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RESEARCH ARTICLE

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# Diurnal H-reflex variation in mice

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**Abstract** Mice exhibit diurnal variation in complex motor behaviors, but little is known about diurnal variation in simple spinally mediated functions. This study describes diurnal variation in the H-reflex (HR), a wholly spinal and largely monosynaptic reflex. Six mice were implanted with tibial nerve cuff electrodes and electrodes in the soleus and gastrocnemius muscles, for recording of ongoing and nerve-evoked electromyographic activity (EMG). Stimulation and recording were under computer control 24 h/day. During a 10-day recording period, HR amplitude varied throughout the day, usually being larger in the dark than in the light. This diurnal HR variation could not be attributed solely to differences in the net ongoing level of descending and segmental excitation to the spinal cord or stimulus intensity. HRs were larger in the dark than in the light even after restricting the evoked responses to subsets of trials having similar ongoing EMG and M-responses. The diurnal variation in the HR was out of phase with that reported previously for rats, but was in phase with that observed in monkeys. These data, supported by those in other species, suggest that the supraspinal control of the excitability of the HR pathway varies throughout the day in a species-specific pattern. This variation should be taken into account in experimental and clinical studies of spinal reflexes recorded at different times of day.

**Keywords** Electromyography · Implanted · Electrodes · Spinal cord · Monosynaptic · Reflex · Circadian rhythm

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## Introduction

Diurnal variation in motor behavior (defined as an action induced by muscle activation in response to a change in an animal's internal or external environment) is a common feature among many species of animals (Turek 1985; Stupfel and Pavely 1990). They exhibit stereotypic variation in frequency and/or intensity of locomotor, feeding, grooming, and sexual activity in the course of the day. This variation is species-specific, and can even differ among strains of a given species.

Diurnal variation also occurs during less complex motor behaviors. For example, the spinal stretch reflex and its electrical analog the H-reflex (HR), which are wholly spinal and mediated largely by monosynaptic pathways, are arguably the simplest motor behaviors of the mammalian nervous system. The size of these reflexes varies throughout the course of the day in monkeys and rats, although the phase of modulation differed between these two species (Wolpaw and Seegal 1982; Dowman and Wolpaw 1989; Chen and Wolpaw 1994). In rats at least, interruption of the corticospinal tract (CST) substantially reduces this rhythm (Chen et al. 2002b).

Operant conditioning of the HR has become a productive paradigm for the study of mammalian learning and memory (Wolpaw 2001). HR conditioning is associated with plasticity at multiple sites within the brain and spinal cord, and it requires an intact CST. The use of mice in studies of learning and memory is growing, due in no small part to the current availability and continuing development of mutant and genetically modified animals (Vaillend et al. 2002; Morgan 2003). In order to take advantage of these experimental tools, our laboratory has recently developed methodology for performing long-term HR recordings in mice (Carp et al. in press). In this study, we present data that demonstrate the existence of diurnal variation in the HR in the mice that is not simply dependent on the expected within-day variations in the ongoing level of motoneuron pool activation. These results provide important information on a potentially

confounding factor in motor system studies that include the HR pathway. A preliminary version of this work has been presented (Tennissen et al. 2004).

## Materials and methods

### Animals and preparation

This report is based on data from six mice (Swiss Webster, male, 9–13 weeks) that were each implanted with a nerve cuff on the right tibial nerve and pairs of recording electrodes in the right soleus muscle (SOL) and the medial and lateral gastrocnemius muscles (GAS) to record spontaneous electromyographic activity (EMG) and evoked responses. All procedures in animals are in accordance with the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Academy Press, Washington, DC, 1996), and Department of Health, Education and Welfare (DHEW) Publ. No. 0309-05377-3, "Guide for the Care and Use of Laboratory Animals."

Surgical procedures and animal care have been described fully elsewhere (Carp et al. in press), and are only briefly described here. Mice were anesthetized with ketamine and xylazine (120 mg/kg and 8 mg/kg i.p., respectively) and received supplemental doses as needed. They also received glycopyrrolate (0.02 mg/kg i.p.) and penicillin G (10,000 units/kg i.p.) immediately prior to surgery. A heating pad under the animal and a lamp above the animal maintained body temperature.

The right SOL, GAS, and tibial nerve were exposed under direct stereomicroscopic visualization and implanted with three pairs of multistranded (7×50 ga) stainless steel Teflon-insulated wires: one pair in each muscle for recording (EMG), and the remaining pair of wires on the tibial nerve within a 3-mm-long silicone rubber cuff (1.65 mm o.d. × 0.76 mm i.d.) for eliciting the HR. The wires were routed under the skin to emerge at a small incision at the nape of the neck. The wires were secured to a small circle of polyester mesh implanted under the skin at the wire exit site. The mesh was secured by suturing it to an external nylon skin button. The wires passed through a 30-cm long stainless steel spring to terminate in an electrical connector.

After surgery, mice received an analgesic (Demerol, 3 mg/kg i.p.) and were transferred to custom-built cages (30.5 cm high on an octagonal base with 16–17 cm between opposing sides). Each mouse received penicillin G on the second and fourth days after surgery. Food and water were available at all times. Lights were on between 0700 and 1900 h.

### Data collection and analysis

The cable plug connected via a low-torque electrical commutator (SL-88-10, Dragonfly Research & Development) to amplifiers (gain = 1,000, bandpass filter = 10–3,000 Hz) and a stimulus isolation unit. Nerve stimulation and acquisition of EMG were under continuous computer control. The computer sampled ongoing EMG at 10 KHz and calculated the absolute value (equivalent to full-wave rectification by analog devices) of EMG from both muscles 24 h/day. When the absolute value of the ongoing EMG remained within a user-defined range (see below) for 3.0–3.6 s (time period varied randomly to minimize voluntary intervention), the computer stored the most recent 50 ms (defined as the background EMG [bEMG] interval), delivered a stimulus to the tibial nerve cuff, and collected and stored EMG for another 100 ms. The pre- and post-stimulus recording periods together comprised a single trial. Pre-stimulus data were decimated by averaging adjacent points and stored at 1,000 Hz resolution; the first 30 ms of the post-stimulus data were stored at the full 10 KHz resolution, and the final 70 ms of post-stimulus data were decimated and stored at 200 Hz resolution.

For each animal, two post-stimulus time intervals encompassed the directly activated muscle response (i.e., the M-response [MR]; typically 1–3 ms after the stimulus for the daily average response) and the spinally mediated HR (typically 4–6 ms after the stimulus for the daily average response). Figure 1a illustrates the typical timing of the bEMG, MR, and HR intervals (delimited by vertical dashed lines) for the average evoked response of one animal for all 10 days of recorded data. Note that the EMG (which is a bipolar, zero-mean signal) is shown after taking its absolute value, which results in a positive ongoing EMG level and positive peaks within the MR and HR intervals above the ongoing EMG level (i.e., EMG prior to time of stimulation [Stim]). The magnitude of the bEMG was quantified as the average EMG amplitude within the bEMG interval (i.e., area of hatched region divided by the bEMG interval duration). The magnitude of the MR and of the HR was quantified as the average EMG amplitude within the MR and HR interval, respectively, above the level of the average bEMG amplitude (dotted line). This analysis assumes that the net level of descending and segmental ongoing input to the motoneuron pool after the stimulus is similar to that occurring before the stimulus, which is likely given that the animals have already maintained an average ongoing EMG level within a given range for at least 3 s in order for the stimulus to be delivered.

Various combinations of stimulus polarity, duration (0.04–0.5 ms), and intensity were assessed prior to the 10-day recording period for determination of the combination that elicited the largest HR. The MR amplitude at which the maximum HR amplitude was elicited was then used as the MR amplitude target value during the computer-controlled experiment. For each trial, the computer determined the average absolute value of the EMG within the SOL MR interval, and then incremented the stimulus amplitude if the average SOL MR amplitude was below the target value, or decremented it if the average SOL MR amplitude was above the target

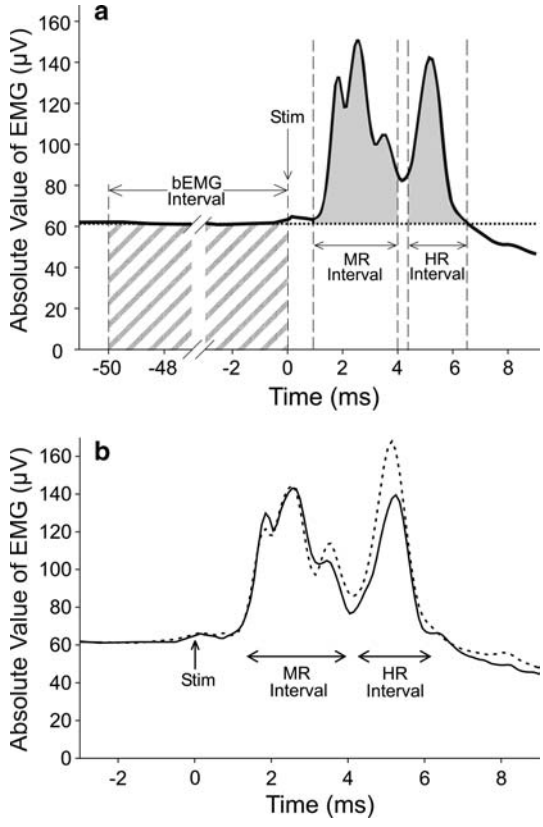


**Fig. 1** **a** regions of interest of the absolute value (equivalent to full-wave rectification) of the average of ten consecutive days of responses evoked by tibial nerve stimulation at time = 0 for one animal. The pre-stimulus ongoing EMG is quantified as the average amplitude of the area under the curve within the bEMG interval (i.e., the hatched area within the 50 ms preceding nerve stimulation). The magnitudes of the MR and of the HR are quantified as the average EMG amplitudes of the grey regions circumscribed by the evoked response within the MR and HR intervals, respectively, and by the dotted line at the level of the average bEMG amplitude. **b** averages of evoked responses recorded from one mouse in the light and in the dark. Averages of absolute values of evoked responses recorded over ten days in the same mouse as in part **a** are shown for trials elicited in the dark between 0100 and 0400 h (*dotted trace*; average of 3,826 trials) and in the light between 1600 and 1900 h (*solid trace*; average of 1530 trials). Average bEMG amplitude was calculated from the EMG recorded during the 50 ms preceding nerve stimulation (Stim at time = 0). MR and HR amplitudes were calculated as the average EMG amplitudes within the MR and HR intervals (*horizontal bars*), respectively, minus the average bEMG amplitude. We selected trials in which the average bEMG and MR amplitudes were closely matched (average bEMG = 62.1 μV during both time ranges and average MR amplitude = 53.3 μV during both time ranges). Even though bEMG and MR amplitudes were comparable (i.e., the traces largely overlap prior to stimulation and after stimulation up until the end of the MR interval), the HR was larger in the dark (average HR amplitude = 66.3 μV) than in the light (average HR amplitude = 49.5 μV)

value. This negative feedback control of the nerve cuff stimulus intensity allowed the computer to adapt to variations in efficacy of nerve stimulation in the freely moving animal, and thereby maintained the SOL MR amplitude near the target value.

The range of bEMG values within which trials were collected was limited so as to require at least a minimal level of activity in both SOL and GAS, and so as to exclude values exceeding the targeted MR amplitude in SOL. The former restriction was necessary to provide sufficient activation of the motoneuron pools to be able to elicit an HR. The latter restriction was necessary to prevent trials from being collected during high-amplitude ongoing EMG. Because of the negative feedback system's control over the stimulus intensity, recording while bEMG exceeded the MR target level could cause the computer to reduce the stimulus intensity below the threshold for nerve stimulation.

In addition to recording the evoked responses to nerve cuff stimulation, a separate data acquisition system recorded, calculated the absolute value of, and stored 1-s epochs of ongoing EMG every 15 s. These data are identified as spontaneous EMG (sEMG) because they were not linked to the HR stimulus nor were there any amplitude limitations, as with the bEMG. The sEMG was recorded at 10 KHz, and then decimated by averaging adjacent samples and stored at a resolution of 200 Hz. In three of these animals, the sEMG recordings were performed during the same 10-day period as that during which the HR recordings were performed. In the other three animals, the sEMG recordings were performed on 6–9 consecutive days subsequent to the days when HR recordings were made.

Data from individual evoked responses and recordings of spontaneous activity (i.e., mean absolute values of bEMG, MR, HR, and sEMG) were grouped into eight 3-h time periods for each day (i.e., 0100–0400, 0400–0700, 0700–1000, 1000–1300, 1300–1600, 1600–1900, 1900–2200, and 2200–0100 h) and averaged. The average values for each time period were expressed as a percentage of the daily mean value. Data from consecutive days were then averaged to give the final daily time courses.

The preceding analysis of diurnal HR variation was based on all trials collected within each of the eight 3-h time periods. In order to address whether HR diurnal variation was simply a function of diurnal variation in the level of excitation of the motoneuron pool (as reflected by bEMG) and/or stimulus strength (as reflected by the MR), we formed subsets of the entire data pool by grouping trials with similar values of average bEMG and MR amplitudes. Comparisons of HR amplitudes among the eight 3-h time periods using trials with similar values of bEMG and MR amplitudes allowed us to determine whether HR diurnal variation occurred independent of the level of bEMG and MR.

One complication in performing this analysis was the differences in average size and range of EMG recorded among the animals. In order to pool data from all animals, we first normalized bEMG, MR, and HR amplitudes based on the characteristics of their distribution in each animal. Individual bEMG values were normalized by subtracting the mean value of all trials and then dividing by the standard deviation of all trials (i.e., z-score). Individual MR and HR amplitudes were nor-

malized by dividing by their standard deviation calculated from all trials. The all-trial mean amplitude was not subtracted from the individual MR or HR amplitude. Accordingly, MR and HR amplitudes of zero (i.e., when the total EMG within the MR or HR intervals was the same as the bEMG value) reflect the MR and HR thresholds, respectively, and thus are comparable among animals. The normalized data are in units of SD. Data from all trials were then pooled into subsets defined by specific ranges of normalized bEMG and MR amplitudes and averaged across animals.

The percentage of the time within the bEMG interval during which motor unit action potentials were present was defined as the bEMG activity percentage. This was calculated as the number of EMG samples within the bEMG interval that were larger than 20 μV divided by the total number of samples within the bEMG interval × 100.

The significance of effects was evaluated by analysis of variance (ANOVA) with time as a within-subjects effect. Post hoc comparisons were made by least squares contrasts. $P$ values $< 0.05$ were considered to be statistically significant.

## Results

All mice recovered rapidly from implantation surgery and exhibited normal feeding, grooming, and locomotor behaviors. EMG recordings were begun within 2 weeks after implantation surgery. Data were recorded continuously for 18–92 days. Ten consecutive days of stable EMG recordings (i.e., $< 10\%$ variation in mean daily bEMG and $< 25\%$ day-to-day variation in the mean daily MR) were selected from each mouse for analysis of diurnal HR variation. The average number of HR trials/day recorded in each animal ranged from 3,643 to 11,045 (median 7,111) during this 10-day period. The time and amplitude criteria for initiating trials (see Materials and methods) precluded data acquisition during SOL and GAS inactivity and during cyclical movements of the hindlimb (e.g., locomotion). Most trials were collected during quadrupedal stance (either horizontally on the cage floor or vertically while holding onto the bars of the food pellet hopper) and bipedal postures (e.g., rearing, upper body grooming). We saw no indication that the animals' behavior was affected by the stimulation. The mice did not appear to attend to the stimulus at the time of its delivery, nor did the stimulus appear to alter or interfere with locomotion, grooming, feeding, rearing, or climbing.

Presentation of the data on diurnal variation in the HR amplitude focuses on the results from SOL, because the computer continuously adjusted nerve stimulation intensity to maintain a constant MR amplitude in this muscle. Even though GAS was not used as the target muscle, data recorded from this muscle were similar to those recorded from SOL, and are described briefly.

### Diurnal variation in SOL HR

Figure 2a shows an example of the time course of the SOL bEMG, MR, and HR amplitudes during five consecutive days from one mouse. The HR amplitude tends to be lower in the light and higher in the dark. For each mouse, the typical time course of this modulation is most clearly shown in the average of all 10 days' data (see example for one mouse in Figure 2b). In this animal, the HR amplitude was largest in the dark between 0100 and 0400 h and smallest in the light between 1600 and 1900 h.

Figure 3 shows the average for all six mice of the within-day time course of the HR amplitude, MR amplitude, bEMG, and number of trials using all available data (filled symbols) and of the sEMG (i.e., average of 1-s EMG epochs recorded at 15-s intervals). Figure 3a shows that there was a significant difference in the HR amplitude among the eight time periods ($p < 0.001$ by ANOVA). The maximum HR value (time period 0100 – 0400 h) was significantly different from the HR amplitudes from the three time periods spanning from 1300 to 2200 ($p < 0.01$ by post-hoc least-squares contrast).

The diurnal variation in the HR amplitude was not simply the result of marked variation in one or two animals, but was present in all six animals. ANOVA of the HR amplitude over the course of the day for ten consecutive days revealed significant differences among eight 3-h time periods ($p < 0.001$ for five animals, $p = 0.02$ for one animal; individual time courses not shown except for one animal in Figure 2b). Figure 4a shows the maximum and minimum within-day modulations of the HR amplitude for each of the six subjects. In the dark, the maximum HR amplitude for the six mice was 12 to 43% larger than its average daily value (mean maximum HR amplitude ± SD = 128 ± 13% of average daily value). The minimum HR amplitude was 14 to 51% less than its average daily value (mean minimum HR amplitude ± SD = 73 ± 13% of average daily value). The peak-to-peak HR (i.e., maximum HR amplitude–minimum HR amplitude) was 26 to 94% of its average daily value (mean peak-to-peak HR ± SD = 56 ± 25% of average daily value). The maximum, minimum, and peak-to-peak HR amplitude were all significantly different from the mean daily value ($p < 0.01$ by paired $t$-test).

Figure 4B shows the within-day timing of the HR amplitude maxima and minima for each of the six mice. The maximum HR typically occurred between 0100 and 0400 h (four of six mice). The minimum HR usually occurred in the light (five of six mice). It was evenly split between 0400–1000 h and 1300–1900 h.

Five of the six mice also exhibited a secondary HR peak amplitude during the light (i.e, it was larger than the HR amplitudes of both adjacent time periods). Figure 2B shows an example of such a secondary HR amplitude peak between 1000 and 1300 h. The amplitudes of the secondary HR peak during the light

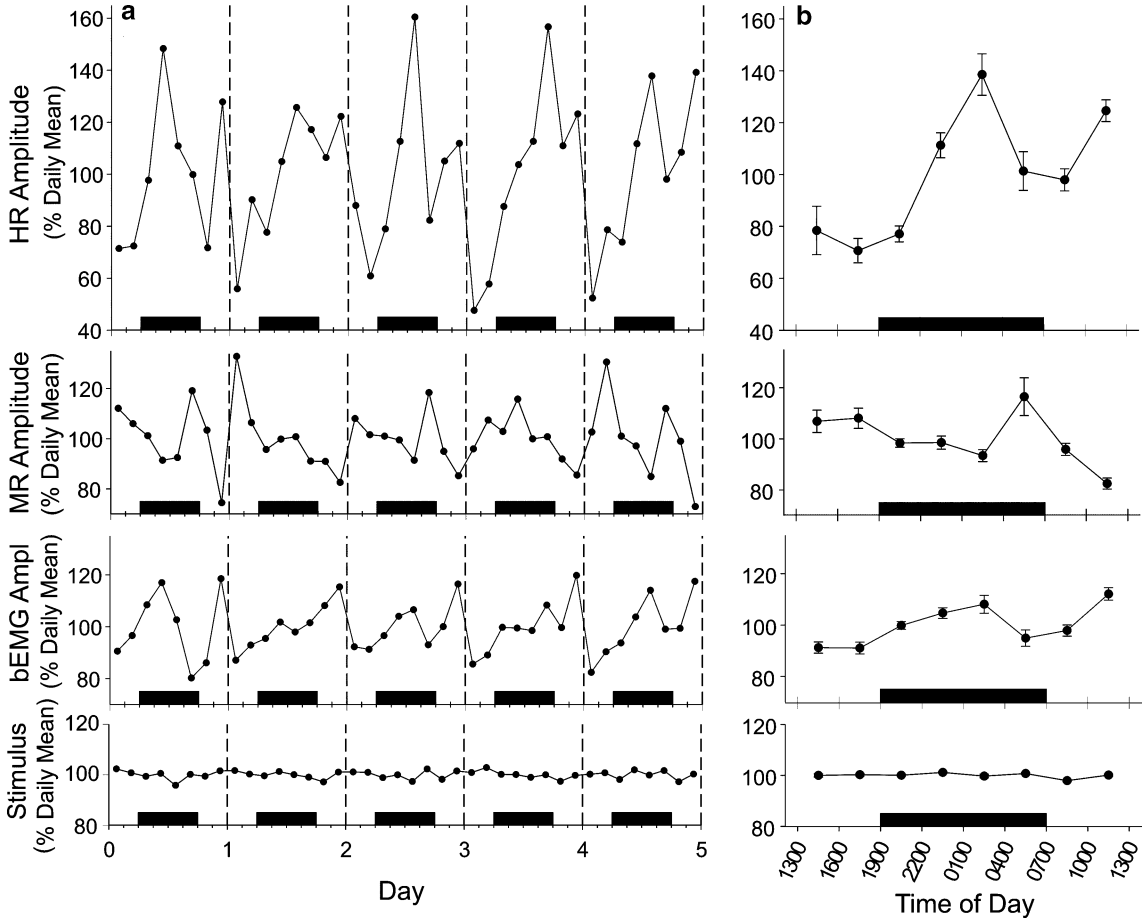


**Fig. 2** Example of diurnal variation in evoked response for one mouse. *a* for one mouse, HR amplitude, MR amplitude, bEMG amplitude, and stimulus intensity are calculated for eight 3-h periods every day for five consecutive days. Each 3-h average is expressed as a percentage of its mean daily value. The HR amplitude shows consistent variation throughout the day, being larger in the dark (indicated by the *black horizontal bars*) and smaller in the light. *b* average of all ten days' data for the same mouse. The averaging reveals the marked modulation of the HR amplitude, which shows a large peak in the dark and a smaller peak in the light. The MR amplitude and bEMG vary modestly and stimulus intensity varies a little. The *black horizontal bars* indicate dark periods. Because the data represent the average of sequential days, the data at the right end of the figure are contiguous with the data at the left end of the figure

ranged from 4 to 33% larger than each animal's average daily HR amplitude (mean secondary peak HR ± SD = 115 ± 13% of average daily HR amplitude). The secondary HR peak amplitude occurred between 1000 and 1300 in four of five mice and between 1300 and 1600 h in one mouse.

The time course of HR variation within the day is complex. Still, the data clearly indicate the presence of diurnal variation in the HR with the maximum value occurring in the dark in all six subjects.

## Diurnal variation in SOL bEMG and sEMG

In addition to the diurnal variation in the HR, there is also a small, but statistically significant diurnal variation in bEMG amplitude ($p < 0.01$ by ANOVA for data shown by filled symbols in Figure 3c). The variation in bEMG parallels that in the HR (see Figs 2, 3). This appears to reflect the diurnal variation in muscle activity.

Figure 3b shows that the MR amplitude varies significantly over the course of the day ($p < 0.05$ by ANOVA), but it is out of phase with the bEMG (Figure 3c). This relationship arises because the computer adjusts stimulation intensity so as to maintain constant average EMG amplitude within the MR interval, which includes both background and stimulus-evoked activity (see *Materials and methods*). Thus, the MR amplitude (calculated by subtracting the average bEMG amplitude from the average EMG amplitude within the MR interval; see Figure 1a) tends to vary inversely with bEMG amplitude. However, the magnitude of the diurnal variation in MR amplitude is small in comparison to that seen in HR amplitude.

The diurnal variation in sEMG is comparable to that observed in locomotor activity (Ticher and Ashkenazi 1995). Figure 3e shows that sEMG has two maxima (the larger one occurring in the dark between 2200 and 0400 h and the smaller one occurring in the light between 0700 and 1300 h) and two minima (the deeper one

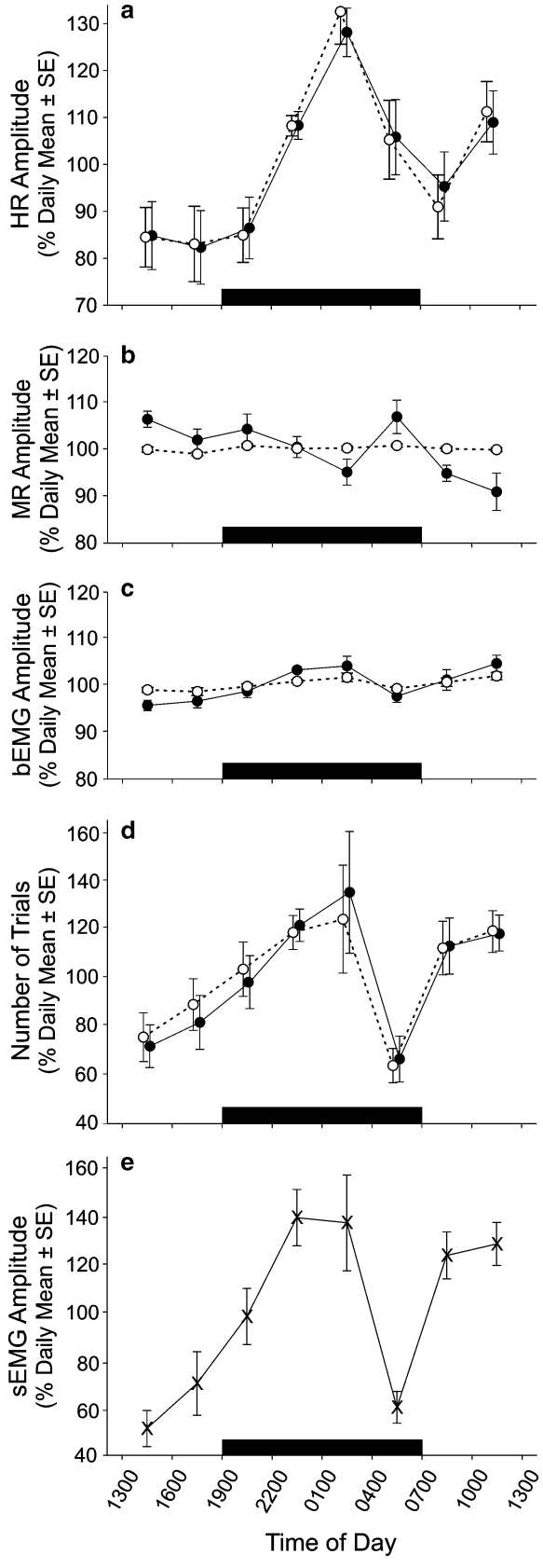


**Fig. 3** Average diurnal variation in all six mice. Average HR amplitude (**a**), MR amplitude (**b**), bEMG (**c**), and the number of trials (**d**) recorded during ten consecutive days are expressed as a percentage of each day's average value for each mouse, and then averaged for each of the eight 3-h periods that comprised a single day across all six mice (*filled symbols*). In addition, spontaneous EMG (sEMG; i.e., the average of one-s epochs of ongoing EMG recorded at 15-s intervals) amplitude recorded separately from the evoked potentials is similarly expressed as a percentage of each day's average value (**e**). The *black horizontal bars* in each panel indicate dark periods. Data at the right end of each figure are contiguous with the data at the left end of the figure. The HR amplitude varies throughout the day, being highest in the dark when the animals are most active (as indicated by the peak in the sEMG between 2200 and 0400 in panel e). The bEMG and MR amplitudes also show modest diurnal variations, which could potentially contribute to the observed daily HR variation. However, diurnal variation in the bEMG and MR amplitudes is not necessary for expression of the diurnal HR variation. The HR variation is not affected when MR and bEMG variation is greatly reduced by forming a subset of the data using only trials with MR and bEMG values limited to narrow ranges (*open symbols*; see text for details). Panel d shows that use of this reduced data set did not alter the distribution of trials recorded throughout the day

occurring in the light between 1300 and 1600 h and the shallower one occurring in the dark between 0400 and 0700 h). This variation in average level of SOL activity closely parallels the average level of EMG prior to HR elicitation (bEMG in Fig. 3; correlation coefficient = 0.95, $p < 0.001$ for sEMG vs bEMG), suggesting that the pre-trial bEMG values recorded are representative of the ongoing EMG throughout the day.

## Independence of diurnal variation in HR from that in bEMG and MR

The presence of diurnal variation in bEMG and MR (albeit modest) raised the possibility that these phenomena are responsible for the diurnal variation in the HR. Thus, after normalizing the data according to each animal's standard deviation (see *Materials and methods*), we extracted from the entire data pool a subset of trials in which bEMG and MR values were closely matched (i.e.,-0.8 SD < normalized bEMG $< +0.8$ SD and $+0.3$ SD < normalized MR $< +1.0$ SD). The data were averaged for each of the eight 3-hour time periods and were plotted as the open circles in Fig. 3a–d. The bEMG and MR amplitudes vary little (as expected), but there is still a clear diurnal variation in the HR ($p < 0.0001$ by ANOVA). Figure 1b illustrates the difference in the HR elicited at two different times of day for one animal. Averages of the evoked responses are shown from one of the dark periods (0100–0400 h) and from one of the light periods (1600–1900 h). The HR is clearly larger in the dark (dotted line) than in the light (solid line), while the pre-stimulus background activity and the MR are well matched.

Diurnal variation in the number of trials performed in each 3-hr time bin using all available trials was similar to that in the bEMG- and MR-limited trials (Fig. 3). Thus, differences in the proportion of trials analyzed in different time bins did not confound this analysis.

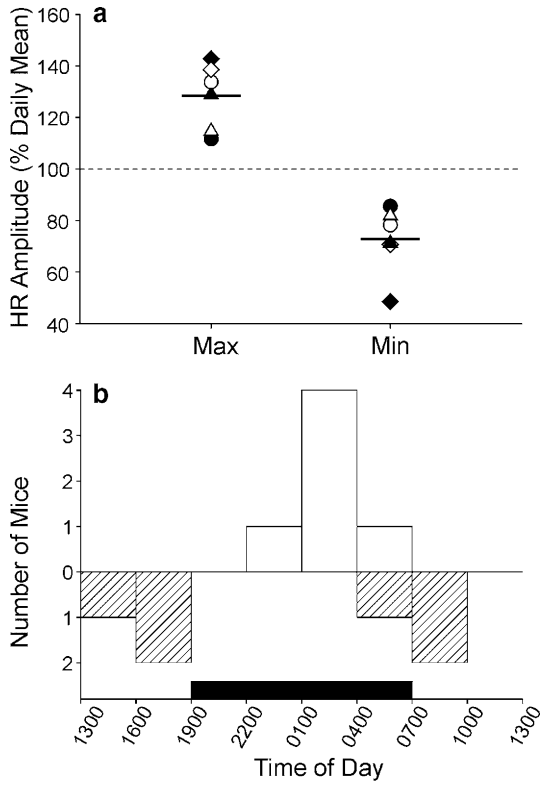


**Fig. 4** Magnitude and timing of maximum and minimum HR amplitudes. **a** distribution of the maximum (Max) and minimum (Min) within-day HR amplitudes for all animals (expressed as a percentage of the daily mean value from each mouse's average daily time course). The *dashed line* at 100% on the ordinate indicates the mean daily value. *Horizontal bars* in each group indicate the mean maximum HR amplitude (128 ± 13% of average daily value ± SE) and the mean minimum HR amplitude (73 ± 13% of average daily value ± SE). Data from a given animal are represented by one symbol type. **b** time of day at which the HR amplitude was at a maximum (*above-abscissa open bars*) or at a minimum (*below-abscissa hatched bars*). The maximum HR amplitude typically occurred between 0100 and 0400 h. The time period of minimum HR amplitude was split between 0400 and 1000 h for three mice and 1300 and 1900 h for the other three mice. The *black horizontal bars* indicate dark periods

The diurnal HR variation is not limited to the specific range of bEMG and MR shown in Fig 1b. The normal variation in bEMG and MR afforded us the opportunity to evaluate the dependence of the HR on these two factors. HR, MR, and bEMG amplitudes were normalized according to each animal's SD for all of its data (see *Materials and methods*), sorted by magnitude, and binned into three ranges of bEMG and four ranges of MR for each of the eight 3-hr time periods. Fig 5 shows the HR as a function of the MR for the three levels of bEMG recorded during one of the dark periods (0100–0400 h; filled circles) and during one of the light periods (1600–1900 h; open circles). The point on the abscissa where the MR is zero represents the motor threshold level (dashed vertical line), just above which the first motor axon is activated by the stimulus. Because both motor and sensory fibers are stimulated within the cuff, the MR amplitude quantifies the degree of activation of group I afferents in addition to that of motor axons (both orthodromically and antidromically). Thus, the curves in Fig. 5 reflect the input-output relationships of the HR pathway at different levels of motoneuron pool activation at two different times of day. Corresponding points (i.e., having the same bEMG and MR amplitudes) in the two families of input–output curves recorded during the two time periods are always larger for the dark than for the light. All pairs were significantly different ($p < 0.02$ for all by least-square contrasts after ANOVA) except for the values in the lowest range of bEMG values (filled square vs open square) at the lowest range of MR values. Thus, diurnal variation in the HR occurs even when variation in bEMG and MR have been minimized.

When comparing HR amplitudes at different times of day, the preceding analyses required the average bEMG values to be similar, but it did not place any restrictions on the pattern of EMG within the bEMG interval. We assessed the activity pattern within the bEMG interval using the bEMG activity percentage, which reflects the portion of this interval that exhibits activity exceeding a

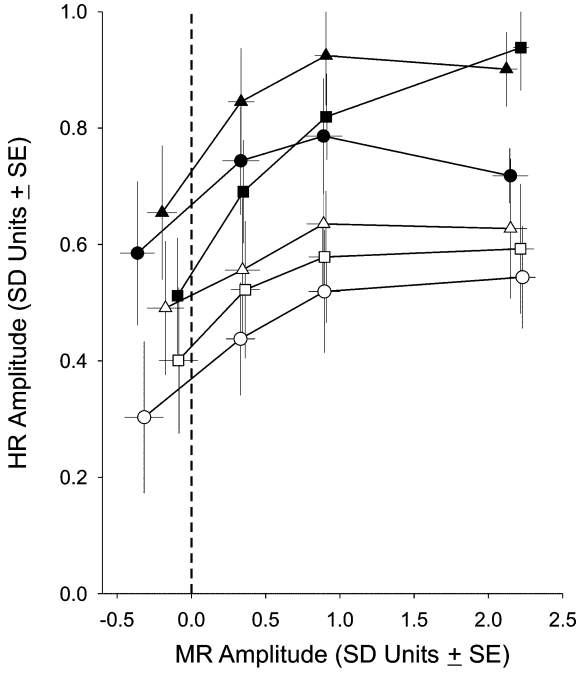


**Fig. 5** HR input–output relationships. Data, normalized according to each animal's standard deviation (SD) of bEMG and MR amplitudes, were rank-ordered so as to form three levels of bEMG (*square, circle*, and *triangle symbols* for the lowest, middle, and highest ranges, respectively) and four levels of MR amplitude (*abscissa*). The average MR amplitude and HR amplitude for each of the 12 combinations of bEMG and MR amplitude were calculated for the two 3-h periods 0100–0400 h (*filled symbols*) and 1600–1900 h (*open symbols*). Each 3-h average is expressed in SD units. The HR amplitude is significantly larger ($p < 0.02$ for all by ANOVA) for 0100–0400 than for 1600–1900, for all levels of bEMG and MR amplitude except the pair of data points representing the lowest MR amplitude range in the lowest bEMG range

**Table 1** Comparison of the bEMG activity percentage at five different mean bEMG amplitude levels between one of the 3-h time periods during the dark and one of the 3-h time periods during the light

| bEMG amplitude quintile | Normalized bEMG amplitude (in units of SD) | | bEMG activity percentage | |
|---|---|---|---|---|
| | 1600–1900 h (light) | 0100–0400 h (dark) | 1600–1900 h (light) | 0100–0400 h (dark) |
| 1st | −1.27 ± 0.05 | −1.32 ± 0.07 | 65 ± 3 | 63 ± 3 |
| 2nd | −0.51 ± 0.04 | −0.50 ± 0.04 | 84 ± 2 | 83 ± 2 |
| 3rd | 0.01 ± 0.06 | 0.01 ± 0.06 | 90 ± 2 | 89 ± 2 |
| 4th | 0.53 ± 0.07 | 0.54 ± 0.07 | 93 ± 2 | 92 ± 1 |
| 5th | 1.47 ± 0.08 | 1.51 ± 0.05 | 96 ± 2 | 95 ± 1 |

*bEMG amplitude quintile*, one of five equal-size groups of trials formed after sorting by ascending average bEMG amplitude; *Normalized bEMG amplitude*, bEMG amplitude minus the overall mean bEMG amplitude and divided by the SD for each animal (i.e., the z-transformation, where the mean value is zero and each full unit represents one SD); *bEMG activity percentage*, percentage of EMG data samples within the bEMG interval that exceed 20 μV

minimal level (20 μV). This variable should be sensitive to changes in the number, duration, and/or frequency of firing of motor units that contribute to the ongoing EMG for a given average level of bEMG (e.g., for two trials having similar average EMG amplitudes, the trial with a bEMG interval containing one motor unit with a large EMG signature firing a given rate would have a lower bEMG activity percentage than would the trial with a bEMG interval containing several motor units with small EMG signatures firing at the same rate). Trials were sorted according to their average bEMG amplitude and binned into five equal subsets of trials. For each of these subsets, we calculated the bEMG activity percentage in the eight 3-h daily time periods. Analysis of the distribution of bEMG values revealed no significant differences among any of the eight 3-h epochs in bEMG activity percentage for any of the five ranges of average bEMG amplitude ($p > 0.4$ by ANOVA). Table 1 shows values calculated for the two time periods that displayed the largest difference in HR amplitude (i.e., 1600–1900 h and 0100–0400 h; see Fig. 3). Sorting and binning the trials into the five groups produces quintiles with increasing average bEMG amplitudes and bEMG activity percentages for both times of day. The average values of bEMG amplitude and bEMG activity percentage in the dark are similar to those in the light. These data are consistent with the hypothesis that the firing patterns during ongoing EMG for a given average level of activity recorded at different times of day are similar.

### Diurnal variation in GAS HR

In the five animals in which GAS EMG was recorded, the GAS HR exhibited diurnal variation similar to that seen in the SOL. The HR maximum in the dark fell in a range between 113 and 167% of the daily mean value (mean ± SD = 137 ± 21% of the mean daily HR) and occurred between 0100 and 0400 h in four out of five animals and between 2200 and 0100 h in one animal. The HR minimum fell in a range between 59 and 83% of the daily mean value (mean ± SD = 70 ± 11% of the mean daily HR) and occurred between 0400 and 0700 h in two animals, between 0700 and 1000 h in two animals between 1600 and 1900 h in one animal. When the data from all five mice were averaged together, the HR varied significantly throughout the day ($p < 0.001$ by ANOVA). The mean maximum HR ± SE was 133 ± 11% of the mean daily HR and occurred in the time period from 0100–0400 h. The mean minimum HR ± SE was 83 ± 9% of the mean daily HR and occurred in the time period from 1300–1600 h. Like SOL, the GAS HR amplitude was larger in the dark than in the light for trials with matched ranges of bEMG and MR amplitude.

## Discussion

This study documents the existence of diurnal variation of the SOL and GAS HRs in mice. The phenomenon was similar in all six mice. The magnitude and timing of within-day HR maxima and minima differed only modestly among animals. The average time course of HR variation was similar to that of the average spontaneous activity level (as assessed by sEMG). However, the diurnal variation in spontaneous activity does not appear to be the primary cause of diurnal variation in the HR, because (1) diurnal HR variation persists even after limiting the data to trials with closely matched values of bEMG amplitude and of MR amplitude and (2) HR amplitude is only weakly dependent on bEMG in the range of values traversed during the day.

The ranges of bEMG and MR at which the HR was evaluated were necessarily limited. The computer controlling the experiment only acquired a trial if the requirement for stable bEMG was met for at least 3 s. Thus, HRs were usually elicited while the animal maintained a relatively stable posture, and not during rapid movements (e.g., locomotion). Maximum MR was determined in four of the six mice. For each animal, the largest MR recorded during a trial (estimated by the value at the 99th percentile of the distribution of all trials) fell in the range between 16 and 48% of the maximum possible MR (mean = 26% for all four ani-

mals). The largest bEMG recorded during a trial (estimated by the value at the 99th percentile of the distribution of all trials) fell in the range between 7 and 12% of the maximum possible MR (mean = 9% for all four animals). The range of permissible bEMG values was sufficiently wide to permit HR acquisition throughout the day.

Tibial nerve stimulation elicits not only strong homonymous SOL and GAS afferent input, but also weak heteronymous inputs from other muscles of the lower hindlimb and foot (Carp 1993; Hongo et al. 1984). Non-SOL and non-GAS tibial nerve inputs to SOL motoneurons are weaker than are those to GAS motoneurons, and yet the diurnal HR variation in SOL is at least as large (if not slightly larger) than in GAS. Thus, these heteronymous inputs are unlikely to play a significant role in the diurnal variation of the SOL and GAS HR.

Diurnal variation in the HR presumably reflects within-day variation in one or more of the many factors that determine the size of the HR. Figure 5 shows that the ongoing level of descending and segmental excitatory input to the motoneurons (as reflected by the SOL and GAS bEMG) and the stimulus-evoked afferent input (as reflected by the MR) are two important determinants of the HR. Both the ongoing SOL EMG and the MR vary throughout the day (Fig. 3b, 3c), and thus theoretically could contribute to the diurnal variation in the HR. However, the data do not indicate a substantial contribution of diurnal variation in the net level of ongoing activation of the motoneuron pool or in the magnitude of the afferent volley to the diurnal variation in the HR. First, restricting the data to a subset of trials having a narrow range of bEMG and MR amplitudes did not affect diurnal HR (Fig. 3). This indicates that variation in bEMG and/or MR is not essential for expression of diurnal HR variation. Second, each input–output relationship in Fig. 5 recorded during the light appears to reach a plateau, suggesting that these curves do not reflect the same underlying relationships (i.e., simply shifted to the right along the MR axis) as those for data recorded in the dark (filled symbols). Third, at the time of maximum HR amplitude (i.e., 0100–0400 in Fig. 3), the MR amplitude is about 5% below its daily average value. Based on the input–output relationships of Fig. 5, a decrease in the MR from the average daily normalized MR value (i.e., 0.76 for all data in Fig. 5) would be expected to decrease the HR. In addition, at the time of minimum HR amplitude (i.e., 1600–1900), the normalized MR amplitude is about 2% above the daily average value, which would be expected to increase in the HR amplitude. These analyses (along with comparable analyses of the other six time points) indicate that while diurnal variation in the MR might make minor contributions to the time course of diurnal HR variation, the diurnal MR variation is not the underlying cause of the daily variation in the MR.

Fourth, the HR amplitude depends on bEMG amplitude in a complex way. Fig. 5 shows that for a given MR amplitude, the HR amplitude is largest for the middle third of bEMG amplitude value distribution; the HR amplitude decreases with decreasing or increasing bEMG amplitude values. At the time of maximum and minimum HR amplitudes, bEMG amplitude is about 4% below and 4% above the daily mean bEMG values, respectively. This range of values corresponds to normalized bEMG values of $\pm 0.25$, in which the HR amplitude displays little or no dependence on bEMG (see curve for middle one-third of MR values in Fig. 4b in Carp et al. [in press], which clearly shows the dependence of the HR on bEMG for the relevant range of MR values). Thus, diurnal variation in bEMG has little impact on the diurnal variation in the HR.

The preceding analysis makes the generally accepted assumptions that motoneurons within a given pool receive ongoing descending and segmental influences that are widely distributed across the entire pool and are recruited in a stereotypic order (Burke 1981; De Luca and Erim 1994; Henneman and Mendell 1981). Synaptic inputs from some descending pathways to motoneurons can theoretically cause modest changes in recruitment order under certain circumstances (Heckman and Binder 1993). During ballistic movements and possibly during locomotion, large deviation from the usually observed recruitment order involves preferential recruitment of large motor units (Smith et al. 1980; Wakeling 2004). In the present study, the EMG amplitude and time requirements would preclude recording trials under these conditions. Even so, it is conceivable that different types of motor units are recruited at different times of the day. For example, the diurnal variation in the HR could reflect preferential recruitment of large motor units (with large EMG signatures) in the dark and preferential recruitment of small motor units (with small EMG signatures) in the light. In order to maintain comparable average levels of ongoing EMG, the frequency and/or number of large motor units firing in the dark would have to be lower than that of small motor units firing in the light. However, we saw no indication of a difference in the bEMG activity percentage (i.e., the fraction of the bEMG interval that contains motor unit action potentials; Table 1). SOL has a relatively uniform distribution of motor unit types, which would tend to blunt any motor unit size-dependent contributions to diurnal variation in the HR. Thus, differences in recruitment order do not provide a likely explanation for the diurnal variation in the HR.

It is likely that factors other than daily variation in bEMG or MR amplitude contribute to the diurnal variation in the HR. For example, pre-synaptic inhibition of Ia afferent input to the spinal cord is under descending control and exhibits task-dependent modulation (McCrea 2001). Within-day differences in the efficacy of transmitter release could readily contribute to HR diurnal variation. Alternatively, modulation of the intrinsic excitability of the motoneuron itself could contribute to diurnal HR variation. For example, descending neuromodulatory pathways affect conduc-

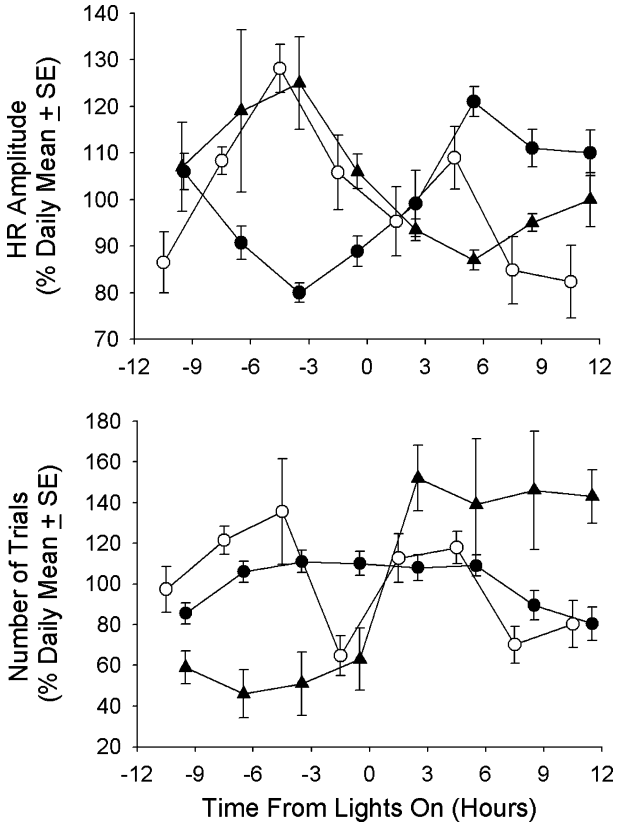


**Fig. 6** Comparison among three species of diurnal variation in reflex size and number of trials. The diurnal variation in the HR amplitude (*upper panel*) and in the number of recorded trials (*lower panel*) are shown for mice (*open circles*; present study), rats (*filled circles*; data from Chen and Wolpaw 1994), and monkeys (*filled triangles*; data from Dowman and Wolpaw 1989). Because monkeys and rats experienced a light period (0600–2100 h) different from that used for the mice (0700–1900 h), the data are shown relative to lights-on time (i.e., time = 0). The timing of the HR amplitude maximum of the mouse is in phase with that of the monkey, despite the species' opposite activity patterns (as indicated by the number of trials). The timing of the HR amplitude maximum of the rat is out of phase with that of the mouse

tances that regulate the motoneuron's responsiveness to synaptic input (Heckmann et al. 2005). Thus, variation throughout the day in brain and spinal cord neuromodulatory influences could contribute to diurnal differences in motoneuron excitability (Reis et al. 1968; Reis and Gutnick 1970).

Stimulation of the motoneuron axons that evoke the MR also elicits an antidromic volley that activates Renshaw cells to provide recurrent inhibitory input to nearby motoneurons. Computer control over the stimulus intensity was designed to maintain a stable MR, and thus minimize variation in recurrent inhibition. That diurnal HR variation occurs even when MR amplitudes are matched at different times of day argues against this mechanism contributing to diurnal HR variation. However, it is conceivable that diurnal variation in the descending control over Renshaw cells could modulate the amount of recurrent inhibition that the nerve stimulation evokes in motoneurons. It is not clear if the conduction time of the relatively small diameter motoneuron recurrent collaterals (Cullheim and Kellerth 1978) and the additional synaptic delay imposed by the Renshaw cell would permit the recurrent inhibition to significantly affect motoneuron recruitment by the more rapidly conducting, largely monosynaptic group Ia fiber input.

Previous studies from this laboratory have demonstrated diurnal HR variation in monkeys (Dowman and Wolpaw 1989) and rats (Chen and Wolpaw 1994). Fig. 6a shows that mice, rats, and monkeys all exhibit diurnal variation in the HR, but the pattern and timing of HR modulation varies among the three species. Mice and monkeys have large HRs in the dark about 4 h prior to light onset. Rats have small HRs in this same time period, but large HRs in the light about 5 h after light onset. The difference between rats and monkeys was attributed previously to the phase difference in their activity cycles (Chen and Wolpaw 1994). Rats are more active in the dark, and monkeys are more active in the light (as indicated by the modulation in the number of HR trials performed at different times of the day; see Fig. 6b), suggesting that HR excitability is highest during periods of rest and lowest during periods of activity. This is clearly not the case for mice, which are not only most active and but also have their highest HR excitability in the dark (i.e., the HR time courses of mouse and rat are out of phase, despite that both species are nocturnal).

The presence of diurnal variation in the HR in three species raises the possibility that this phenomenon is common to mammals in general, but that the timing of HR modulation within the day varies among species. The data presented here from mice, along with those from two other species, forms a unique pattern that is not easily explained by a single factor. The results cannot be explained simply as a rodent versus primate difference or as a daytime versus nighttime activity difference. The pattern of HR diurnal variation appears to be specific to each species.

The HR variation in mice shows a time course similar to that of their overall activity level (as assessed by sEMG). However, HR magnitude does not depend simply on the gross spontaneous activity level, because the HR variation is evident at different times of the day comparing only trials with similar bEMG levels and with similar MR amplitudes. Similarly, HR magnitude does not appear to depend simply on the illumination level, because mice often exhibit a secondary HR peak in the light. Alternatively, the modulation of the excitability of the HR pathway may be linked to the types of behaviors that the animals perform at different times of the day. The daily pattern of activity is temporally more complex in mice than in rats (Fig. 6b; Ticher and Ashkenazi 1995). Modulation of stretch reflex excitability at different points in the light/dark cycle could be advantageous to the animals' performance of certain types of tasks (e.g., when activation of an extensor reflex could interfere with body weight support or execution of spe-

cific voluntary commands). In addition, the biomechanical differences imposed by the difference in mass that is greater than tenfold between rats and mice could influence the need for proprioceptive reflex contribution to the performance of specific behaviors (James et al. 1995; Clarke and Still 1999).

Rats and mice also exhibit diurnal variations of spinally mediated withdrawal reflexes. In rats, the tail flick latency to noxious thermal input increases at night (Wright 1981). In mice, injection of formalin in the paw induces a higher rate of flinching (a spinally mediated reflex response) at night than during the day (Perissin et al. 2000). Although the sensory modalities tested were different (thermal vs chemical), these data are consistent with a phase difference between rats and mice in the diurnal variation in spinally mediated oligosynaptic withdrawal reflexes. This inter-species difference is comparable to that seen between the mice (present study) and rats for the largely monosynaptic HR, in which spinal excitability in the dark was high in mice and low in rats.

Humans also exhibit diurnal variation in spinal reflexes. In normal subjects, the electrically evoked flexion reflex has a higher threshold at night than during the day (Sandrini et al. 1986). However, people with restless legs syndrome experience a higher frequency of symptoms (including flexion reflex-like EMG patterns) at night (Bara-Jimenez et al. 2000; Hening 2004). Because altered dopaminergic transmission may contribute to this nocturnal enhancement of abnormal segmental reflexes (Clemens and Hochman 2004; Trenkwalder and Paulus 2004), it may also play a physiological role in modulation of spinal reflex pathways throughout the day.

In addition, at least part of the diurnal variation in HR amplitude may be under the control of the motor cortex. Mid-thoracic lesion of the main CST, but not of other pathways, reduces the magnitude of diurnal variation to about one-half of the pre-lesion value (Chen et al. 2002b). In addition, the CST is essential for acquisition of operantly conditioned plasticity in the HR in rats (Chen and Wolpaw 1997, 2002; Chen et al. 2002a). Motor cortex exerts control over pre-synaptic inhibition of Ia afferent transmission to spinal motoneurons (Meunier 1999) and Renshaw cells (MacLean and Leffman 1967). Thus, the substantial influence exerted by the CST over the HR suggests that the CST plays a large role in the diurnal variation in HR amplitude.

The present study, like those carried out in rats and monkeys, was performed during alternating light–dark cycles. Thus, it cannot be determined whether the intra-day HR variation reflects diurnal variation in the animal's daily behavioral repertoire or whether it instead reflects the circadian activity of an intrinsic neuronal oscillator interacting with the light–dark cycle. The latter possibility is supported by two observations. First, the reduction in HR diurnal variation in rats after CST lesion was not accompanied by gross changes in animal behavior (Chen et al. 2002b). Second, the sensory evoked potential recorded over cortex in response to the HR stimulus in monkeys also exhibited diurnal variation that was out of phase with the HR amplitude (Dowman and Wolpaw 1989). The similarity of the modulation of the segmental and supraspinal responses to sensory input, along with the marked difference in the timing of this reflex amplitude modulation, suggests that a central mechanism controls the within-day variation in the HR. Assessment of HR variation using differing photoperiods would help to clarify this issue.

Because the excitability of the HR pathway differs throughout the day, studies of simple reflex function (e.g., HR testing in chronically instrumented animals) would be expected to have different HR amplitudes at different times of day. This diurnal variation could also be relevant to studies that evaluate more complex motor behaviors that involve the HR pathway, and thus, should be taken into consideration in such studies. An obvious example is our laboratory's studies of operant conditioning of the HR, where evoked responses are averaged over 24 h to minimize contribution of the diurnal variation in HR size to the long-term conditioning-induced change in the HR. Assuming that humans exhibit diurnal HR variation comparable to that seen in monkeys, clinical studies involving assessment of spinal reflexes should be performed at a consistent time of day among subjects.

In summary, the murine HR is larger in the dark and smaller in the light. Diurnal variation in the HR is present in several species, although the relationships among reflex size, photoperiod, and activity pattern are complex, and differ among the species examined. These data emphasize the importance of taking diurnal variation into account, in both experimental and clinical studies of spinal reflex excitability.

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