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Ultradian components in the locomotor activity rhythms of the genetically normal mouse, *Mus musculus*

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SUMMARY

Ultradian periodicities in physiological processes have been reported for a wide variety of organisms and may appear as bouts in locomotor activity. In some instances, this temporal organization can be related to some ethological strategy. In mice, however, ultradian rhythms have been reported largely in animals with circadian pacemakers disrupted either by genetic or surgical manipulation. Using analysis techniques capable of resolving periodicities in the ultradian range in the presence of strong diel periodicity, we found unequivocal evidence of ultradian rhythms in mice entrained to an light:dark cycle. We collected locomotor activity data of individuals from 11 genetically disparate strains of mice whose activity was recorded in 12 h:12 h L:D photoperiods for 3 days. Data were subjected to maximum entropy spectral analysis and autocorrelation, both before and after filtering to remove the 24-h periodicity. We found that every strain had a majority of individuals with strong ultradian rhythms ranging from ~3 to ~5 h. These periodicities were commonly visible in individual animals both in high-pass-filtered and in unfiltered data. Furthermore, when all raw data from a given strain were pooled to get a 24-h ensemble average across all animals and days, the rhythms continued to be discernable. We fitted Fourier series to these form estimates to model the frequency structure of each strain and found significant effects of strain and an interaction between period and strain indicating significant genetic variation for rhythmicity in the ultradian range. The techniques employed in this study should have wider use in a range of organisms and fields.

Key words: mouse, ultradian periodicity, locomotor activity, genetic variation.

INTRODUCTION

With few exceptions, the activities of living systems occur in temporal cyclical patterns (for reviews, see Palmer, 2002; Young, 2005). This largely occurs on an approximately 24-h or circadian basis, but other periodicities are common, e.g. the 12.4/24.8-h tidal/lunar daily cycle (Palmer, 1995). All are adaptations to environmental variables, but persist in constant conditions (Palmer, 2002; Young, 2005). The molecular basis of the persistent biological clock is generally held to be a transcription–translation feedback oscillator (TTO) (Dunlap, 1999; Young, 2005). Less commonly observed are persistent cycles with periods between about 1 h and up to about 18 h, with tidal periodicities excluded, referred to as 'mid range ultradian' periodicities (Dowse, 2008). These should be distinguished from the 'basic rest activity cycles' or BRAC (Kleitman, 1963) which are hypothesized to be related to the alternation between REM and non-REM sleep and which have periods commonly less than an hour, but which may be slightly longer. Such periodicities have been reported, for example, in mice (D'Olimpio and Renzi, 1998).

A number of species exhibit fairly clear mid-range ultradian periodicities in their locomotor activity. For example, the vole, *Microtus arvalis*, has very clear ultradian rhythms that interact with the circadian clock and drive bouts of foraging which may have an adaptive value (Gerkema and van der Leest, 1991; Gerkema et al., 1993). However, in rats, mice and hamsters, all three of which have been intensively studied for rhythmicity, ultradian periodicity has not been well-characterized (Schibler, 2008). Honma and Hiroshige (Honma and Hiroshige, 1978) found ultradian periodicity in a number of physiological variables, including locomotor activity, with periods from 4–6 h in rats exposed to constant light sufficiently intense and of long enough duration to eliminate the circadian component. Rhythms in the locomotor activity of rats in the range of about 4–6 h have been reported with significant differences among strains (Buttner and Wollnik, 1984). These strain differences proved to be heritable (Wollnik et al., 1987). With regard to mice, Del Pozo et al. (Del Pozo et al., 1978a; Del Pozo et al., 1978b) recorded locomotor activity in individuals for either 90 min or 23 h and noted ultradian rhythms. They found that there were significant differences in the amplitudes and periods of these rhythms in isolated individuals compared with groups. Amphetamine injection altered this periodicity, which was also seen to vary with environment. Ticher and Ashkenazi (Ticher and Ashkenazi, 1995) observed both rats and mice, and reported the organization of the locomotor activity into bouts with ultradian rhythmical characteristics. Poon et al. (Poon et al., 1997) showed that cage size affects the extent of mouse ultradian rhythmicity.

In another intensively studied organism, the fly, *Drosophila melanogaster*, ultradian rhythms have also been found in individuals with circadian clocks disrupted either genetically or by environmental manipulation, once proper analytical techniques are applied (for a review, see Dowse, 2008). Undisturbed, wild-type individuals may also display these short period rhythms (Dowse et al., 1987). In this instance, it has been argued that they may represent a manifestation of a population of even shorter-period cellular

oscillators that are the actual frequency standard of the clock system, relegating the TTO to a mechanism that couples the ultradian clocks to behavioral and physiological periodicities with environmental relevance (Dowse, 2008). Whether or not this is so, it still is of interest to study these short period clocks as they must certainly reflect a deep cellular temporal order essential for regulation of cellular processes (for a review, see Lloyd and Rossi, 2008). Strong, heritable ultradian rhythms in intact, wild-type individuals in an important animal model like the mouse would be further evidence of this importance.

We had previously studied behavior patterns of 11 inbred strains of *Mus musculus* (Linnaeus), including eight that were wild-derived (Koide et al., 2000) (Table 1). These wild-derived strains fall into three subspecies groups: *domesticus*, *musculus* and *castaneus* (Bonhomme et al., 1984; Moriwaki et al., 1994). These were genetically normal individuals, as none of the strains were identified as carrying observable pathological mutant phenotypes. 'Home-cage activity' was recorded, i.e. detectable movements of a given individual as a function of time (see below, Materials and methods). It has been reported that most laboratory strains have limited genetic polymorphism since the original population of laboratory strains derives from a relatively homogeneous group of mice belonging to the sub-species *Mus musculus domesticus* (Yonekawa et al., 1981; Ferris et al., 1982; Bonhomme and Guénet, 1996). This lack of heterogeneity may result in limited diversity of observable behavioral phenotypes within the ensemble of strains. Comparing inbred strains which are derived from different subspecies groups of *M. musculus* is thus potentially highly useful for discovering and elucidating behavioral differences and identifying the genes that underlie them.

The results reported by Koide et al. (Koide et al., 2000) showed substantial diversity of behavioral phenotypes among strains. Analysis of home-cage activity over a 3-day period revealed a high degree of difference among strains (Koide et al., 2000). During the dark phase, two wild strains, NJL and KJR, were hyperactive, while three wild strains BFM/2, HMI and BLG2, and the fancy strain JF1were hypoactive compared with the laboratory strain DBA/1. Most of the strains were consistently nocturnal but the high level of activity during dark phase was often interrupted with periodic resting phases in many cases in all the strains and low-amplitude bouts of activity still occurred during the daylight phase. Thus simple inspection of the activity data over a 3-day period strongly suggested ultradian rhythmicity was present and we elected to probe more deeply into this large collection of data from mice comprising a wide range of genetic backgrounds to confirm this. Given that *Drosophila*'s ultradian periodicities were not seen using the common techniques of the 'actogram' and the 'periodogram' (Whitaker and Robinson, 1924; Enright, 1965; Enright, 1990; Dowse and Ringo, 1991; Dowse, 2007; Dowse, 2009), we subjected the locomotor activity records to analyses sufficiently powerful to find them and allow subsequent comparisons.

Table 1. Origins and pedigrees of the strains tested

Strain	History	Subspecies group	Origin
BFM/2	Wild derived	*domesticus*	France
BLG2	Wild derived	*musculus*	Bulgaria
B6	Laboratory	*domesticus*	West Europe
CAST/Ei	Wild derived	*castaneus*	Thailand
DBA/1	Laboratory	*domesticus*	West Europe
HMI	Wild derived	*castaneus*	Taiwan
JF1	Fancy derived	*musculus*	Japan
KJR	Wild derived	*musculus*	Korea
MSM	Wild derived	*musculus*	Japan
NJL	Wild derived	*musculus*	Denmark
SWN	Wild derived	*musculus*	Korea

JF1 originally came from Denmark, but was characterized as a Japanese strain after genetic study (Koide et al., 1998).

MATERIALS AND METHODS

Mice

Eight strains, NJL, BFM/2, HMI, KJR, SWN, BLG2, MSM and JF1, had been established as inbred over 20 generation of brother-sister mating at the National Institute of Genetics (NIG, Mishima, Japan) (Koide et al., 1998; Koide et al., 2000). C57BL/6J (B6), DBA/1J (DBA/1) and CAST/Ei were obtained from the Jackson Laboratory, Bar Harbor, ME, USA (Table 1). Mice were maintained according to NIG guidelines, and all procedures were carried out with approval from the institutional animal care and use committee. All strains were (and continue to be) maintained at NIG in a 12 h:12 h light:dark cycle with lights coming on at 08:00 h and food and water available *ad libitum*. All the mice were group-housed until time of test for spontaneous activity. Ten females from each strain, aged between 8 and 13 weeks, were used in measuring spontaneous home cage activity. Animals at this age are sexually mature.

We chose to use only females in this experiment despite the potential complication of the estrous cycle owing to our decision to separate the sexes early post weaning and house the experimental subjects communally. In some of the wild-derived strains, males are aggressive and harm, even kill, one another when housed in this manner (Takahashi et al., 2010). Therefore, we were unable to keep male littermates of some of the wild-derived strains in the same cage. It has been reported that housing the mice for long periods in isolation tends to induce excessive stress that may interfere with our observations (Motoyama et al., 2009). Furthermore, we surmised that the social interaction would tend to synchronize all the individuals in a group, potentially making our subsequent form estimates of daily activity across subjects within a co-housed group more coherent (e.g. Mrosovsky, 1988).

Activity recording

Spontaneous activity of individually housed mice in a 12 h:12 h L:D environment was acquired previously and the overall activity level was calculated for each strain and compared in the previous report (Koide et al., 2000). The environmental conditions were identical to those from which they had been raised since birth. 'Home cage' activity data were acquired using an infra-red sensor (AB-system 24, Neuroscience Co. Ltd, Tokyo, Japan) and all movements registered by the sensor were counted. Data were initially summed over a 60-s interval, i.e. in 1-m 'bins', as events per unit time. Activity was recorded continuously over a 4-day period. The first day was allotted to a time of habituation to the new environment and those data were omitted from the record. The input stream to be analyzed started at 08:00 h with lights-on on day 2 and continued for 72 h. We offset the time scale such that the hour of initiation of data collection, 08:00 h on day 1, becomes t=0 of the experiment.

Analysis of data

To attenuate the noise in the signal, we first re-summed the 1-min data to 10-min bins. This has the effect of clarifying any periodicity (Dowse and Ringo, 1994). The data were then subjected to autocorrelation analysis and maximum entropy spectral analysis (MESA) to assess any periodicity in any range (Burg, 1967; Burg, 1968; Levine et al., 2002; Dowse, 2009). Autocorrelation is a straightforward technique involving producing correlation

coefficients between the data vector and itself as it is sequentially 'lagged' out of phase one time unit at a time. Recurring peaks in coefficients indicate that the signal is periodic and how robust that periodicity might be (Chatfield, 1989; Dowse, 2009). MESA is a very high resolution, high sensitivity technique that is well-suited to short noisy time series such as are seen in biological rhythms (Ables, 1974).

To enhance further the chances of discerning any ultradian periodicity, we next removed the circadian range of the signal using a Butterworth recursive high-pass filter (3 dB attenuation at a period of 18 h) (Hamming, 1983) and conducted the autocorrelation and MESA a second time. Use of recursive filters causes phase shifts in the output compared with the original series, and this should be kept in mind when viewing the activity plots. Original phase can be restored by running the filter in 'reverse', but filtering multiple times can actually amplify noise (Hamming, 1983) so we elected not to do so. These differences are consistent and identical within a given analysis. We also produced 'form estimates' or 'ensemble averages' of the activity profiles of each strain by summing the activity across all individuals and days, producing a mean composite 24-h plot that represents all data for all individuals within a group (e.g. Hamblen-Coyle et al., 1989; Poon et al., 1997). The net effect of this treatment is to emphasize important characteristics of the time course of the day's activity by letting spurious bursts of activity cancel one another out in the averaging process. Only the most robust features remain common to all members of the group.

To establish the acrophase of activity, we used the method of Batschelet (Batschelet, 1965). The data for each strain were first summed across each bin and across all 3 days and the mean calculated as above. These values are treated as vectors in the polar coordinate plane and were assigned phase angles in radians corresponding to the time of day. The vectors were decomposed into X and Y components by taking the cosine and sine respectively, and these values were summed. A composite vector $\mathbf{R}$ was calculated using $\mathbf{R}=\sqrt{(X^2+Y^2)}$ and the angle by the arctangent (X/Y) in the usual manner (Batschelet, 1965).

To elucidate further any potential differences in spectral character among the strains, we fitted Fourier series to the form estimates for each strain (Chatfield, 1989). In this sense, we are not doing spectral analysis, *per se*, rather we are modeling the structure of the functions in the frequency (period) domain using the Fourier series of sine and cosine terms; all the information from the time domain is present, simply in a transformed state (Lanczos, 1956). Only coefficients in the ultradian range are useful, as we have only one full 24-h cycle represented in these form estimates, and we went down to 20 min, recognizing the Nyquist limit requiring sampling at half the period of the shortest cycle in the analysis, 10 min (Hamming, 1983).

In analysis of variance (ANOVA) statistical tests, we set $\alpha=0.05$. Parametric ANOVAs were done with the GLM (general linear models) procedure, and nonparametric ANOVAs with the NPAR1WAY procedure (Statistical Analysis System; SAS).

RESULTS

As expected, all mice were active with a clear circadian pattern. Fig. 1A shows the raw data in 1-min bins for a typical individual (chosen arbitrarily as being representative) from the SWN strain (SWN135A), followed by autocorrelation and MESA. We follow this single data set through all phases to illustrate the development of the analysis and the effects of the various techniques. The correlogram is robust and the MESA peak at about 24 h is clear (Fig. 1B,C). Fig. 2 shows the same data after rebinning. Note the clarification of the 24-h pattern in the data and general reduction in random noise. Again, the correlogram is clear and a MESA peak at about 24 h is observed (Fig. 2B,C). Given that the animals were kept in an L:D cycle, this is expected. Means for entrained 24-h periodicity and acrophases, calculated as above, from rebinned,

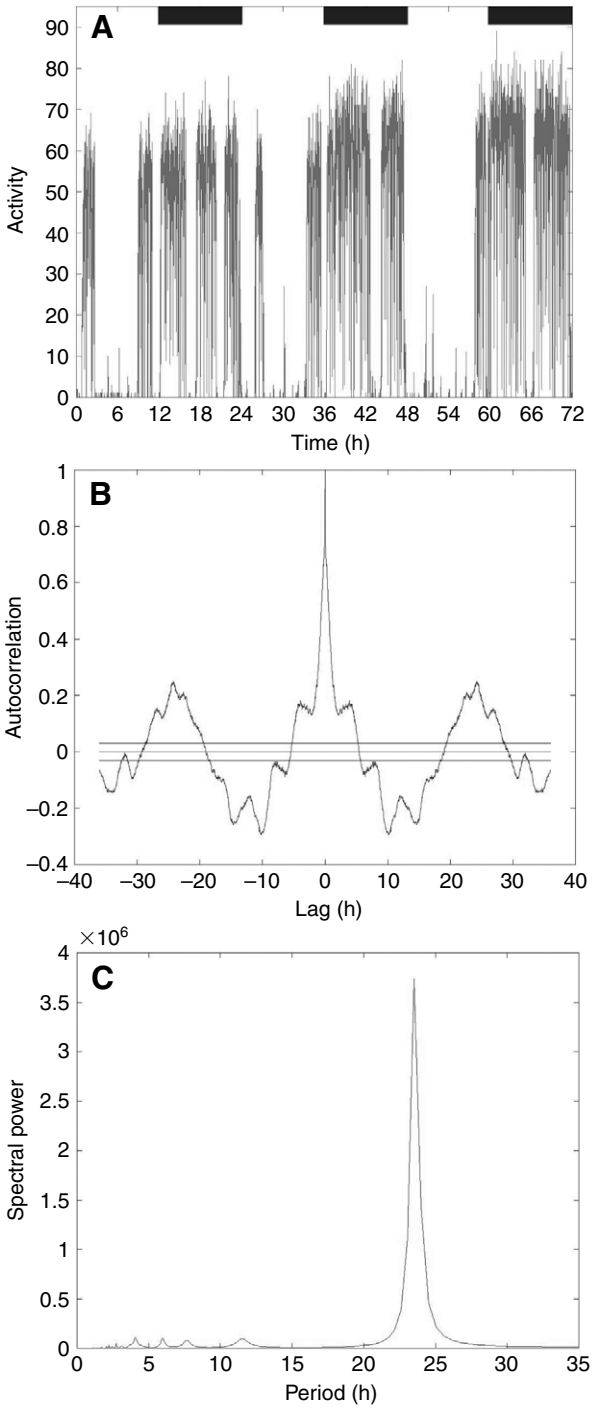

Fig. 1. (A) Actogram from 3 days of raw data, summed over one-minute bins, from an individual from strain SWN (SWN135A). Lights on is at hour zero, which was at civil time 0800 h. The organization of the activity is most strongly seen in the 24-h range, but higher-frequency bouts of activity may also be observed despite the amplification of noise by the small bin size. (B) Autocorrelogram (see text) corroborating the 24-h periodicity. (C) Maximum entropy spectral analysis (MESA) picks up a period of 23.5 h.

unfiltered data, are given for all strains in Table 2. There is no difference in period among the strains ($F_{10,118}$=1.42, P=0.18; GLM procedure, SAS). The mean period across all strains is 23.4 h. It is not surprising that this is not precisely 24 h given that only 3 days of recording were available and the number of cycles in the analysis affects accuracy; even with longer records, variation from the entraining period is seen (cf. Dowse, 2008). The robust short ultradian periodicities may also be influencing these numbers. There was no discernable effect of the estrous cycle in the records, and given that at least 14 days of recording would be necessary to allow spectral analysis for cycles of this length, we did not attempt to control for it and it clearly did not interfere with results in either the circadian or ultradian range. In any event, the high pass filter, when applied, would have removed it for that section of the analysis.

Data from the same individual whose activity is depicted in Figs 1 and 2 are shown after the high-pass Butterworth filter was applied to the re-binned data in Fig. 3. The presence of an ultradian periodicity now becomes easy to see even in the raw data (Fig. 3A). This is picked up by autocorrelation (Fig. 3B) and MESA (Fig. 3C). There is a 4.0-h periodicity corroborated unequivocally by the autocorrelogram. We next fitted a sinusoid with the same period as assessed by MESA to the data using nonlinear regression (NLIN procedure, SAS). Fig. 4A shows the rebinned raw data from this mouse with the fitted sinusoid superimposed. This procedure was done for several other individuals and are shown in Fig. 4B and C for comparison.

Fig. 5 shows the form estimates as calculated for four strains, including the one from which the individual was chosen for the above suite of analyses (Fig. 5C). We chose a range of clarity of the ultradian rhythmicity to display. Remarkably, in three of the four, even after the creation of the form estimate, ultradian

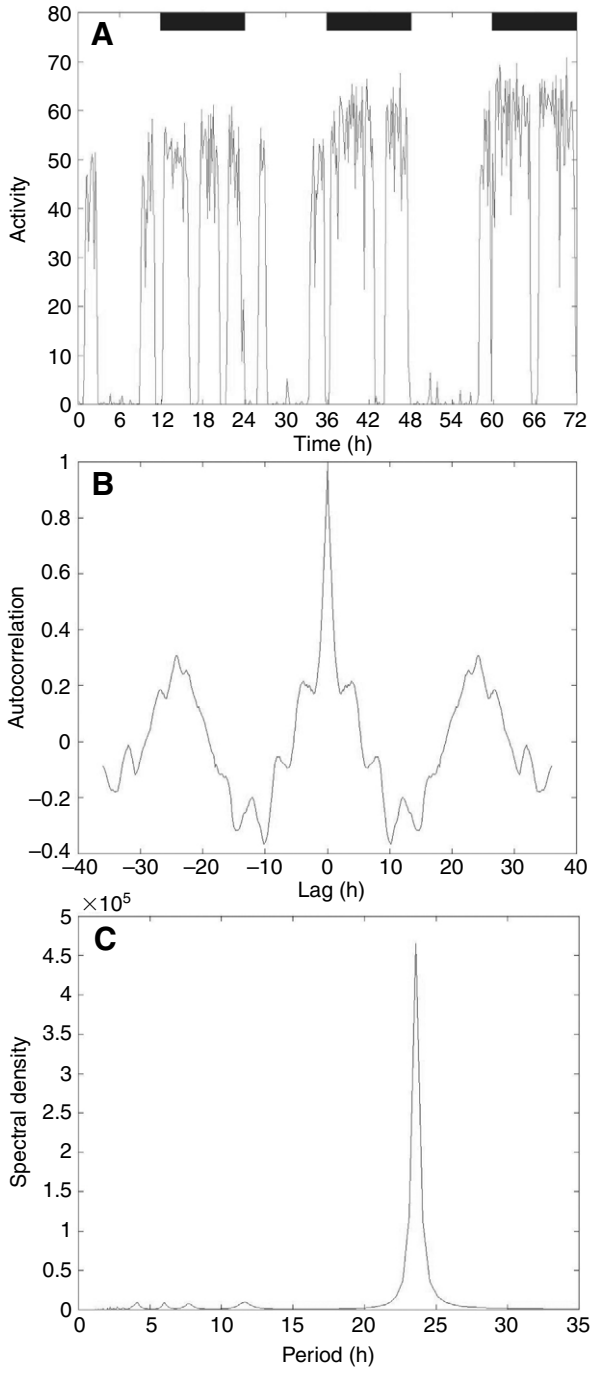

Fig. 2. (A) The same data as in Fig. 1, but with the bin size increased to 10 min. This has the immediate effect of removing a substantial fraction of the noise in the series. The higher frequencies are more readily apparent. (B) Autocorrelation and (C) MESA from the re-binned data. Again, the improvement in apparent signal-to-noise ratio is clear.

Table 2. Periodicities by strain while entrained to a 12 h:12 h light:dark cycle

Strain	τ (h; mean ± s.e.m.)	Acrophase	*N*
BFM/2	23.8±0.57	18.1	15
BLG2	22.9±0.16	16.1	14
B6	24.1±0.25	18.0	12
CAST/Ei	24.3±0.80	15.3	10
DBA/1	23.3±0.12	18.5	8
HMI	23.8±0.20	16.1	11
JF1	23.7±0.26	16.2	12
KJR	22.8±0.34	18.2	11
MSM	23.1±0.32	17.0	14
NJL	23.5±1.40	12.0	12
SWN	22.5±1.00	17.4	10

Values as obtained by MESA in hours for unfiltered data, emphasizing the near 24-h periodicity in the animals' activity.
τ, periodicity.

Table 3. Ultradian periodicities reported by MESA after a high-pass Butterworth digital filter was employed to remove the circadian-range periodicities

Strain	τ (h; mean ± s.e.m.)	*N*	RI (mean ± s.e.m.)	*N*
BFM/2	4.4±0.38	9	0.16±0.018	8
BLG2	3.5±0.37	6	0.16±0.014	6
B6	4.1±0.40	12	0.15±0.011	9
CAST/Ei	4.0±0.31	9	0.14±0.067	6
DBA/1	4.3±0.48	3	0.11±0.004	2
HMI	3.4±0.30	8	0.14±0.016	7
JF1	4.3±0.48	11	0.14±0.014	10
KJR	4.0±0.35	9	0.15±0.014	8
MSM	3.6±0.64	5	0.14±0.025	5
NJL	4.2±0.31	9	0.14±0.014	9
SWN	4.5±0.43	7	0.15±0.032	7

All periods shown were corroborated by autocorrelation analysis. The rhythmicity indices reported reflect only those plots from which they could be retrieved, hence in several instances, *N* for RIs is lower than *N* for total rhythmic animals. Arrhythmic animals do not appear in RI results.
RI, rhythmicity index; τ, periodicity.

organization in activity bouts is quite discernable from simple inspection.

Ultradian periodicities were found in a majority of records (Table 3). There were no differences among strains in either the number of individuals displaying ultradian rhythms ($F_{10,220}$=1.12, P=0.35) (NPAR1WAY procedure), or in the values of the primary periods as discerned by MESA and backed by autocorrelation ($F_{10,118}$=1.1, P=0.37) or RIs (rhythmicity index; $F_{10,81}$=0.58, P=0.83; GLM procedure). However, when we derived coefficients by fitting a Fourier series and compared them, we obtained results as follows: coefficient magnitude depended strongly on frequency, which is expected, and trivial ($F_{1,220}$=57.04, P=0.0001). There was also a

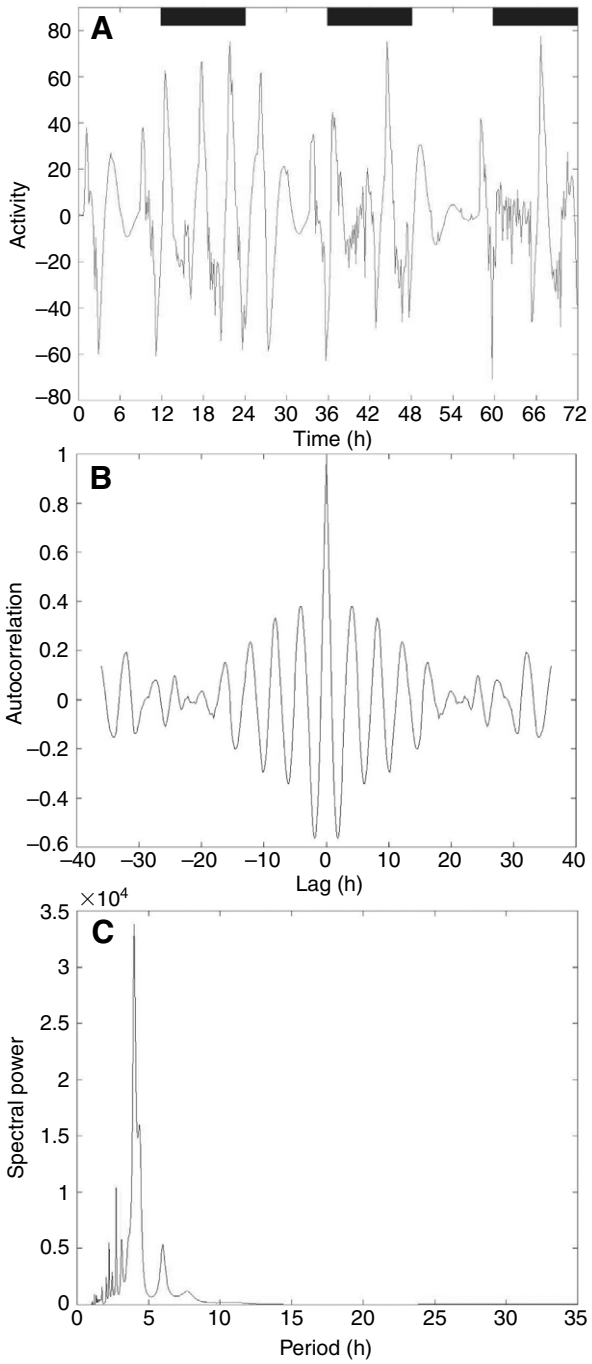

Fig. 3. (A) The same data as in Fig. 1 were subjected to a high pass Butterworth filtering to remove the circadian periodicity and this further emphasizes the ultradian temporal organization as seen in the autocorrelation (B) and MESA (C). The period found by MESA and verified by autocorrelation is 4.0 h, and the rhythmicity index (RI), the height of the third autocorrelation peak, is 0.33.

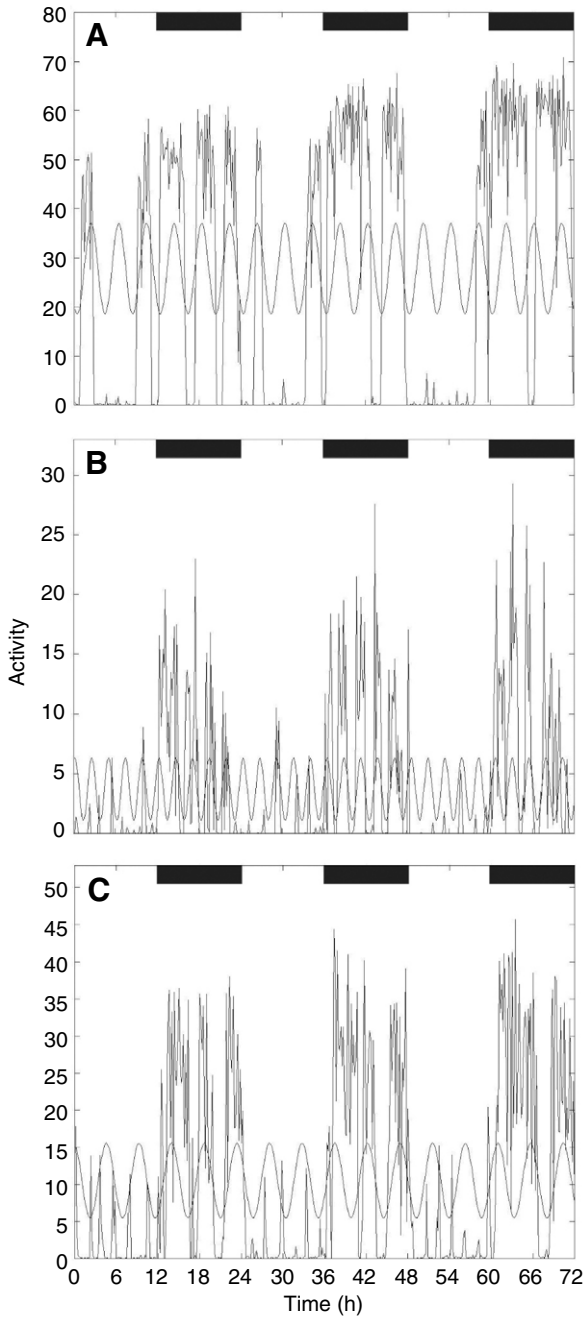

Fig. 4. (A) The actogram of data from the same mouse from strain SWN as in Fig. 1, with data re-binned to 10 min intervals as above; however, the periodicity value garnered from MESA and corroborated by autocorrelation from the high-pass filtered data was used to fit a cosine to the *un*filtered data using nonlinear regression (NLIN). The resulting curve was superimposed on these unfiltered data showing the good fit over all 3 days of recording, including peaks evident during the light periods. (B) The same treatment for a mouse from the JF1 strain (JF1248e, ultradian periodicity, τ=2.42 h). (C) As above for a mouse from strain B6 (B6-44a, ultradian periodicity, τ=4.7 h).

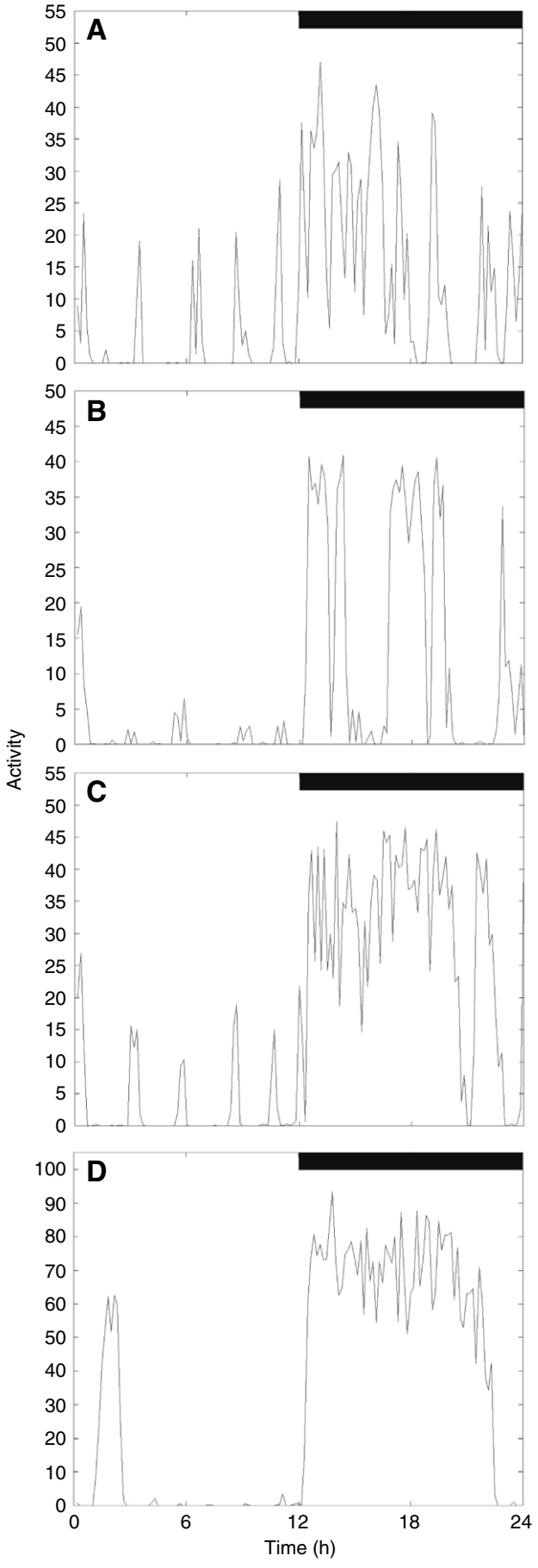

Fig. 5. 'Form estimates' of the activity patterns constituting all mean hourly data from all mice in a given strain, superimposed to give a single 24-h (144-bin) profile. These profiles were constructed from re-binned, unfiltered data. This process is equivalent to 'time averaging' in electrophysiology. The profiles are arranged in decreasing levels of coherent ultradian activity across strains. (A) Strain HMI, (B) strain BFM/2, (C) strain B6, (D) strain SWN. It is remarkable that despite the pooling of data, the strong amplitude and phase coherence across individuals shows clear evidence of the rhythmicity.

dependence on strain ($F_{10,220}$=4.83, P=0.0001). Critically, there was a clear interaction between strain and frequency ($F_{10,220}$=20.58, P=0.0001; GLM procedure).

DISCUSSION

By applying sufficiently robust analytical techniques to mouse activity data, we have shown that intact, genetically normal animals in an entraining light–dark cycle display strong ultradian periodicities. These periodicities persist with unchanging phase throughout the duration of the experiment, and can be seen even during the light portion of the cycle at reduced amplitude. In several instances, the periodicities are still visible when all animals of a given strain are pooled to make the form estimates, indicating extraordinary regularity and strength. There was no systematic interference by the estrous cycle. Poon et al. (Poon et al., 1997) found similar results in mice, also recorded for 72 h. In their case, however, they used the continuous wavelet transform (Daubechies, 1990) to emphasize transient rather than long-range periodicities and thus were unable to look at ultradian periodicities in individual animals, only in the ensemble averages (Poon et al., 1997).

In our study, there were no systematic differences in the primary periods of the ultradian rhythms among the 11 genetic strains, with all showing values in the same range. The strength of the rhythmicity was also uniform, with all strains having roughly the same percentage of animals with clear rhythms as assessed by inspection of the correlograms. However, we did find significant variation among genotypes when we looked at the structure of this periodicity in the full ultradian period domain. Multiple ultradian periodicities are not uncommon, for example, in the fly (Dowse et al., 1987). There was significant variation among strains for the computed Fourier coefficients and an interaction between period and strain. Given this genetic variation in the structure of the ultradian periodicity within a highly genetically heterogeneous ensemble of strains, it seems warranted to hypothesize that the period is subject to selection, although narrow-sense heritability studies would have to be done to confirm this possibility (Falconer, 1960). Such a study in fly heartbeat serves as an example of heritability in a high-frequency cyclical process (Jennings et al., 2009). If, however, these high-frequency oscillations are solely involved in maintenance of what Bünning (Bünning, 1973) has called 'internal temporal order', meaning the time-dependent marshalling of intracellular functions, then their periods and amplitudes would be independent of environmental influences and this would also be important to know.

It has been thought that in mammals such as rats and mice, it is only after disruption of the circadian clock occurs that the higher frequency rhythms appear (Rosenwasser and Adler, 1986; Redlin and Mrosovsky, 1999) and they have been considered 'epiphenomena', simply wreckage from a disrupted clock or other artifact (e.g. Rosenwasser and Adler, 1986). We hypothesize that

the robustness of these high-frequency cycles reported here is a measure of the importance of the ultradian periodicity to the animal. The range of periods around roughly 4-h is not attuned to any known relevant geophysical variable. Since the periodicity persists during the time of the animal's normal rest time as well as during activity, it is unlikely to be a manifestation of a short-period timer driving bouts of foraging as is seen in the vole (Schibler, 2008). Rather the possibility exists that it is an external manifestation of a critical internal timekeeping mechanism (Dowse, 2008).

The natural question to ask is what is the underlying oscillator driving these curious rhythms? They are clear, persistent, and visible even in the presence of massive signal in the circadian range. The results reported here form a basis for further work. Given that we now see there is genetic variation for the trait in question it becomes essential to investigate the differences systematically by, for example, looking more closely at home-cage activity of strains like B6 (ultradian τ=4.1 h) and MSM (ultradian τ=3.6 h) using the parent strains as well as chromosome substitution strains derived from them (Takahashi et al., 2008). This work is underway and, ultimately, the use of knockout strains will prove invaluable to extend it. The goal is to identify individual genes central to these important cellular oscillators.

One intriguing possibility for a mechanism lies in the synthetic oscillator reported by Tigges et al. (Tigges et al., 2009). The oscillator is driven by both negative and positive feedback loops in a circuit that controls transcription in hamster ovary cells. The periods, as monitored by Green Fluorescent Protein, could be 'tuned' by altering experimental conditions, but were seen to be in the range of ~2.8 h to ~5.5 h. Although there is no reason to connect the two phenomena directly, this serves as evidence that molecular oscillators can certainly function in exactly the range of periods we report here.

The combination of digital signal analysis techniques we employ here are powerful tools for looking at multiple periodicities in a wide range of organisms and their physiology and behavior. They have been used to analyze systems ranging from heartbeat to *Drosophila* mating song, in addition to circadian and ultradian rhythms (Dowse, 2009). Critically, they enable the search for cycles of one frequency embedded in a much stronger signal in another range. It is expected that these could be of utility to others, and the programs are freely available from the corresponding author upon request. Instructions and full discussions of their use can be found in Dowse (Dowse, 2007; Dowse, 2009).

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