1.88 $\pm$ 0.03	
		Serotonin (μ mol/L)	1.4E-15	5.0E-01	5.5E-01	7.55 $\pm$ 0.21		5.99 $\pm$ 0.14	
		Melatonin (pmol/L)	1.0E-08	2.5E-95	6.1E-02	128.97 $\pm$ 7.07		171.85 $\pm$ 9.61	
		Cortisol (nmol/L)	1.6E-01	1.5E-01	5.0E-01	14.68 $\pm$ 0.87		13.16 $\pm$ 0.62	
Ewes	7	Tryptophan (μ mol/L)	1.7E-08	1.3E-40	4.1E-01	38.24 $\pm$ 0.41	14	40.32 $\pm$ 0.39	16
		Kynurenine (μ mol/L)	2.1E-15	9.0E-16	2.3E-05	1.76 $\pm$ 0.03		1.95 $\pm$ 0.03	
		Serotonin (μ mol/L)	2.4E-01	3.4E-01	4.9E-02	7.23 $\pm$ 0.12		7.05 $\pm$ 0.16	
		Melatonin (pmol/L)	2.8E-39	7E-114	1.4E-02	188.75 $\pm$ 10.34		343.28 $\pm$ 15.13	
		Cortisol (nmol/L)	1.9E-04	1.2E-02	2.1E-03	35.61 $\pm$ 1.80		28.48 $\pm$ 1.50	
Ewes Repeated	7	Tryptophan (μ mol/L)	1.4E-27	7.3E-36	5.0E-01	37.30 $\pm$ 0.51	10	41.82 $\pm$ 0.47	12
		Kynurenine (μ mol/L)	2.0E-02	1.1E-08	5.6E-04	1.89 $\pm$ 0.03		1.95 $\pm$ 0.03	
		Serotonin (μ mol/L)	8.0E-14	5.6E-04	4.6E-02	7.42 $\pm$ 0.15		6.25 $\pm$ 0.11	
		Melatonin (pmol/L)	6.5E-22	1.6E-74	4.9E-01	187.46 $\pm$ 12.63		325.22 $\pm$ 18.28	
		Cortisol (nmol/L)	2.0E-02	3.6E-01	2.0E-02	35.77 $\pm$ 2.24		30.12 $\pm$ 1.87	

^a P -values were corrected for multiple comparisons according to the Benjamini-Hochberg False Discovery Rate (FDR). Metabolites significant at a FDR-adjusted P value $< .05$ are highlighted in bold

melatonin offset in HD sheep (10.3 ± 0.5 dec.hours) compared to the controls (9.0 ± 0.4 dec.hours). The effect of age on melatonin timing is best studied in the sub-cohort of sheep that were sampled at both 5 and 7 years (Figure 2). Neither the control nor HD sheep showed significant differences in DLMO or

DLMOff with age although there was a trend ($P = .076$) to a phase delay of DLMO in 7 year old HD ewes compared to that seen at 5 years of age.

Analysis of the melatonin rhythms revealed a significant fit to a cosine curve in all the sheep studied, irrespective of

TABLE 3 Cosinor rhythm analysis (acrophase/amplitude) for each analyte in each study group. The number of sheep (n) showing robust 24 h rhythms in each group is also shown

Sex	Age (y)	Metabolite	Control			Huntington's disease		
			n	Peak time (dec. h)	Amplitude	n	Peak time (dec. h)	Amplitude
Rams	5	Tryptophan (μmol/L)	9	22.20 ± 0.55	6.9 ± 1.0	11	23.38 ± 1.9	6.3 ± 0.9
		Kynurenine (μmol/L)	6	24.34 ± 0.72	0.58 ± 0.07	12	24.51 ± 1.5	0.52 ± 0.06
		Serotonin (μmol/L)	4	21.43 ± 2.2	1.0 ± 0.5	0		
		Melatonin (pmol/L)	10	4.90 ± 1.0	132.2 ± 20.2	12	2.76 ± 0.56	192.0 ± 22.0
		Cortisol (nmol/L)	0			0		
Ewes	5	Tryptophan (μmol/L)	15	23.11 ± 0.53	6.49 ± 0.64	14	22.81 ± 0.24	6.79 ± 0.54
		Kynurenine (μmol/L)	11	24.36 ± 0.38	0.31 ± 0.05	14	24.63 ± 0.49	0.31 ± 0.03
		Serotonin (μmol/L)	5	21.69 ± 2.57	1.69 ± 0.23	4	18.14 ± 2.57	1.13 ± 0.10
		Melatonin (pmol/L)	15	2.71 ± 0.44	127.65 ± 10.85	16	1.94 ± 0.51	179.18 ± 16.10*
		Cortisol (nmol/L)	0			0		
Ewes Repeated	5	Tryptophan (μmol/L)	10	22.83 ± 0.37	6.56 ± 0.62	11	23.01 ± 0.28	6.97 ± 0.68
		Kynurenine (μmol/L)	7	24.71 ± 0.51	0.29 ± 0.06	11	24.80 ± 0.57	0.32 ± 0.03
		Serotonin (μmol/L)	4	23.81 ± 1.87	1.70 ± 0.29	3	20.31 ± 1.95	1.06 ± 0.10
		Melatonin (pmol/L)	10	2.87 ± 0.62	131.95 ± 8.48	12	2.28 ± 0.65	187.10 ± 19.59*
		Cortisol (nmol/L)	0			0		
Ewes	7	Tryptophan (μmol/L)	11	20.93 ± 1.48	5.49 ± 0.40	15	20.69 ± 0.83	5.73 ± 0.58
		Kynurenine (μmol/L)	12	22.90 ± 1.50	0.26 ± 0.04	14	21.72 ± 0.68	0.28 ± 0.03
		Serotonin (μmol/L)	4	16.09 ± 0.80	1.03 ± 0.16	3	15.92 ± 3.46	2.00 ± 1.33
		Melatonin (pmol/L)	14	2.46 ± 0.41	216.08 ± 18.25	16	3.56 ± 0.48	294.82 ± 34.05
		Cortisol (nmol/L)	5	13.26 ± 3.56	17.29 ± 6.49	5	16.38 ± 2.64	9.31 ± 4.60
Ewes Repeated	7	Tryptophan (μmol/L)	7	22.25 ± 0.76	4.90 ± 0.49	11	20.16 ± 1.06	5.64 ± 0.73
		Kynurenine (μmol/L)	8	20.87 ± 1.53	0.25 ± 0.04	10	21.57 ± 0.90	0.30 ± 0.03
		Serotonin (μmol/L)	2	15.70 ± 1.81	1.27 ± 0.19	2	19.37 ± 0.44	0.67 ± 0.05
		Melatonin (pmol/L)	10	2.75 ± 0.50	217.67 ± 24.50	12	3.54 ± 0.60	298.73 ± 42.15
		Cortisol (nmol/L)	3	15.65 ± 5.92	23.66 ± 9.37	3	17.31 ± 3.68	11.01 ± 6.37

genotype, sex or age. In agreement with the DLMO and DLMOFF data, there were no significant changes in the timing of the melatonin peak between genotypes in any of the study groups (Table 3). The cosinor-derived amplitude of the melatonin rhythm, however, was significantly increased in the 5 year old HD ewes ($P < .05$) with a trend to be increased in HD rams ($P = .06$) and 7 year old HD ewes ($P = .06$).

3.4 | Decreased cortisol secretion in HD ewes

Cortisol levels in control and HD rams were similar at 5 years, but significantly lower in HD compared to control ewes at both ages (Table 2). Cortisol was secreted in a pulsatile manner throughout the day. Cosinor analysis showed that none of the 5 year old rams or ewes displayed significant

24 hour cortisol rhythms (Table 3). In the 7 year old ewes, however, a few control and HD sheep (25%-36%) exhibited 24 hour cosine rhythms. The cortisol acrophase times were highly variable, with no significant differences in timing or amplitude between the control and HD ewes.

3.5 | Significant changes in melatonin pathway metabolites in HD sheep

Three metabolites from the melatonin biosynthetic pathway were measured, tryptophan, kynurenine and 5-hydroxytryptamine (Figures 3-5 respectively, and Figures S1-S3 respectively). There were significant changes in all three metabolites in HD compared to control sheep (see below). There were no qualitative differences in metabolite profiles when data were corrected for DLMO (Figures 3-5, right panels).

FIGURE 3 Plasma tryptophan levels in control and Huntington's disease (HD) sheep. Plasma tryptophan levels measured in rams (A, B); 5 y ewes (C, D) and 7 y ewes (E, F), plotted against clock (A, C, E) or DLMO-corrected (B, D, F) time. Data from control (black symbols) and HD (red symbols) sheep are shown as mean $\pm$ SEM. Where error bars are not visible they are occluded by the symbol

Tryptophan was significantly higher in HD than control sheep in all groups (Figure 3, Figure S1, Table 2). The increase in tryptophan occurred across the 24 hour day in the 5 and 7 year old ewes and was not restricted to the night-time, when the increase in melatonin was seen. The elevation of tryptophan in HD ewes was greater at 5 years than it was at 7 years. There was also a significant time of day variation in tryptophan levels in all the study groups (Table 2). Cosinor analysis revealed robust 24 hour tryptophan rhythms in all study groups with a high proportion of sheep (70%-100%) exhibiting cosine rhythms (Table 3). The peak times of the tryptophan rhythms occurred around 23.00 hours, with most peaking between melatonin onset and peak.

Kynurenine levels were significantly higher in HD than control sheep for all groups (Figure 4, Figure S2). As with tryptophan, the increase in kynurenine in the 5 year old sheep was global across the 24 hour period (Figure 4B; Figure S2B, Table 2). In all groups, there was a significant time of day variation in kynurenine levels. In both 5 and 7 year old ewes (Figure 4C, 4, Figure S2C, E), the increase in kynurenine in HD was greatest in the afternoon and

the first half of the night. As with tryptophan, kynurenine exhibited significant fits to a cosine curve in most of the sheep, irrespective of genotype (Table 3). The kynurenine acrophase times ($\approx$ 24.00 hours) occurred after the tryptophan peak in 90% of sheep.

Serotonin levels were similar in HD and control rams (Figure 5A, B; Figure S3; Table 2). At both 5 and 7 years serotonin was lower in HD than control ewes (Figure 5C, E, Table 2, Figure S3C, E). There was no effect of time of day on serotonin levels in the 5 and 7 year old ewe groups. The rams and 7 year old repeated ewes, however, showed a small significant variation across time (Table 2). Only a few sheep showed significant cosine rhythms (0%-40%), with no effect of genotype, sex or age on the prevalence of these 24 hour rhythms (Table 3).

4 | DISCUSSION

Circadian production of plasma melatonin was significantly elevated in HD sheep of both sexes at 5 years. By

FIGURE 4 Plasma kynurenine levels in control and Huntington's disease (HD) sheep. Plasma kynurenine levels measured in rams (A, B); 5 y ewes (C, D) and 7 y ewes (E, F), plotted against clock (A, C, E) or DLMO-corrected (B, D, F) time. Data from control (black symbols) and HD (red symbols) sheep are shown as mean $\pm$ SEM. Where error bars are not visible they are occluded by the symbol

7 years, elevated melatonin levels were still evident, and in addition, baseline melatonin had increased in HD sheep. Metabolites in the melatonin pathway were also dysregulated. There were significant increases in kynurenine and tryptophan in HD sheep (although the pattern/timing of this did not coincide with the increased night-time melatonin production), and plasma levels of serotonin and cortisol were significantly lower in some HD compared to control ewes.

The changes in melatonin were opposite to those we expected, given that HD patients have been reported to show normal or reduced levels of melatonin.⁴³⁻⁴⁵ However, the sheep used for this study were several years away from predicted symptomatic onset of disease, whereas disease was already manifest in most HD patients used in these studies. It is, of course, possible that the changes we see in the sheep are species-specific. Nevertheless, this raises the intriguing possibility that the increase in melatonin in the sheep is part of a compensatory process in the presymptomatic HD brain that confers endogenous neuroprotection against the mutant protein. There is considerable evidence for the existence of such a phenomenon in presymptomatic HD mice that, despite

ongoing expression of the mutation that eventually causes disease, are less susceptible to neurotoxins than control mice.⁴⁶ Melatonin, with its well-documented neuroprotective properties,⁴⁷⁻⁴⁹ is a prime candidate for playing a role in such autoprotection.

The sheep used in this study were aged 7 years at the second sampling period. The age at onset of disease is not yet known for the HD sheep. The natural lifespan of a domestic sheep depends on its lifestyle, but sheep kept as pets can easily live for 10-12 years, and often live much longer (We have several brains obtained *post mortem* from pet sheep aged >16 years). The oldest sheep in the OVT73 flock are 11 years. Both control and HD sheep are now showing signs of ageing (unpublished observations), but the HD sheep in this group do not show HD-specific signs. The sheep used in this study should therefore be considered prodromal/presymptomatic with at least 4 years to age of onset. Nevertheless, there is progressive HD-like brain pathology (aggregates) in the HD sheep at 3-5 years,⁵⁰ and aggregate load increases with age. By 7 years of age, there are numerous aggregates in HD sheep cortex and increasing numbers in neostriatum (A. J. Morton, unpublished data).

FIGURE 5 Plasma serotonin levels in control and Huntington's disease (HD) sheep. Plasma serotonin levels measured in rams (A, B); 5 y ewes (C, D) and 7 y ewes (E, F), plotted against clock (A, C, E) or DLMO-corrected (B, D, F) time. Data from control (black symbols) and HD (red symbols) sheep are shown as mean $\pm$ SEM. Where error bars are not visible they are occluded by the symbol

The stage of HD is likely to be critical and may explain why some of the human melatonin data are so variable. In an early study, Aziz et al⁴⁴ found that serum melatonin levels in early stage HD patients were phase-advanced, although peak levels were not different from those of control subjects. A study of end-stage patients found that DLMO was absent in 5/10 and delayed or diminished in another four patients.⁵¹ A larger study showed that plasma melatonin was reduced in stage II/III HD patients, with a trend for a decrease being seen in premanifest patients.⁴⁵ In this latter study, the timing of melatonin onset was more variable in patients than in control subjects, with onset advanced in some patients, as shown by Aziz et al,⁴⁴ but delayed in others. A fourth study showed a phase delay with a lowered melatonin amplitude in stage III HD patients.⁴³ All of these studies measuring melatonin in HD patients suffer from small numbers, lack of age- and sex-matched controls, and medication confounds. In addition, inadequate control of the light conditions before and during melatonin sampling (for which dim light conditions are needed) may have confounded the measurements. Alternatively, if melatonin increases in presymptomatic HD (as it does in HD sheep) before decreasing after the onset of disease, this would confound the results.

Only two human studies included presymptomatic patients^{44,45} and neither found a significant difference between melatonin levels in presymptomatic patients and control subjects. Interestingly, one of these studies⁴⁴ showed a melatonin rhythm that resembled the one we obtained from HD sheep, with the peak amplitude of melatonin being higher and occurring later in HD patients. This, however, did not reach statistical significance, perhaps because of the low sample number ($n = 9$) as well as the well-known inter-individual difference in melatonin amplitude.⁵²⁻⁵⁴ It would be interesting to repeat this clinical study using a larger sample size and to also include patients "at risk and near-to-onset" that would be equivalent to that of HD sheep.

Blood levels of melatonin correlate well with the CSF levels⁵⁵ so it is likely that the brains of HD sheep were exposed to elevated levels of melatonin. All the evidence to date suggests that an endogenous increase in melatonin is likely to be beneficial. In vitro, melatonin has direct antioxidative actions mediated by its free radical scavenging activity.²⁵ Melatonin may also act indirectly. For example, numerous studies report an increase in sirtuins after melatonin treatment.⁵⁶⁻⁵⁸ There is convincing evidence from a number of HD studies that SIRT1 protects both cellular and animal models from mutant huntingtin toxicity.^{59,60}

In addition to melatonin, plasma levels of tryptophan and kynurenine were significantly increased in the 5 and 7 years HD sheep. There is some evidence in HD patients that tryptophan pathways are disturbed.^{61,62} Whilst there are biochemical pathways linking both tryptophan and kynurenine with melatonin biosynthesis, our study was not specifically designed to show a cause-effect relationship with melatonin. The source of the increased melatonin is unknown. The high baseline levels of plasma melatonin as well as the higher night-time levels observed in the 7 years HD sheep suggest an adrenergic activation of the pineal via the superior cervical ganglia (SCG) postsympathetic innervation in the older sheep. This would be worthy of further investigation. It is also possible that the altered amplitude arises as a consequence of disruption of the efferent pathways from the SCN, within the SCN itself, or downstream to the pineal. Finally, an intriguing possibility is that melatonin may not be coming from the pineal itself, but may be being produced directly by mitochondria in the brain.⁶³ The relevance of this to HD is not yet clear, although it is interesting to note that there is increased energy metabolism in presymptomatic HD mice,⁶⁴ and they are also more resistant to mitochondrial toxins at presymptomatic stages.⁴⁶

Huntington's disease is a late onset disease, and we speculate that the endogenous increase in plasma melatonin in HD sheep is part of a neuroprotective mechanism that may contribute to delaying the onset of disease. Onset of disease may coincide with the decrease in melatonin and phase delay in production seen in HD patients. There has not been a clinical trial of melatonin as a therapy for HD patients with sleep disturbances, but our data support the existing clinical evidence to suggest there are important HD-related changes in melatonin levels at play and that further investigation in HD patients is warranted.

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CONFLICT OF INTEREST

AJM, TRK, CSB, SR have no conflict of interest. DJS and BM are co-directors of Stockgrand Ltd.

AUTHOR CONTRIBUTIONS

AJM and DJS designed the study, collated the data and wrote the paper. SR, CSB and TRK organised the sample

collection. DJS and BM analysed the data. All authors critically reviewed the manuscript and approved the final article.

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REFERENCES

1. Brzezinski A. Melatonin in humans. *N Engl J Med.* 1997; 336(3):186-195.
2. Krauchi K, Cajochen C, Pache M, Flammer J, Wirz-Justice A. Thermoregulatory effects of melatonin in relation to sleepiness. *Chronobiol Int.* 2006;23(1-2):475-484.
3. Ansari Dezfouli M, Zahmatkesh M, Farahmandfar M, Khodaghali F. Melatonin protective effect against amyloid beta-induced neurotoxicity mediated by mitochondrial biogenesis; involvement of hippocampal sirtuin-1 signaling pathway. *Physiol Behav.* 2019;204:65-75.
4. Marra A, Mcgrane TJ, Henson CP, Pandharipande PP. Melatonin in critical care. *Crit Care Clin.* 2019;35(2):329-340.
5. Sulkava S, Muggalla P, Sulkava R, et al. Melatonin receptor type 1a gene linked to Alzheimer's disease in old age. *Sleep.* 2018;41(7), zsy103. <https://doi.org/10.1093/sleep/zsy103>
6. Vincent B. Protective roles of melatonin against the amyloid-dependent development of Alzheimer's disease: a critical review. *Pharmacol Res.* 2018;134:223-237.
7. Burgess HJ, Emens JS. Drugs used in circadian sleep-wake rhythm disturbances. *Sleep Med Clin.* 2018;13(2):231-241.
8. Cardinali DP, Vigo DE, Olivar N, Vidal MF, Brusco LI. Melatonin therapy in patients with Alzheimer's disease. *Antioxidants (Basel).* 2014;3(2):245-277.
9. Sanchez-Barcelo EJ, Rueda N, Mediavilla MD, Martinez-Cue C, Reiter RJ. Clinical uses of melatonin in neurological diseases and mental and behavioural disorders. *Curr Med Chem.* 2017;24(35):3851-3878.
10. Shi Y, Fang YY, Wei YP, et al. Melatonin in synaptic impairments of Alzheimer's disease. *J Alzheimers Dis.* 2018;63(3):911-926.
11. Zhang W, Chen XY, Su SW, et al. Exogenous melatonin for sleep disorders in neurodegenerative diseases: a meta-analysis of randomized clinical trials. *Neurol Sci.* 2016;37(1):57-65.
12. Diago EB, Martinez-Horta S, Lasasa SS, et al. Circadian rhythm, cognition, and mood disorders in Huntington's disease. *J Huntington's Dis.* 2018;7(2):193-198.
13. Morton AJ, Wood NI, Hastings MH, Hurelbrink C, Barker RA, Maywood ES. Disintegration of the sleep-wake cycle and circadian timing in Huntington's disease. *J Neurosci.* 2005;25(1):157-163.
14. Hansotia P, Wall R, Berendes J. Sleep disturbances and severity of Huntington's disease. *Neurology.* 1985;35(11):1672-1674.
15. Lazar AS, Panin F, Goodman AO, et al. Sleep deficits but no metabolic deficits in premanifest Huntington's disease. *Ann Neurol.* 2015;78(4):630-648.
16. Morton AJ. Circadian and sleep disorder in Huntington's disease. *Exp Neurol.* 2013;243:34-44.
17. Piano C, Mazzucchi E, Bentivoglio AR, et al. Wake and sleep EEG in patients with Huntington disease: an eLORETA study and review of the literature. *Clin EEG Neurosci.* 2017;48(1):60-71.

18. Aziz NA, Anguelova GV, Marinus J, Lammers GJ, Roos RA. Sleep and circadian rhythm alterations correlate with depression and cognitive impairment in Huntington's disease. *Parkinsonism Relat Disord*. 2010;16(5):345-350.
19. Bates G, Tabrizi S, Jones L. *Huntington's Disease*, 4th edn. Oxford: Oxford University Press; 2014.
20. Carmo C, Naia L, Lopes C, Rego AC. Mitochondrial dysfunction in Huntington's disease. *Adv Exp Med Biol*. 2018;1049:59-83.
21. Essa MM, Moghadas M, Ba-Omar T, et al. Protective effects of antioxidants in Huntington's disease: an extensive review. *Neurotox Res*. 2019;35(3):739-774.
22. Milczarek R, Hallmann A, Sokolowska E, Kaletha K, Klimek J. Melatonin enhances antioxidant action of alpha-tocopherol and ascorbate against nadph- and iron-dependent lipid peroxidation in human placental mitochondria. *J Pineal Res*. 2010;49(2):149-155.
23. Reiter RJ, Cabrera J, Sainz RM, Mayo JC, Manchester LC, Tan DX. Melatonin as a pharmacological agent against neuronal loss in experimental models of Huntington's disease, Alzheimer's disease and parkinsonism. *Ann NY Acad Sci*. 1999;890:471-485.
24. Reiter RJ, Tan DX, Rosales-Corral S, Galano A, Zhou XJ, Xu B. Mitochondria: central organelles for melatonin's antioxidant and anti-aging actions. *Molecules*. 2018;23(2):pii: E509.
25. Galano A, Tan DX, Reiter RJ. Melatonin: a versatile protector against oxidative DNA damage. *Molecules*. 2018;23(3):pii: E530.
26. Ganie SA, Dar TA, Bhat AH, et al. Melatonin: a potential anti-oxidant therapeutic agent for mitochondrial dysfunctions and related disorders. *Rejuvenation Res*. 2016;19(1):21-40.
27. Wang X, Sirianni A, Pei Z, et al. The melatonin mt1 receptor axis modulates mutant Huntingtin-mediated toxicity. *J Neurosci*. 2011;31(41):14496-14507.
28. Antunes WE, Jesse RC, Bortolatto FC, Nogueira WC. Correlations between behavioural and oxidative parameters in a rat quinolinic acid model of Huntington's disease: protective effect of melatonin. *Eur J Pharmacol*. 2013;701(1-3):65-72.
29. Chakraborty J, Nthenge-Ngumbau DN, Rajamma U, Mohanakumar KP. Melatonin protects against behavioural dysfunctions and dendritic spine damage in 3-nitropropionic acid-induced rat model of Huntington's disease. *Behav Brain Res*. 2014;264:91-104.
30. Zhao Z, Lu C, Li T, et al. The protective effect of melatonin on brain ischemia and reperfusion in rats and humans: in vivo assessment and a randomized controlled trial. *J Pineal Res*. 2018;65(4):e12521.
31. Bartlett DM, Cruickshank TM, Hannan AJ, Eastwood PR, Lazar AS, Ziman MR. Neuroendocrine and neurotrophic signaling in Huntington's disease: implications for pathogenic mechanisms and treatment strategies. *Neurosci Biobehav Rev*. 2016;71:444-454.
32. Maywood ES, Fraenkel E, Mcallister CJ, et al. Disruption of peripheral circadian timekeeping in a mouse model of Huntington's disease and its restoration by temporally scheduled feeding. *J Neurosci*. 2010;30(30):10199-10204.
33. Kudo T, Schroeder A, Loh DH, et al. Dysfunctions in circadian behavior and physiology in mouse models of Huntington's disease. *Exp Neurol*. 2011;228(1):80-90.
34. Wang HB, Loh DH, Whittaker DS, Cutler T, Howland D, Colwell CS. Time-restricted feeding improves circadian dysfunction as well as motor symptoms in the q175 mouse model of Huntington's disease. *eNeuro*. 2018;5(1):pii: ENEURO.0431-17.2017.
35. Whittaker DS, Loh DH, Wang HB, et al. Circadian-based treatment strategy effective in the BACHD mouse model of Huntington's disease. *J Biol Rhythms*. 2018;33(5):535-554.
36. Ouk K, Hughes S, Potthecary CA, Peirson SN, Morton AJ. Attenuated pupillary light responses and downregulation of opsin expression parallel decline in circadian disruption in two different mouse models of Huntington's disease. *Hum Mol Genet*. 2016;25(24):pii: ddw359.
37. Goto M, Oshima I, Tomita T, Ebihara S. Melatonin content of the pineal gland in different mouse strains. *J Pineal Res*. 1989;7(2):195-204.
38. Morton AJ, Rudiger SR, Wood NI, et al. Early and progressive circadian abnormalities in Huntington's disease sheep are unmasked by social environment. *Hum Mol Genet*. 2014;23(13):3375-3383.
39. Skene DJ, Middleton B, Fraser CK, et al. Metabolic profiling of presymptomatic Huntington's disease sheep reveals novel biomarkers. *Sci Rep*. 2017;7:43030.
40. English J, Arendt J, Poulton A, Symons AM. Short-term variations of circulating melatonin in the ewe. *J Pineal Res*. 1987;4(4):359-366.
41. Sletten TL, Revell VL, Middleton B, Lederle KA, Skene DJ. Age-related changes in acute and phase-advancing responses to monochromatic light. *J Biol Rhythms*. 2009;24(1):73-84.
42. Isherwood CM, Van Der Veen DR, Johnston JD, Skene DJ. Twenty-four-hour rhythmicity of circulating metabolites: effect of body mass and type 2 diabetes. *FASEB J*. 2017;31(12):5557-5567.
43. Adamczak-Ratajczak A, Kupsz J, Owecki M, et al. Circadian rhythms of melatonin and cortisol in manifest Huntington's disease and in acute cortical ischemic stroke. *J Physiol Pharmacol*. 2017;68(4):539-546.
44. Aziz NA, Pijl H, Frolich M, et al. Delayed onset of the diurnal melatonin rise in patients with Huntington's disease. *J Neurol*. 2009;256(12):1961-1965.
45. Kalliolia E, Silajdzic E, Nambron R, et al. Plasma melatonin is reduced in Huntington's disease. *Mov Disord*. 2014;29(12):1511-1515.
46. Morton AJ, Skillings EA, Wood NI, Zheng Z. Antagonistic pleiotropy in mice carrying a CAG repeat expansion in the range causing Huntington's disease. *Sci Rep*. 2019;9(1):37.
47. Liu J, Clough SJ, Hutchinson AJ, Adamah-Biassi EB, Popovska-Gorevski M, Dubocovich ML. Mt1 and mt2 melatonin receptors: A therapeutic perspective. *Annu Rev Pharmacol Toxicol*. 2016;56:361-383.
48. Osier N, McGreevy E, Pham L, et al. Melatonin as a therapy for traumatic brain injury: a review of published evidence. *Int J Mol Sci*. 2018;19(5):1539.
49. Shukla M, Govitrapong P, Boontem P, Reiter RJ, Satayavivad J. Mechanisms of melatonin in alleviating Alzheimer's disease. *Curr Neuroparmacol*. 2017;15(7):1010-1031.
50. Huntington's Disease Sheep Collaborative Research Group, Reid SJ, Patassini S, et al. Further molecular characterisation of the OVT73 transgenic sheep model of Huntington's disease identifies cortical aggregates. *J Huntingtons Dis*. 2013;2(3):279-295.
51. Alders J, Smits M, Kremer B, Naarding P. The role of melatonin in sleep disturbances in end-stage Huntington's disease. *J Neuropsychiatry Clin Neurosci*. 2009;21(2):226-227.
52. Burgess HJ, Fogg LF. Individual differences in the amount and timing of salivary melatonin secretion. *PLoS ONE*. 2008;3(8):e3055.
53. Gunn PJ, Middleton B, Davies SK, Revell VL, Skene DJ. Sex differences in the circadian profiles of melatonin and cortisol in plasma and urine matrices under constant routine conditions. *Chronobiol Int*. 2016;33(1):39-50.
54. Skene DJ, Swaab DF. Melatonin rhythmicity: effect of age and Alzheimer's disease. *Exp Gerontol*. 2003;38(1-2):199-206.

55. Rousseau A, Petren S, Plannithin J, Eklundh T, Nordin C. Serum and cerebrospinal fluid concentrations of melatonin: a pilot study in healthy male volunteers. *J Neural Transm (Vienna)*. 1999;106(9-10):883-888.
56. Jeong JK, Lee JH, Moon JH, Lee YJ, Park SY. Melatonin-mediated beta-catenin activation protects neuron cells against prion protein-induced neurotoxicity. *J Pineal Res*. 2014;57(4):427-434.
57. Mayo JC, Sainz RM, Gonzalez Menendez P, Cepas V, Tan DX, Reiter RJ. Melatonin and sirtuins: a "not-so unexpected" relationship. *J Pineal Res*. 2017;62(2):e12391.
58. Naia L, Rego AC. Sirtuins: double players in Huntington's disease. *Biochim Biophys Acta*. 2015;1852(10 Pt A):2183-2194.
59. Jiang M, Wang J, Fu J, et al. Neuroprotective role of sirt1 in mammalian models of Huntington's disease through activation of multiple sirt1 targets. *Nat Med*. 2011;18(1):153-158.
60. Parker JA, Vazquez-Manrique RP, Tourette C, et al. Integration of beta-catenin, sirtuin, and foxo signaling protects from mutant huntingtin toxicity. *J Neurosci*. 2012;32(36):12630-12640.
61. Christofides J, Bridel M, Egerton M, et al. Blood 5-hydroxytryptamine, 5-hydroxyindoleacetic acid and melatonin levels in patients with either Huntington's disease or chronic brain injury. *J Neurochem*. 2006;97(4):1078-1088.
62. Ruddick JP, Evans AK, Nutt DJ, Lightman SL, Rook GA, Lowry CA. Tryptophan metabolism in the central nervous system: medical implications. *Expert Rev Mol Med*. 2006;8(20):1-27.
63. Suofu Y, Li W, Jean-Alphonse FG, et al. Dual role of mitochondria in producing melatonin and driving GPCR signalling to block cytochrome c release. *Proc Natl Acad Sci USA*. 2017;114(38):E7997-E8006.
64. van der Burg JM, Bacos K, Wood NI, et al. Increased metabolism in the R6/2 mouse model of Huntington's disease. *Neurobiol Dis*. 2008;29(1):41-51.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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