Early and progressive circadian abnormalities in Huntington’s disease sheep are unmasked by social environment


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Insidious changes in behaviour herald the onset of progressive neurodegenerative disorders such as Huntington’s disease (HD), sometimes years before overt symptoms are seen. Sleep and circadian disturbances are particularly disruptive symptoms in patients with neurological disorders, but they are difficult to measure in humans. Here we studied circadian behaviour in transgenic HD sheep expressing the full-length human huntingtin protein with an expanded CAG repeat mutation in the juvenile range. Young HD sheep with no other symptoms exhibited circadian behavioural abnormalities that worsened with age. The most obvious change was a disturbed evening behaviour reminiscent of ‘sundowning’ that is seen in some patients with dementia. There were no structural abnormalities seen with magnetic resonance imaging, even in 5-year-old HD sheep. Interestingly, detection of the circadian abnormalities depended upon their social grouping. Abnormalities emerged in sheep kept in an ‘HD-only’ flock, whereas the behaviour of HD sheep kept mixed with normal sheep was relatively normal. Sleep–wake abnormalities in HD patients are also likely to be hidden, and may precede overt symptoms by many years. Sleep disruption has deleterious effects, even in normal people. The knock-on effects of sleep–wake disturbance may exacerbate, or even cause symptoms such as irritability and depression that are common in early stage HD patients. HD sheep will be useful models for probing the mechanisms underlying circadian behavioural disorder in HD.

INTRODUCTION

Huntington’s disease (HD) is a late-onset neurodegenerative disorder that typically does not manifest until the fourth decade of life (1). There is limited treatment and no cure for the disease, and it follows a relentless progression to an inevitable premature death. Much effort is being put into developing treatments for HD, but the hurdles are immense. The onset of symptoms in HD patients is very variable, and by the time a patient is diagnosed there is typically already significant degeneration present (2). One of the greatest challenges for clinicians and scientists in developing therapies for HD is determining when the disease starts, since ideally treatment would be given after the onset of symptoms but before there is significant neurodegeneration. Finding a marker of disease that indicates the earliest changes would not only inform us about early pathological mechanisms, but would also help to pinpoint a starting point for treatment.

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The mouse is the species most widely used to model HD. Nevertheless, while they are extremely valuable for preclinical research, mice have significant limitations when it comes to translating findings to humans. Of particular relevance to late-onset neurodegenerative diseases such as HD, mice have a natural lifespan that is much shorter than humans (for discussion, see 3 and 4). This makes it impractical to study aspects of neurodegenerative disease that take many years to appear in a relevant time frame. Furthermore, their usefulness is limited by the fact that mouse brains are much smaller than human brains, and they also differ significantly from human brains in their neuroanatomical organization (3). These limitations are becoming increasingly important as we move towards the development of novel therapies that need to be introduced directly into the brain, such as cell replacement or gene-targeting therapies (5,6). For example, it is not clear to what extent gene therapies that are effective in mice will need to be ‘scaled up’ for delivery to humans. To overcome some of these limitations, a number of large animal models of HD have been developed (3) including the HD sheep (7,8). In this study, we report the first abnormal behavioural phenotype in the HD sheep.

RESULTS

Huntington’s disease sheep show no overt symptoms up to 5 years of age

We used the ovine model of HD for our study (OVT73) (7). These animals carry copies of a full-length cDNA transgene for HTT with an expanded CAG repeat that combined with CAA codons result in an uninterrupted 73 amino acid polyglutamine tract in the protein. This is in the range of repeat length that would typically cause disease in juvenile humans (1). We have been studying these sheep for > 5 years, and to date, have seen no overt signs of disease. They grow and develop normally, and so far, show no gait abnormalities. Their wool and hoof growth is normal, and their behaviour in the field is indistinguishable from that of normal sheep. None of the HD animals have died from unknown causes.

Huntington’s disease sheep have early circadian abnormalities

One of the advantages of using sheep as a model is that they are social animals that can be kept under naturalistic conditions for long periods. Here we measured circadian behaviour in sheep kept in outdoor flocks. All sheep were the offspring of a single transgenic sire and born to normal breeding ewes. The first groups of sheep we used were born in October 2010. At weaning, lambs were distributed into one of three flocks: a ‘normal-only’ flock comprising 59 animals, an ‘HD-only’ flock comprising 48 animals or a ‘mixed-genotype’ flock comprising 107 animals (58 normal and 49 HD animals). When they were 18-months old we measured their general patterns of activity using Actiwatches worn on collars. All sheep showed a clear diurnal pattern of activity (Fig. 1), with a shortening of the main activity period (a) corresponding to the shortening of the day length with the approach of the winter solstice. Midday temperatures were high (25–39°C), so daytime activity of normal sheep was biphasic, with a morning phase that started just before sunrise and lasts for 2–3 h, and a late afternoon phase that lasted for ≏4 h until an hour or so after dusk (Figs 1 and 2). The shallow activity peak, visible in all groups in late morning/early afternoon (Fig. 2A and B), was caused by ‘feeding out’ of supplementary feed (see Materials and Methods for details).

Figure 1. Double plotted actograms showing activity of representative individual sheep from each of the test flocks measured outdoors under natural light conditions in South Australia in February–April 2012. Lines on activity traces show sunrise and sunset times.
Figure 2. Daily activity patterns differ in HD and normal sheep at 18-months of age. Total activity from normal (closed symbols) and HD sheep (open symbols) is shown as averaged across the data collection period without correction for change in day length in the mixed- (A) or single-genotype flocks (B), or as the mean across the whole-time period of total activity of each sheep in the period either side of sunrise (C) or sunset (D). Mean daily activity of sheep in different flocks was normalized to data from either sheep in the single-genotype normal flock (E, G, H) or to normal sheep in the mixed-genotype flock sheep (F). In A, B and E–H night-time is shown as light grey, and the period including the range of sunrise/sunset times over the data collection period is shown in dark grey. Dotted lines in A and B indicate times of supplementary feeding. The dotted lines in C and D indicate sunrise (C) and sunset (D). The arrow in G shows the time corresponding to the peak of activity seen in the single-genotype HD flock. Data in A–D are means ± SEM, in E–H means only. Where error bars are not visible, they are obscured by the symbols. Closed diamonds = mixed-flock normal sheep; open diamonds = mixed-flock HD sheep; closed circles = single-flock normal sheep; open circles = single-flock HD sheep (except in H, where for clarity closed diamonds represent mixed-flock HD sheep). * P < 0.05, ** P < 0.01, *** P < 0.001. (Comparisons are between HD groups only).
There were major differences between activity of HD sheep held in single-genotype flocks and either HD sheep in the mixed flock or normal sheep in either single or mixed-genotype flocks. Sheep in the HD-only flock had significantly longer acrophase, the acrophase was significantly later in the day, and their day–night activity ratio was significantly lower (Table 1). The lower day–night activity ratio was due to a combination of a shortening of activity at sunrise (Fig. 4A and B) that had not been seen in the mixed flock or normal sheep until 3 h after sunset (Fig. 2D).

When HD sheep data were normalized to that of the normal sheep, the differences in activity described above were seen clearly as a trough in the morning and a peak in the evening (Fig. 2E). In addition, three other significant differences in behaviour emerged. First, HD animals within the mixed flock were less active than normal animals in the same flock (Fig. 2F). Second, HD sheep in the single-genotype flock were significantly more active in the small hours compared with either normal animals in the single-genotype flock (Fig. 2E) or to sheep of either genotype in the mixed flock (Fig. 2G and H). Finally, there was a small increase in activity of both HD and normal sheep in the mixed flock (Fig. 2G) that coincided with the timing of the large evening activity peak seen in the HD-only flock (Fig. 2E and H). This is unlikely to be due to neighbour effects (i.e. the HD sheep in the single-genotype flock disturbing all the sheep in the mixed flock) since the sheep were not in adjacent paddocks, and there was no change in activity in the single-genotype normal flock at this time. Rather, it seems that although circadian abnormalities of HD sheep are ‘masked’ in the mixed flock, they still have residual abnormalities that emerge as an increase in late-evening activity. The ‘knock-on’ effects in behaviour of normal sheep in the mixed flock, that showed disturbed behaviour in the evening and in the small hours (Fig. 2G and H), is reminiscent of disturbed sleep—wake activity patterns seen in care-givers of HD patients (9).

### Brain structures of Huntington’s disease sheep are normal

We wondered if there were structural abnormalities in the brains of the HD sheep, since significant brain atrophy is seen in presymptomatic patients close to onset (2,10,11). Mutant protein is expressed in the HD sheep brains, and definite but very sparse aggregate pathology is seen in HD sheep brain at 18 and 36 months (8). Because we are planning to study the social behaviours of the present cohort until they show overt symptoms, we did not want to disrupt the social structure of the flocks by removing individuals. We reasoned that if structural changes were present in 18-month sheep, then these would be of the same (or greater) magnitude in older sheep. We used a separate cohort of 5-year-old HD sheep (six HD and six age-matched normal) to examine brain morphology using magnetic resonance imaging (MRI). MRI scans showed that all sheep have well-defined cerebral cortices and subcortical structures, with the brains of normal sheep (Fig. 3A) morphologically indistinguishable from those of age-matched HD sheep (Fig. 3B). We compared the size of brain substructures of HD and normal sheep using manual morphometry of HD-relevant brain regions (Fig. 3D). We also conducted voxel-based morphometry using a sheep brain library we have constructed from 60 sheep brain scans (Fig. 3C). No differences were seen between the brain subregions of the two genotypes (Table 2).

### Behavioural abnormalities emerge in HD sheep after separation of subflocks by genotype

The sheep we used for the first part of this study were the only ones we have reared in single-genotype flocks. We wondered if the behavioural phenotype seen at 18 months would emerge if HD sheep that had been raised in a mixed flock from weaning were separated from their normal flockmates. We took a group of 5-year-old ewes that had been living together from weaning and separated them into two subflocks (one of 41 TG sheep, the other of 53 normal sheep). We used a separate cohort of 5-year-old HD sheep (six HD and six age-matched normal) to examine brain morphology using magnetic resonance imaging (MRI). MRI scans showed that all sheep have well-defined cerebral cortices and subcortical structures, with the brains of normal sheep (Fig. 3A) morphologically indistinguishable from those of age-matched HD sheep (Fig. 3B). We compared the size of brain substructures of HD and normal sheep using manual morphometry of HD-relevant brain regions (Fig. 3D). We also conducted voxel-based morphometry using a sheep brain library we have constructed from 60 sheep brain scans (Fig. 3C). No differences were seen between the brain subregions of the two genotypes (Table 2).

### Table 1. Circadian measures in 18-month-old and 5-year-old sheep

<table>
<thead>
<tr>
<th>Flock type</th>
<th>Genotype</th>
<th>Acrophase (time of day, h:min)</th>
<th>LD activity ratio (%)</th>
<th>Total activity/24 h (counts)</th>
<th>Alpha (h:min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-Month-old sheep</td>
<td>Normal</td>
<td>15:24 ± 0:29 **</td>
<td>75.0 ± 1.0 ***</td>
<td>23601.4 ± 27703.1</td>
<td>13:51 ± 0:05 ***</td>
</tr>
<tr>
<td></td>
<td>HD</td>
<td>17:20 ± 0:31</td>
<td>66.0 ± 0.7</td>
<td>217970.3 ± 21059.7</td>
<td>14:36 ± 0:09</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>14:39 ± 0:32 **</td>
<td>76.4 ± 0.8 ***</td>
<td>251343.8 ± 32407.7</td>
<td>13:48 ± 0:06 ***</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>14:29 ± 0:29 **</td>
<td>76.8 ± 0.7 ***</td>
<td>209563.8 ± 23698.5</td>
<td>13:49 ± 0:07 ***</td>
</tr>
<tr>
<td>5-Year-old sheep</td>
<td>Normal</td>
<td>13:32 ± 0:08</td>
<td>78.60 ± 1.55</td>
<td>46634.1 ± 1829.9</td>
<td>10:59 ± 0:07</td>
</tr>
<tr>
<td></td>
<td>HD</td>
<td>15:03 ± 0:12 ***</td>
<td>67.23 ± 1.33 ***</td>
<td>56776.1 ± 1426.3</td>
<td>10:47 ± 0:13 ***</td>
</tr>
</tbody>
</table>

Statistical comparisons for 18 month-old sheep are single-genotype flock HD sheep versus all other groups *P < 0.05, **P < 0.01 and ***P < 0.001.
Statistical comparison for 5 year-old sheep is HD sheep versus normal sheep *P < 0.05, **P < 0.01 and ***P < 0.001.

Acrophase is the time at which the peak of a rhythm occurs; LD activity ratio is the ratio of total activity in the light and dark periods; alpha is the portion of the circadian cycle during which the sheep were active.
The maintenance of a normal phase relationship between the internal circadian clock, sleep–wake activity and the light–dark cycle is essential for normal physiological function in mammals. We found that the relationship between these factors, the phase angle of entrainment \((\phi)\), was significantly different in HD and normal animals at both sunrise and sunset (Fig. 5D). Finally, the abnormal evening activity lasted for much longer in the older HD sheep (Fig. 4C) than it did in 18-month HD sheep (Fig. 2E).

DISCUSSION

It is very difficult to measure accurately circadian behaviour in humans in the home environment, because life-style factors, in particular employment and children, mask endogenous rhythms. Nevertheless, there is a growing acceptance that circadian dysfunction is part of the spectrum of disorder in HD as well as other neurological diseases such as Alzheimer \((AD; 12–14)\) and Parkinson disease \((PD; 12,15)\). We report an early and progressive behavioural deficit with evening/night-time behavioural disturbance in the HD sheep that is reminiscent of disturbed sleep–wake activity reported in HD patients \((9,16;\text{ for other references, see 17})\). Our study strongly supports the idea that the disruption of circadian and sleep–wake behaviour described in HD patients is part of the earliest repertoire of symptoms in HD, rather than a knock-on effect of the disease. The HD sheep should be useful, not only for studying the mechanisms underlying early behavioural abnormalities in HD, but also for studying the consequences of such abnormalities on the progression of the disease. A measurable phenotype in the HD sheep means that they can be used for testing long-term therapies aimed at preventing or slowing the course of the disease.

Circadian rhythms are easy to measure in laboratory rodents, and clear circadian abnormalities in both behaviour and gene expression have been reported in a number of rodent models of HD \((\text{for references, see 17})\). However, not only are rodents nocturnal, but also, most HD models carry a mutation with a CAG repeat that is considerably longer than is typically seen in humans. Furthermore, the best-characterized line \((R6/2)\) carries only a fragment of the HD gene. Thus, the relevance of data from mice to the human condition has frequently been questioned. The fact that there are significant circadian behavioural changes in the HD sheep—that are not only diurnal mammals, but also carry the HD mutation in a full-length protein with a CAG repeat directly relevant to the human disease—suggests that the changes in circadian behaviour measured in mice and sheep are directly relevant to those seen in HD patients.

Light is the most potent stimulus for entraining mammalian circadian rhythms. There is, nevertheless, considerable evidence that other stimuli modify circadian behaviour. The evidence is best for food-entrainment \((18)\), but there is also evidence that social stimuli function as ‘Zeitgebers’ \((19–21)\). Our data

Figure 3. MRI shows no structural brain abnormalities in HD sheep at 5 years of age. A single representative coronal slice from each sheep taken at the level of the caudate nucleus is shown for all normal \((A)\) and HD sheep \((B)\). A labelled coronal and sagittal slice from the MRI library is shown in \((C)\). The position of the coronal section is indicated by the white line in the sagittal section. 3-D representation of the regions analyzed by manual morphometry is shown in \((D)\). Amyg: amygdala, CN: caudate nucleus (red in \(D\)), Hf: hippocampal formation (yellow), lv: lateral ventricle (blue), P: putamen (green), ic: internal capsule, CB: cerebellum.

Table 2. Structural volumes measured from MRI scans

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Volume (ml) mean ± SD</th>
<th>Genotype (number of brains)</th>
<th>Library (60)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HD (OVT 73) (6)</td>
<td>Normal (6)</td>
<td>Library (60)</td>
</tr>
<tr>
<td>Caudate</td>
<td>1.37 ± 0.06</td>
<td>1.47 ± 0.12</td>
<td>1.33 ± 0.11</td>
</tr>
<tr>
<td>Putamen</td>
<td>1.19 ± 0.07</td>
<td>1.25 ± 0.09</td>
<td>1.13 ± 0.09</td>
</tr>
<tr>
<td>Lateral ventricles</td>
<td>2.90 ± 0.54</td>
<td>3.40 ± 1.10</td>
<td>2.65 ± 0.75</td>
</tr>
<tr>
<td>Hippocampal formation</td>
<td>1.65 ± 0.11</td>
<td>1.79 ± 0.23</td>
<td>1.64 ± 0.15</td>
</tr>
<tr>
<td>Amygdaloid nuclei</td>
<td>0.70 ± 0.04</td>
<td>0.75 ± 0.11</td>
<td>0.69 ± 0.05</td>
</tr>
<tr>
<td>Whole-brain grey matter</td>
<td>79 ± 7</td>
<td>83 ± 6</td>
<td>79 ± 8</td>
</tr>
<tr>
<td>Whole-brain white matter</td>
<td>60 ± 1</td>
<td>64 ± 3</td>
<td>55 ± 6</td>
</tr>
<tr>
<td>Whole-brain CSF</td>
<td>8.0 ± 2.0</td>
<td>9.6 ± 4.0</td>
<td>8.4 ± 3.0</td>
</tr>
<tr>
<td>Total intracranial</td>
<td>147 ± 9</td>
<td>157 ± 13</td>
<td>142 ± 13</td>
</tr>
</tbody>
</table>
support the idea that social stimuli modulate circadian behaviour. First, the behavioural abnormalities of the HD sheep are ‘masked’ when they live in a mixed genotype flock. Second, there was a small but significant ‘knock-on’ disturbance in behaviour of normal sheep that lived in the mixed flock. The fact that the behaviour of the HD sheep in the mixed flock was more similar to that of normal sheep, rather than the other way around, makes ethological sense; it would be advantageous for an individual flock animal if social cues normalize mildly abnormal behaviour. In this case, the abnormality is caused by a transgene. In a natural population, behavioural abnormalities might be caused by genetic mutations, but are more likely to be caused by developmental abnormalities, illness or injury. Interestingly, we have previously observed a similar ‘social’ phenomenon in a study using HD mice (22). In that study, R6/2 mice housed in single-genotype groups were treated with vehicle or drugs that modified their sleep–wake behaviour and improved cognitive function. Improvements in cognition were seen not only in the drug-treated mice, but also in the vehicle-treated R6/2 mice living in the same cage. Circadian abnormalities are not typically noticed in patients until they are severe enough to interfere with daily living. If we extrapolate our data to HD patients, we predict that mild abnormal circadian behaviour would be masked by normal social environment. Furthermore, if the behavioural manifestations of circadian disturbance are modulated by social context, then life-style factors may influence the timing of when symptoms emerge, and how they are manifest.

We do not know the mechanism underlying the abnormal behaviour of the HD sheep. There is no reason to suppose that the delay in ‘settling’ of the HD sheep at night is due to abnormal responsiveness to light, since onset of activity at sunrise in the 18-month sheep was similar for both genotypes in the single-genotype flocks. It is possible that the HD sheep that are separated from normal sheep do not give and/or respond correctly to cues generated within the flock that signals night-time. Nevertheless, the phase delay in the older sheep suggests that there is also progressive deterioration of circadian clock control. We have not yet been able to measure circadian markers such as temperature or melatonin levels in the sheep—this would be particularly interesting to study, given the delay in onset of rise of melatonin in HD patients (16), and the evidence of post-transcriptional neuropeptide deficits in the suprachiasmatic nuclei of patients with HD (23). The sheep we used for this study are still alive, and we plan to follow them longitudinally until they show overt symptoms. The fact that there are no significant changes in brain structure of sheep is not particularly surprising. Even in the most rapidly advancing HD mouse models, the earliest changes seen are in synaptic physiology (24,25) and behaviour (26,27). Although the circadian abnormalities are measurable, the oldest HD sheep currently show no obvious

Figure 4. Circadian disruption is more pronounced in HD sheep aged 4–5 years. Total activity from normal (closed symbols) and HD (open symbols) sheep is shown as either mean activity averaged across the data collection period without correction for change in day length (A), or as the mean across the whole-time period of total activity of each sheep in the period either side of sunrise (B). Mean daily activity of HD sheep was normalized to data from normal sheep (C). In A and C, night-time is shown as light grey, and the period including the range of sunrise/sunset times over the data collection period is shown in dark grey. Data in A and B are means ± SEM, in C they are means only. Where error bars are not visible, they are obscured by the symbols. The dotted line in C indicates sunset. \( P < 0.05, ** P < 0.001. \)
symptoms. It will be interesting to see if the HD sheep maintained in the mixed flock eventually show major circadian dysfunction, and if they do so, whether or not this correlates with the emergence of other symptoms.

Circadian disruption in normal people has deleterious effects on mental and physical health. As well, there is growing awareness that the circadian clock influences disease states (20,21,28,29), with defects in clock control giving rise to pathophysiological states, including depression (30,31), sleep disorders (32,33), cardiac disease (34), mood and cognitive dysfunction (35,36). It is notable that at some stage in their disease all of these domains are deleteriously affected in HD patients. More attention should be paid to the role that circadian dysfunction plays in symptomology of HD. It is possible that poor circadian/sleep management may cause some of the early symptoms in HD.

It is interesting that the first behaviour to emerge in the HD sheep is a sleep–wake disturbance that is reminiscent of a behavioural disturbance seen in AD and other dementias that is known as ‘sundowning (37–39). Sundowning is characterized by late afternoon exacerbation of behavioural symptoms, where patients exhibit increased confusion, anxiety and agitation late in the day, and it is a common cause of caregiver ‘burnout’. Sundowning has not been described in HD patients, although this may be due to the fact that by the time HD patients are institutionalized with dementia they typically also have profound movement disorders that limit potentially disruptive behaviour. Circadian behavioural disorder is one of the primary causes of institutionalization of AD patients (40). However, circadian behaviour disturbance is a difficult issue for management of all patients with neurodegenerative diseases, regardless of the disease (17,41,42). Severe circadian disturbance is particularly difficult to manage once a patient has been placed in a facility, since, other than antipsychotic medications, off-label medications and restraint, there are no real treatment options available. Developing treatments that target circadian disturbance will be beneficial for such patients. At the very least, preventing or delaying the onset of circadian dysrhythmia in neurodegenerative diseases may delay the appearance of knock-on symptoms such as depression, irritability and mild cognitive impairment. The HD sheep, with its clear circadian abnormalities, will make an interesting preclinical research model to study these phenomena.

MATERIALS AND METHODS

Animals
We used six groups of South Australian merino sheep for this study. All were born to normal breeding ewes. All of the HD
sheep had the same sire. Experiments were conducted under PIRSA Animal Ethics Approval #41/09.

Cohorts 1–3: Actimetry of 18-month-old sheep
Sheep were born in October 2010 and tested in February–April 2012. After weaning, lambs were distributed into one of three flocks. A ‘normal-only’ flock comprised 59 normal animals; a ‘HD-only’ flock comprised 48 HD animals. The third ‘mixed’-genotype flock comprised 107 animals (58 normal and 49 HD animals). Sheep were ewes or wethers (rams castrated before weaning), with sexes mixed evenly within each subflock.

Cohort 4: MRI
Sheep used for MRI were born in October 2007. T1-weighted MRI was performed in 2012 when they were 5-years-old. six normal and six TG sheep were used.

Cohorts 5–6: Actimetry of 5-year-old sheep
Sheep were born between March 2008 and March 2009 and tested in May 2013 when they were 4–5-years-old. Only ewes were available. Sheep were reared in two flocks of mixed genotype. These sheep were raised as separate flocks, but had been mixed together every 6 months, for up to 2 months at a time, in the previous 3 years. They were grouped as a single flock for 6 weeks before this experiment started. In March 2013, the single large flock was segregated into two subflocks by genotype (53 normal and 40 HD animals). A month later, Actimetry data collection was started.

Actiwatch data collection
We measured general patterns of activity in the sheep using Actiwatch data collection (Actiwatch Mini; Linton Instruments) worn on collars. For the 18-month sheep, we recorded activity simultaneously from 10 sheep of each genotype in each flock, in 5 min bins, for 4 weeks in May–June 2013. During the recording period in 2012, time of sunrise advanced by 35 min, while time of sunset was earlier by 56 min. For May–June 2013, time of sunrise advanced by 24 min, while time of sunset was earlier by 15 min. For the measurement of activity around the times of sunrise and sunset, data from individual sheep were zeroed around actual time of sunrise or sunset each day. Data (in 20 min bins) were then averaged across all days, first by sheep then by cohort.

Acrophase was estimated for each sheep each day using ClockLab (Actimetrics, Wilmette, IL, USA). Activity in the light and dark periods (for LD ratios) was calculated daily, correcting for changes in sunrise and sunset times. Times of onset and offset of activity were generated day-by-day using Clocklab, and corrected where necessary following visual inspection of the actograms by an experimenter blinded to flock details. Alpha (the portion of the circadian cycle during which the sheep were active) was calculated daily for each sheep by subtracting the time of activity onset from the time of activity offset.

For acrophase, LD ratios and alpha, daily results obtained from individual animals were averaged across each week of the recording period, meaned by flock, then averaged across the entire 6-week recording period.

For normalization of total activity by flock, at each timepoint, group means of activity were divided by the mean of activity from the normal animals in the single or mixed-genotype flocks, as appropriate.

Statistical analyses were performed using StatSoft Statistica 19.0 (StatSoft Inc., Tulsa, USA) or Prism 5 (GraphPad Software Inc., San Diego, USA).

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Conflict of Interest statement. The authors declare that they have no conflict of interest.

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