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Sensorimotor-Correlated Discharge Recorded From Ensembles of Cerebellar Purkinje Cells Varies Across the Estrous Cycle of the Rat

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SUMMARY AND CONCLUSIONS

1. In the present study, locomotor-correlated activity of cerebellar Purkinje cells, recorded using arrays of microwires chronically implanted in adult female rats, was examined across estrous-cycle-associated fluctuations in endogenous sex steroids. Ongoing studies from this laboratory have shown that systemic and local administration of the sex steroid 17 β-estradiol (E_2) augments excitatory responses of cerebellar Purkinje cells to iontophoretically applied glutamate, recorded in vivo from anesthetized female rats. In addition, this steroid potentiated discharge correlated with limb movement. For the present study, extracellular single-unit activity was recorded from as many as 5–11 Purkinje cells simultaneously during treadmill locomotion paradigms. Motor modulation of activity was recorded across three to five consecutive estrous cycles from behaviorally identified cohorts of neurons to test the hypothesis that fluctuations in endogenous sex steroids alter motor modulation of Purkinje cell discharge.

2. Locomotor-associated discharge correlated with treadmill locomotion was increased by a mean of 47% on proestrus, when E_2 levels are elevated, relative to diestrus 1. These changes in discharge rate during treadmill locomotion were of significantly greater magnitude than corresponding cyclic alterations in discharge during stationary periods.

3. Correlations with the circadian cycle were also significant, because peak levels of locomotor-associated discharge on the night of behavioral estrus, following elevations in circulating E_2, were on average 67% greater than corresponding discharge recorded during the light (proestrus).

4. Alterations in the step cycle were also observed across the estrous cycle: significant decreases in the duration of the flexion phase (by 265 ms, $P < 0.05$) were noted on estrus compared with diestrus.

5. When recorded on estrus, Purkinje cell discharge correlated with the stance or flexion phase of the step cycle was greater in magnitude and preceded the event by an average of 130 ms, compared with values determined on diestrus.

6. On estrus, responses of Purkinje neurons to iontophoretically applied quisqualate were enhanced fourfold after administration of exogenous E_2, assessed in urethan-anesthetized female rats.

7. In addition, systemic administration of E_2 (30 ng iv) potentiated responses of cerebellar Purkinje cells to electrical stimulation of the forepaw by an average of 150%, recorded in anesthetized female rats.

8. These results are consistent with the hypothesis that elevations in circulating E_2 are associated with enhanced discharge of cerebellar Purkinje cells in response to pharmacological or electrical stimuli or associated with locomotor behavior.

INTRODUCTION

Elevations in the female reproductive hormones 17 β-estradiol (E_2) and progesterone in the circulation of the rat not only are followed on the night of behavioral estrus (BE) by full reproductive capacity, but are also associated with facilitation of an entire array of sensorimotor functions (Beatty 1979). These include increases in locomotor activity and sensory perception, as well as improved limb coordination. Rats in estrus perform more accurately in hurdle negotiation tasks (Smith 1994), balance beam walking (Becker et al. 1987), and running in square wheels (Broverman et al. 1968). All of these tasks require split-second timing and accurate limb placement. In humans, certain tests of manual dexterity, such as the peg-and-board test, are performed faster and more accurately during cyclic elevations in E_2 (Hampson and Kimura 1988).

Although multiple CNS areas act together to produce smooth, coordinated target-directed movement, the cerebellum is one likely target for hormonal effects that may have an impact on performance. The paravermal cerebellum plays a role in coordinating movement of the distal limbs (Brooks and Thach 1981; Ebner and Bloedel 1987; Eccles et al. 1967; Houk and Gibson 1987). Ongoing studies from the present laboratory have demonstrated that E_2 is able to significantly enhance responses of cerebellar Purkinje cells to iontophoretically applied glutamate (Smith et al. 1987b, 1988), the primary excitatory transmitter in the cerebellum. Both local and systemic administration of the steroid, at physiological doses, were previously shown to amplify excitatory amino acid sensitivity by an average of 85% (Smith et al. 1987b, 1988). This effect was shown to be specific for quisqualate (QUIS)-receptor-mediated responses of Purkinje cells (Smith 1989a). The QUIS receptor is located at Purkinje cell–parallel fiber synapses (Llano et al. 1991), as well as at Purkinje cell–climbing fiber synapses (Llano et al. 1991).

More recent studies from this laboratory have sought to examine the physiological outcome of steroid treatment by examining sex steroid effects on behaviorally correlated discharge of Purkinje cells, recorded individually with a drivable tungsten electrode during treadmill locomotion (Smith and Chapin 1987; Smith et al. 1989). Purkinje cell discharge is modulated by the step cycle (Apps and Lidierth 1989; Armstrong and Edgley 1984; Ebner and Bloedel 1981; Orlovsky 1972). Results from the present laboratory suggest that 15–20 min after acute administration of E_2 (30 ng ip), Purkinje cell discharge correlated with movement was enhanced by an average of 117% (Smith et al. 1989). Conversely, treatment with progesterone depressed movement-correlated Purkinje cell discharge, an effect that was, however, dependent on the stage of the estrous cycle (Smith et al. 1989).

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During the estrous cycle, elevations in both E_2 and progesterone precede the observed improvements in limb coordination. Therefore, in the present study, movement-correlated activity of cerebellar Purkinje cells was examined across estrous-cycle-associated fluctuations in endogenous sex steroids using chronically implanted bundles of microwires. In addition, the ability of E_2 to enhance excitatory amino acid responses of these neurons was also tested across the estrous cycle as a possible mediating factor for the changes in behaviorally correlated discharge.

METHODS

Animals

Female Long-Evans rats (200–250 g) were employed for all experiments. Rats were housed individually in a temperature-controlled facility with a constant 14:10 light:dark cycle (lights on at 0500). All but one group of rats tested during the dark phase of the light:dark cycle were housed in a facility with a reverse light:dark cycle, and were acclimated for 2 wk before testing. Food and water were available continuously.

Estrous cycle determinations

Vaginal smears from adult female rats were examined microscopically on a daily basis until a pattern of cycles was established (Smith et al. 1987b). Briefly, these are: proestrus, characterized by a predominance of nucleated cells; estrus, characterized by squamous, cornified epithelial cells; and diestrus 1 and 2, which are identified by the presence of leukocytes in the vaginal lavage. The night following proestrus is referred to here as the night of BE. On the day of proestrus and the night of BE, circulating levels of both E_2 and progesterone reach their peak; diestrus is associated with low levels of sex steroids. BE is the period when full reproductive capacity is attained and enhanced sensorimotor function is observed.

Chronic recording paradigm

For recording of sensorimotor-correlated discharge from multiple single units on a chronic basis, rats were first surgically implanted with headplugs containing Amphenol microconnectors crimped to arrays of Teflon-coated stainless steel microwire electrodes (25 μm tip diam, California Fine Wire). Wire tips were cut to a length of 1–5 mm and were implanted as an array of 5–11 wires into the paravermal cerebellum, lobules 1–3 (1.5 mm from midline). This paradigm allows stable recording of up to 11 individual neurons for a period of weeks to several months. (See Fig. 2*G* for examples of waveforms from neurons recorded simultaneously with this technique.) Purkinje cells were identifed because of their unique discharge pattern of simple and complex spikes (Smith and Chapin 1987).

During the recording sessions, the implanted rat was placed in the treadmill enclosure and the multiple-wire recording harness was plugged into the connector chronically implanted on the rat's head. The plug at the end of the harness contains one field effect transistor to be used as a cathode follower for each recording microwire. (See Nicolelis et al. 1993a,b for additional details.) Individual Purkinje cells were then identified and recorded from 7 to 10 days postsurgery. Simple spike discharge, but not complex discharge, has been shown to be modulated by the step cycle (Armstrong and Edgley 1984; Armstrong et al. 1988). Therefore changes in recorded Purkinje cell discharge are indicative of changes in simple spike discharge. (Both, however, were monitored during recording paradigms.) In all cases, recorded Purkinje cells exhibited receptive fields from the distal limbs, and discharge frequencies ranged from 2 to 60 spikes/s. Sensorimotor correlates of a particular cell were identified as the limb and step cycle phase that elicited a burst of discharge from that neuron. When correlating changes across the days of the estrous cycle, it was important to ensure that neurons from the same functionally identified cohort were being recorded from day to day. To accomplish this, several criteria have been devised. *1*) The spike height of each neuron was compared with that from the previous day. *2*) The sensorimotor correlates of each neuron were tested and verified on a daily basis. *3*) The discharge pattern of each neuron was compared from day to day. Data were then obtained from at least three estrous cycles; only data exhibiting a consistent change across the estrous cycle were included in this study. (This applied to 95% of the data.) However, the statistical procedures used to determine the significance of changes across the estrous cycle, the nonparametric Friedman's analysis of variance (ANOVA), made no assumptions that the same neurons were recorded over different days.

The paradigm employed for all behavioral studies required that rats locomote on a computer-controlled treadmill device (alternating between 5 s on, 5 s off) for a period of 20–40 min/day during continuous recording of 5–11 single units. This paradigm was not unduly stressful for the rats, as noted by their ability to follow the treadmill easily without slipping back and by the absence of stress-related behaviors (grooming, rearing, vocalization). The maximal speed required (11 cm/s) produced a quadrupedal gait, rather than a gallop, indicating that this is clearly a submaximal speed. Initially rats were trained to locomote on a treadmill over a period of 4 days. Rats tested during the light cycle were tested on a daily basis at a treadmill speed of 11 cm/s over at least three consecutive estrous cycles, assessed by inspection of vaginal cytology. Rats tested in the dark phase of the light:dark cycle were evaluated at treadmill speeds of either 4 or 11 cm/s 1–4 h after dark onset over three or four consecutive estrous cycles, every other day (on BE and diestrus) for 20-min periods. At both speeds, rats locomote with a rhythmic quadrupedal gait. Data were analyzed only for those sequences when treadmill locomotion was consistent and regular.

During the recording session, a Motorola computer stored digitized extracellular multiple single-unit activity for off-line analysis (using software written by J. K. Chapin, Philadelphia, PA). The video counter timer (Thalner Electronics) provides a digital pulse (30 cycles/s) and allows frame-by-frame analysis of the video record with 10-ms accuracy. Unit activity was recorded and correlated with locomotor (treadmill on) or stationary (treadmill off) periods. In some cases, the relative treadmill on:off value was obtained by calculating (discharge during treadmill on period)/(discharge rate during treadmill off period). Videotape analysis of data was also utilized to correlate unit activity "bursts" with movement of specific limbs during particular phases of the step cycle. Perievent histograms were then constructed around the time of the step cycle correlate (see Fig. 5, *top panel*) or the time of treadmill onset and were compared across the days of the estrous cycle. The nonparametric Friedman's ANOVA was used to test the statistical significance of changes in behaviorally correlated discharge obtained across hormone and circadian cycles.

Iontophoresis study

Responses of cerebellar Purkinje cells to iontophoretically applied QUIS were obtained across the estrous cycle before and after systemic administration of E_2 (30 ng in 0.01% propylene glycol iv) through an implanted jugular cannula. This paradigm has previously been described in detail (Smith 1989a; Smith et al. 1987b). For this protocol, extracellular unit discharge of Purkinje cells was obtained from the paravermal area, 1–1.5 mm lateral to midline, lobules 3–5, recorded from urethan-anesthetized (1.2 g/kg ip)

rats. Electrophysiological recordings were made using five-barrel micropipettes with 4- to 6-μm tips, a saline-filled central barrel (3 M NaCl), and a side barrel containing QUIS (20 mM, pH 8.0, Sigma). QUIS was iontophoretically ejected as an anion by 20-s pulses every 50 s, and retained by applications of 15-nA current of opposite polarity.

Action potentials of Purkinje cells identified by their characteristic discharge pattern of simple and complex spikes were monitored on an oscilloscope and converted to uniform voltage pulses by a window discriminator. From these gated pulses, peridrug histograms were constructed. To quantify QUIS-evoked excitation, the discharge rate during QUIS application was compared with the rate between QUIS pulses and the difference was expressed as percent excitation above spontaneous activity. Pre- versus poststeroid QUIS excitation were then compared for the population of cells tested on each day of the estrous cycle. Differences between pre- and poststeroid data were evaluated using the paired *t*-test. In addition, the one-way ANOVA and Student-Newman-Keuls tests were used to statistically assess significant differences between groups.

Forepaw stimulation study

The forepaw was stimulated bipolarly using subcutaneous 26-gauge hypodermic needles, 2 mm apart, placed in the upper limb flexors of a female rat anesthetized with urethan (1.2 g/kg ip). Two to three monophasic rectangluar pulses of 0.3–1.2 MA, each lasting 0.2 ms, were given at a rate of 500 Hz every 2 s. Responses of Purkinje cells were recorded using tungsten metal electrodes to record single-unit extracellular activity from the forelimb area of the paravermal cerebellum (lobules 3–5). Poststimulus histograms were constructed using data obtained before and at 10- to 12-min intervals after systemic injection of E_2 (30 ng in 0.01% propylene glycol ip). Pre- and poststeroid responses were then evaluated using the paired *t*-test.

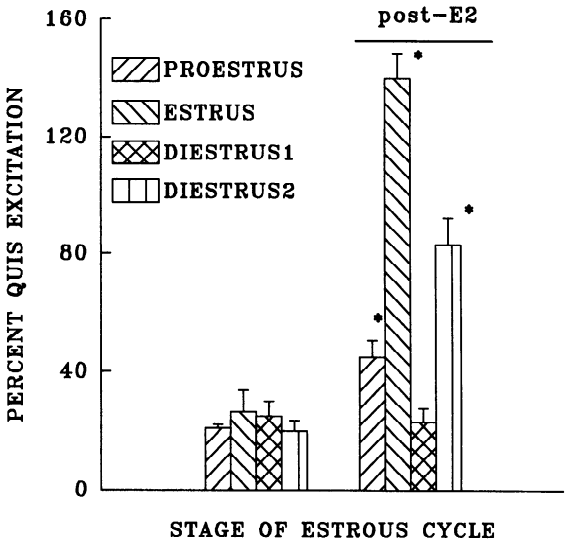

FIG. 1. Ability of 17 β-estradiol (E_2) to modulate excitatory amino acid function is dependent on the stage of the estrous cycle. This summary graph presents alterations in the ability of quisqualate (QUIS) to increase the discharge of cerebellar Purkinje cells above the spontaneous discharge level (PERCENT QUIS EXCITATION) before and after systemic administration of E_2 (30 ng iv) across the 4 days of the estrous cycle. (Horizontal bar: post-E_2 data.) E_2 administration signficantly enhanced QUIS-induced levels of excitation, above the presteroid level, over all the days of the estrous cycle, except on diestrus 1. Asterisk: $P < 0.05$ vs. the presteroid value. In all cases, vehicle was tested before steroid injection and produced no effect on the QUIS response. All poststeroid groups differed significantly from each other ($P < 0.05$), as determined by analysis of variance (ANOVA) and Student-Newman-Keuls statistical procedures. ($n = 35$, proestrus; $n = 45$, estrus; $n = 32$, diestrus 1; $n = 15$, diestrus 2.)

RESULTS

Effects of the estrous cycle on E_2 modulation of QUIS responsiveness

This study was conducted to determine whether changes in the background circulating level of E_2 across the estrous cycle could alter the ability of exogenously administered E_2 to enhance QUIS responses of Purkinje cells, a finding well established in ovariectomized rats (Smith 1989a; Smith et al. 1987b, 1988). The data obtained suggest that E_2 is able to enhance QUIS-induced responses of Purkinje cells to a differential degree across the estrous cycle. As illustrated by Fig. 1, the ability of exogenously administered E_2 to enhance QUIS responses of Purkinje cells, at a physiological dose (Di Paolo et al. 1985), was maximal on the night of BE (estrus), following peak levels of endogenous E_2 in the circulation. At this time, E_2 was able to enhance a 26 ± 7.2% (mean ± SE) control QUIS excitation to a 140 ± 8.5% excitation by 20 min poststeroid ($P < 0.001$). (In all cases, control responses were obtained with ejection currents of QUIS that produced firing 20–30% above the spontaneous level.) Intravenous injection of the steroid produced a significantly greater QUIS response ($P < 0.05$) than the presteroid level on all nights of the estrous cycle, except on diestrus 1 (a time of low circulating hormone levels).

Changes in movement-correlated Purkinje cell discharge across the estrous cycle

Discharge recorded from individual Purkinje cells during treadmill locomotion (tread on) was significantly greater than discharge recorded during the stationary phase (tread off) in 85% of the cells recorded. Although the data (Fig. 2) represent the average discharge for both simple and complex spikes, only increases in simple spike discharge were noted during treadmill locomotion compared with the nonlocomotor period. Both the circadian and estrous cycles were associated with changes in the magnitude of this locomotor-correlated discharge relative to discharge during rest. This effect is illustrated by the population of five cells simultaneously recorded across the estrous cycle and presented in Fig. 2. When assessed during the light phase of the estrous cycle, a threefold increase in the locomotor-correlated activity of all recorded neurons was noted on the day of proestrus (Fig. 2*C*), during the peak in circulating E_2, compared with diestrous values (Fig. 2, *A* and *B*). At this time, locomotor-correlated discharge (tread on) simultaneously recorded from a population of five neurons increased to 10–19 Hz relative to 3–7 Hz (tread off). Maximal levels of locomotor-correlated discharge, however, were noted for most neurons during the dark, on the night of BE (0100 until 5 h after dark onset), after elevations in circulating E_2. At this time (Fig. 2*D*), discharge during tread on varied from 10–27 Hz relative to 1–8 Hz (tread off). [During the light phases of diestrus 1 and 2 (Fig. 2, *A* and *B*), locomotor-correlated discharge varied from 0.2 to 8 Hz compared with 0.4–3 Hz during the tread off period.] Interestingly, discharge during the stationary phase decreased for all but one neuron compared with values recorded on the previous day of proestrus. On the following day of estrus (Fig. 2*E*), discharge during both behavioral states decreased, with the exception of *cell*

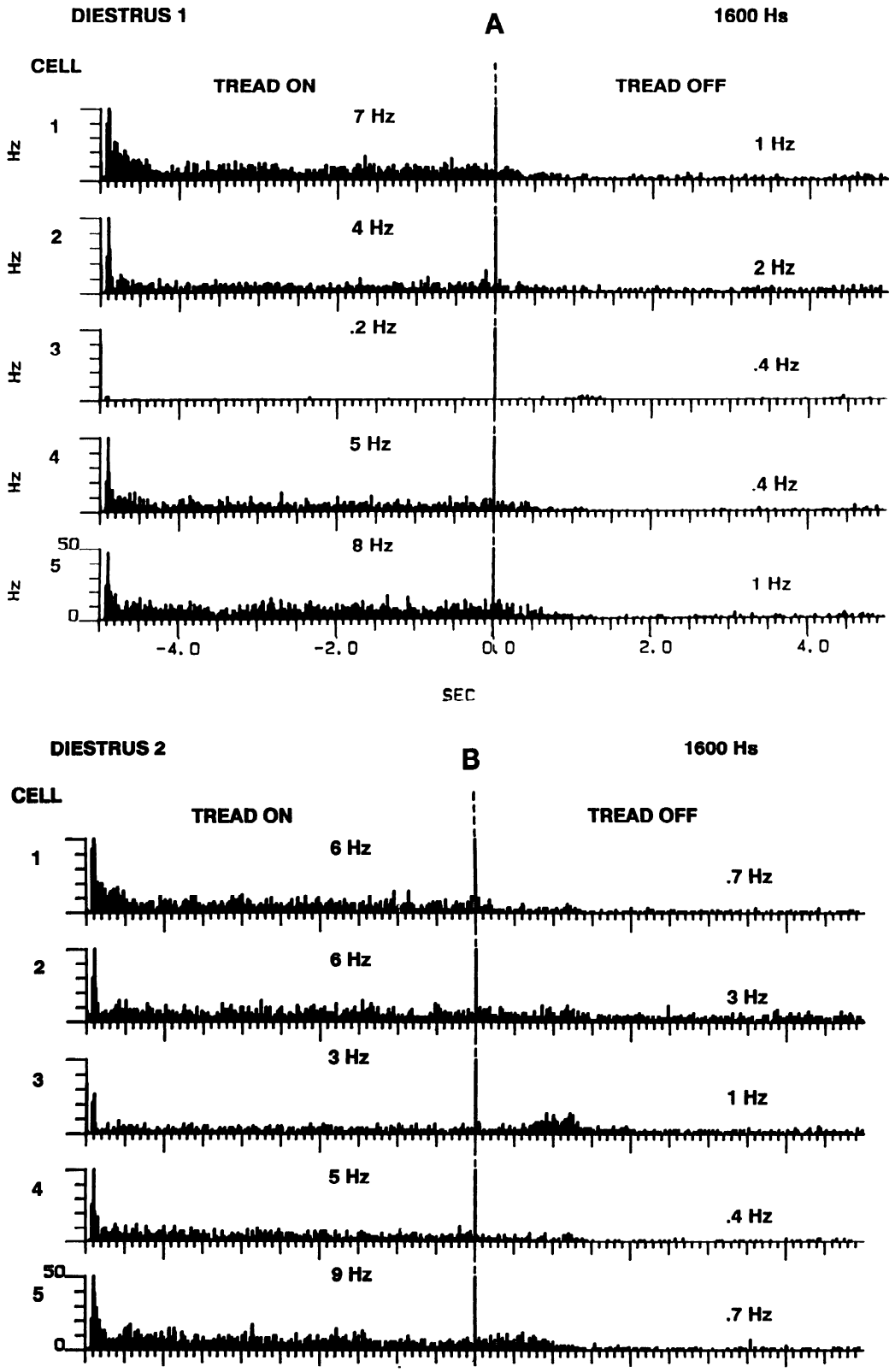

FIG. 2. Locomotor-correlated cerebellar discharge increases on the night of behavioral estrus (BE) relative to other days of the cycle. *A–F*: changes in activity of the same 5 cerebellar neurons of 1 rat recorded from the paravermal area (lobule 3) during both light (1600) and dark (0100) across the estrous cycle. In this and the following figure, perievent histograms constructed around times of treadmill activity demonstrate changes in cerebellar discharge during locomotor (TREAD ON) and stationary (TREAD OFF) periods. [each histogram sums activity of 120 cycles (DIESTRUS 1), 85 cycles (DIESTRUS 2), 85 cycles (PROESTRUS), 65 cycles (BEHAVIORAL ESTRUS—dark), 60 cycles (ESTRUS), and 120 cycles (DIESTRUS—dark).] Numbers above histograms: average discharge during the depicted tread on or tread off period (minus the initial spike). These results are representative of 43 cells (10 rats, 3–5 cells per rat) for the light phase and 49 cells (10 rats, 2–6 cells per rat) for the dark phase of the light:dark cycle. *G*: waveforms of 6 Purkinje cells recorded simulataneously. (Amplitude, polarity, and time base are indicated.)

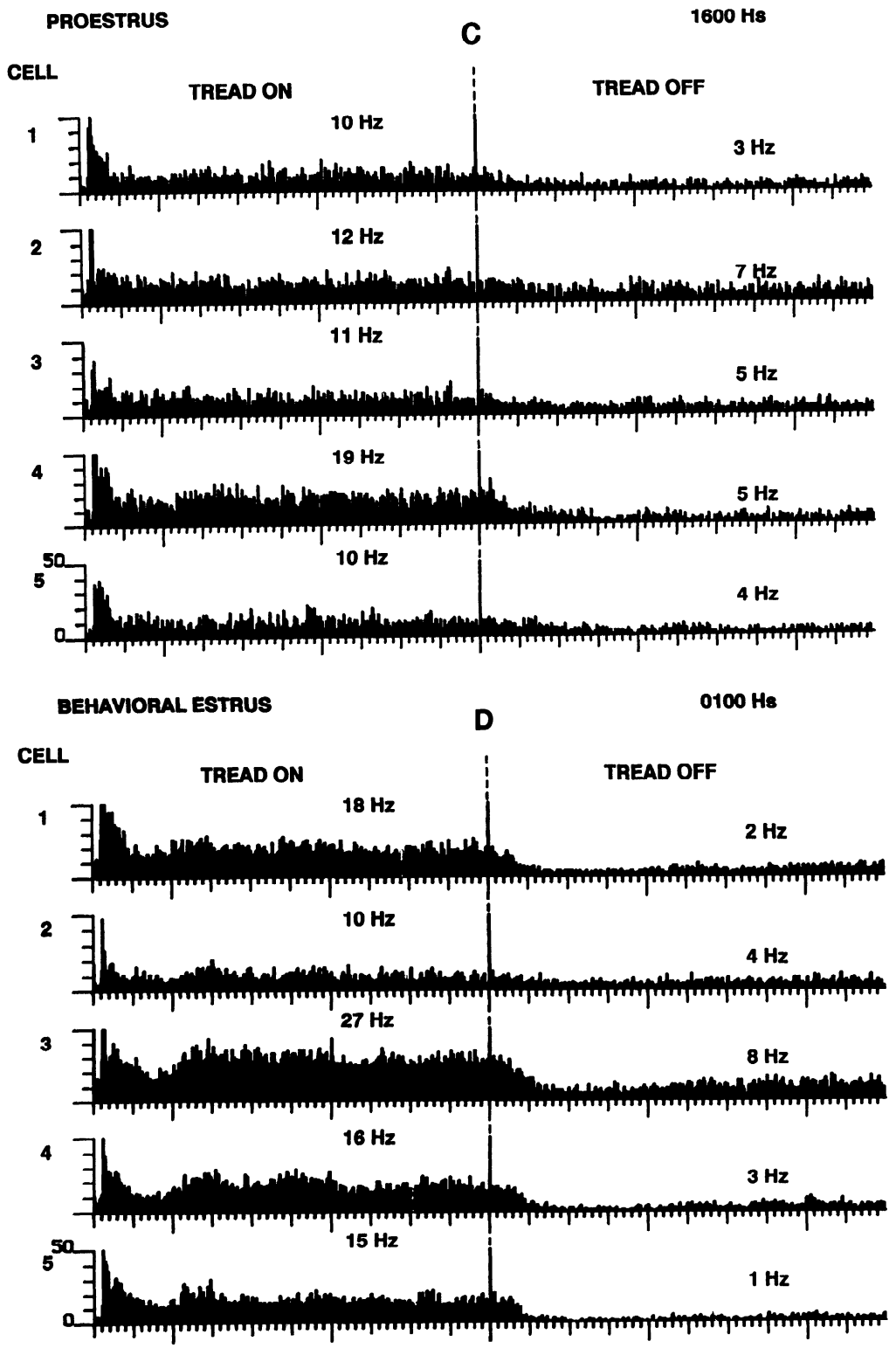

FIG. 2. (*continued*)

1, to 1–20 Hz (tread on) relative to 0.1–4 Hz (tread off). However, significant bursts of discharge were noted for *cells 3* and *4* at 1 s after the termination of treadmill locomotion when recorded on estrus (Fig. 2*E*). By 0100 the following evening on diestrus (Fig. 2*F*), discharge during both treadmill periods was markedly reduced to 2–9 Hz (tread on) and 0.2–2 Hz (tread off). Thus increases in circulating sex steroids and the ensuing dark phase of the circadian cycle are both associated with enhanced discharge during treadmill locomotion. These results are representative of 43 cells (10 rats, 3–5 cells per rat) for the light phase and 49 cells (10 rats, 2–6 cells per rat) for the dark phase of the light:dark cycle.

When the population data are summarized (Fig. 3, *A* and

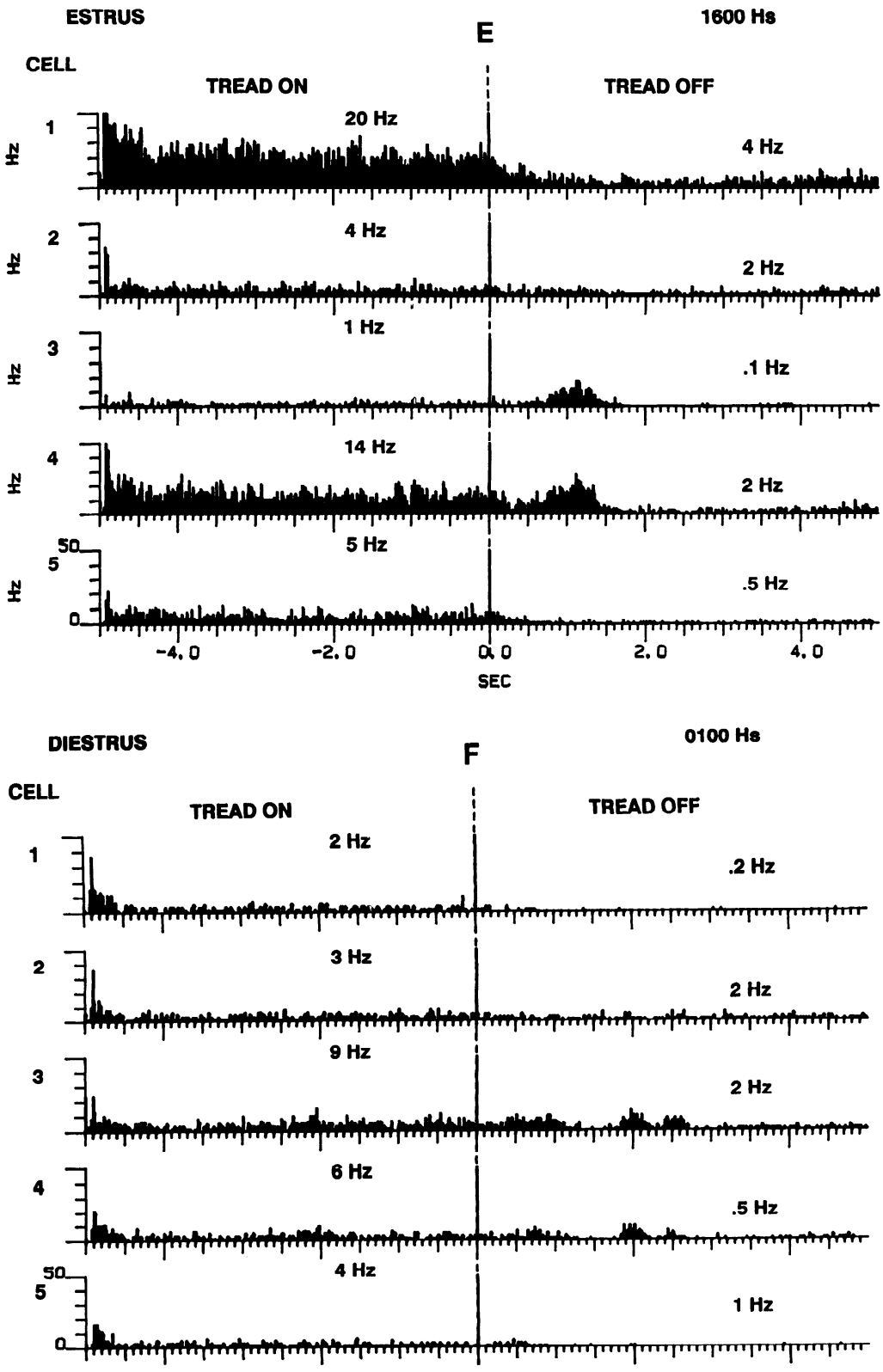

FIG. 2. (*continued*)

B), a number of significant changes can be noted across the estrous and circadian cycles. The maximal locomotor-correlated discharge (TRD ON) recorded from a Purkinje cell occurred on the night of BE (means: fast speed—22.1 ± 3.8 Hz; slow speed—15.7 ± 2.9 Hz) relative to other days of the estrous cycle (5.8–13.2 Hz, $P < 0.05$, Fig. 3*B*). In the light, locomotor discharge was greater during proestrus (13.2 ± 2.1 Hz), the day when circulating E_2 levels peak, compared with similar discharge recorded during other phases of the cycle (5.8–8.3 Hz, $P < 0.01$, Fig. 3*A*). In

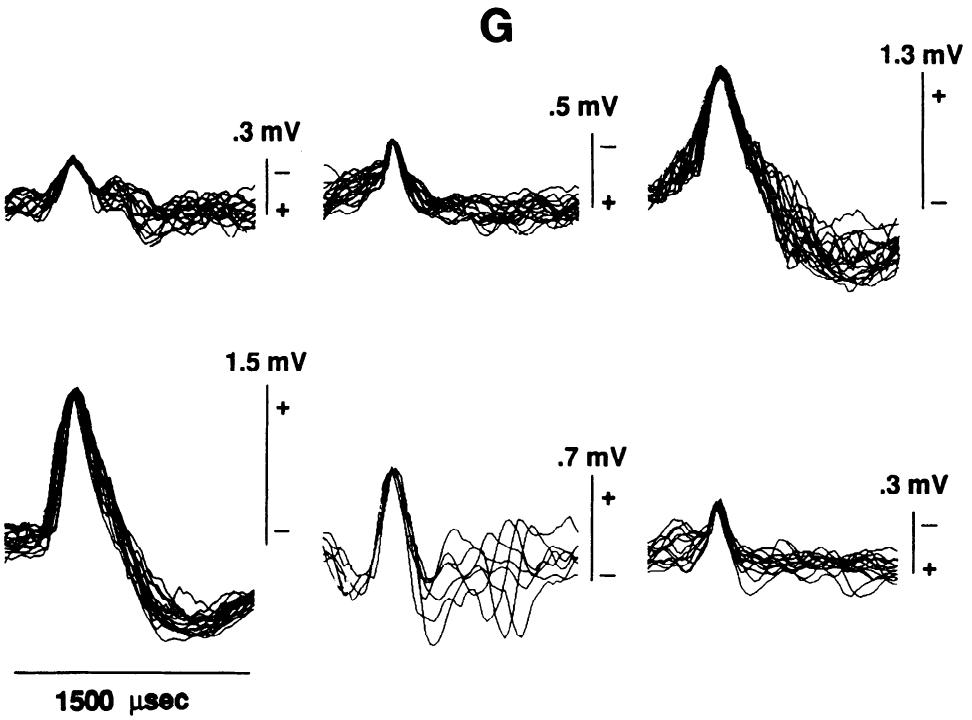

FIG. 2. (*continued*)

contrast, the circadian cycle was not associated with alterations in the magnitude of locomotor-correlated discharge recorded on diestrus. On estrus, locomotor-correlated discharge was also greater at the faster treadmill speed (22.1 ± 3.8 Hz, $P < 0.001$, Fig. 3*B, right panel*) versus the slower speed (15.7 ± 2.9 Hz, Fig. 3*B, left panel*), and estrous cycle changes in locomotor-correlated discharge were also 50% greater when tested with rats run at 11 cm/s rather than 4 cm/s. In addition, tread off discharge was threefold greater on estus versus diestrus (dark phase, fast speed, Fig. 3*B*), but not during the light or when tested at the slower speed.

Effects of training and habituation on locomotor-correlated discharge

Other factors that could have an impact on locomotor-correlated Purkinje cell discharge include training and habituation. Irrespective of the stage of the estrous cycle on the initial day of testing, cerebellar neurons from all rats tested demonstrated a significant increase ($P < 0.05$) in relative locomotor-correlated activity by day 2 (mean: 10.1 ± 0.83 Hz) compared with day 1 (mean: 6.4 ± 1.0 Hz) of the initial treadmill training period (Fig. 4*A*). (Rats were trained 30–60 min/day.) The average change in cerebellar discharge across 2 days of treadmill running was an increase of 3.6 Hz, a change of 56.3%. This increase in locomotor-correlated discharge was accompanied by a more regular gait pattern; stance and swing phases were significantly less variable ($P < 0.05$) on the 2nd day of treadmill running compared with the 1st day. Thus initial adaptation to the treadmill locomotor paradigm produced a significant increase in movement-correlated discharge. Estrous cycle modulation of this parameter was observed in all cases after training was complete (see previous section).

In contrast to sensorimotor training, long-term habituation to the treadmill after an initial training period produced a gradual decline in locomotor-correlated cerebellar discharge (Fig. 4*B*). For this study, ensembles of Purkinje cells were recorded from rats run on a treadmill (11 cm/s) for 20-min periods every hour over a 10-h session. The relative locomotor-correlated activity averaged from five sessions decreased from 8.75 ± 3.0 Hz for the 1st h to 4.2 ± 2.2 Hz by the 3rd h of treadmill running. From this time and progressing to the next day, locomotor-correlated activity was not significantly different. In addition to decreases in relative locomotor-correlated activity, the variability of the discharge rate also increased by the 5th h of locomotion, further complicating comparisons between data obtained during the duration of this prolonged treadmill locomotion session. However, these data suggest that long-term sessions of treadmill locomotion are accompanied by a gradual decline in locomotor-correlated discharge such that there are no significant differences noted due to hormone state on that day.

Estrous cycle changes in the stance-swing cycle

The step cycle can be described as a five-stage event, as illustrated in Fig. 5 (*top panel*), and for this study it is made up of footfall, stance, early thrust, late thrust (or flexion), and swing phases. The duration of these step cycle phases for the hindlimb was assessed for rats on estrus and diestrus. Results of videotape analysis of behavior indicate that the early and late extensor thrust phases of the step cycle are shortened by ~180 and 85 ms, respectively, when evaluated on the night of BE compared with values obtained on diestrus (Fig. 5, *bottom panel*).

Further evaluation of the gait pattern exhibited by rats on different days of the estrous cycle revealed that the timing

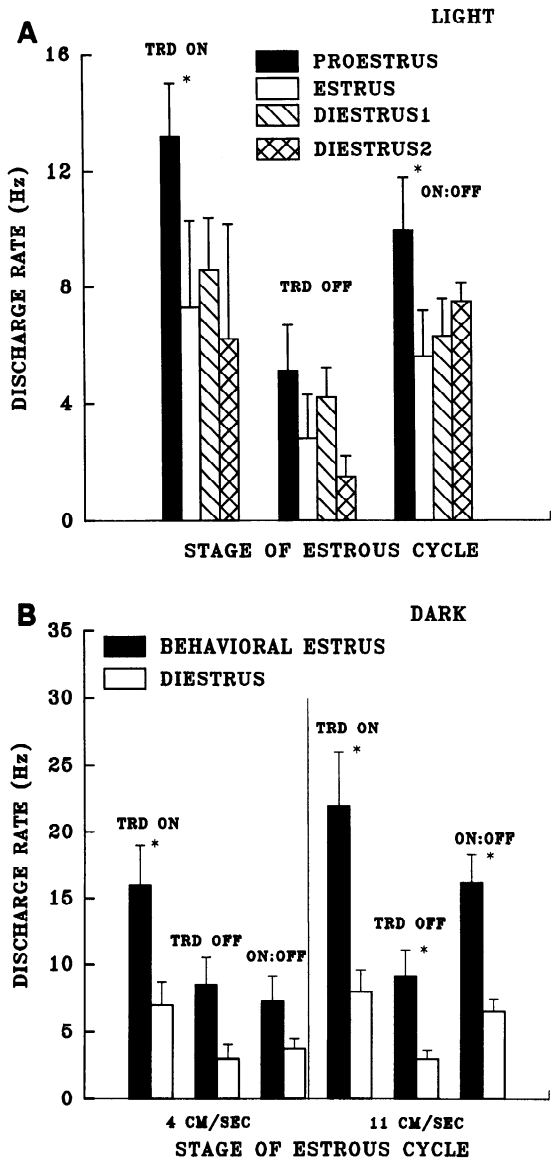

FIG. 3. Locomotor-correlated discharge varies both with the estrous and the light/dark cycles. These charts summarize the alterations in locomotor-correlated discharge (TRD ON), non-movement-correlated discharge (TRD OFF), and the ratio between the two across the estrous cycle in the light (*A*) or dark (*B*) phases of the light:dark cycle. In *B*, rats were also tested at 2 treadmill speeds, 4 or 11 cm/s; in *A*, rats were always tested at the faster speed. Several changes are significant: maximal discharge occurred on proestrus or BE relative to the other stages of the cycle ($*P < 0.05$). In addition, when tested at the same speed, locomotor discharge was greater during the dark phase of the cycle ($P < 0.01$), and comparisons between BE and diestrus were greater at this time than in the light ($*P < 0.05$). These charts summarize data from 43 cells (*A*) or 49 cells (*B*) recorded from 10 rats, in both cases across 3 estrous cycles. Each group was compared with every other group using standard ANOVA and Student-Newman-Keuls statistical procedures.

of alternate limb movements during footfall and foot-off phases of the step cycle did not differ significantly when these parameters were evaluated on BE compared with diestrus (data not shown).

Estrous cycle changes in step-cycle-correlated discharge

Purkinje cell discharge recorded during treadmill locomotion in all cases was correlated with either the stance or the late thrust (or flexion) phase of the stance-swing cycle, as has been described by others (Apps and Lidierth 1989). The representative cell in Fig. 6 illustrates the effect of the estrous cycle on this step-cycle-correlated discharge. The correlated discharge rate of this cell averaged around the late thrust timepoint of the ipsilateral hindlimb (±200 ms) increased markedly on BE (from 70 to 92 Hz) compared with its rate recorded on diestrus, when the rat was run at the faster treadmill speed. In addition, the timing of this correlated discharge was altered, because the cell began to fire 50 ms earlier and decreased discharge 100 ms earlier than when

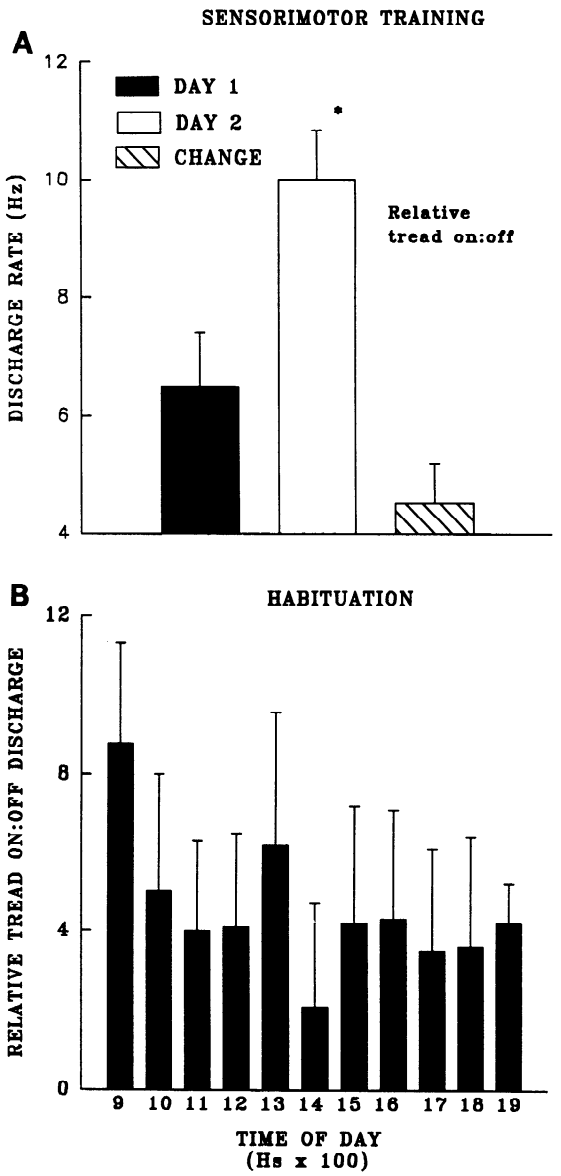

FIG. 4. Locomotor-correlated discharge exhibits changes associated with habituation and training. *A*: relative locomotor-correlated activity (tread on:off), averaged for all 49 cells across the days of the estrous cycle, is presented for the 1st 2 days of treadmill running, as is the difference between the 2 values. A significant increase ($*P < 0.05$) in locomotor-correlated activity was noted for all cells by the 2nd day of the treadmill locomotion paradigm. *B*: for this procedure, 6 cells from 1 trained rat were recorded during intermittent treadmill locomotion (20 min/h) for 10 h on the day of proestrus. Food and water were provided during the rest period. In contrast to sensorimotor training, habituation produced decreases in locomotor-correlated cerebellar discharge across the 10-h period.

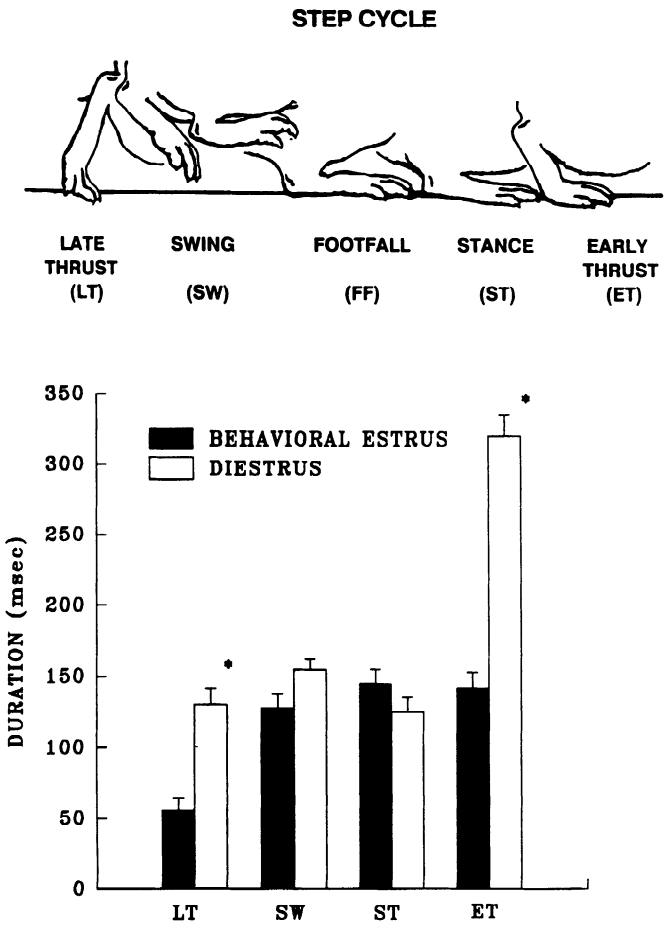

FIG. 5. Estrous-cycle-correlated alterations in the step cycle of the rat. The step cycle (*top panel*) is depicted here as a 5-stage event, with 1 flexion phase (swing, SW), a stance phase (ST), and several extension phases (late swing to footfall, FF; early thrust, ET; late thrust, LT). In most cases, cerebellar discharge can be correlated with ≥1 of these events for a particular limb(s), with the use of videotape analysis of firing patterns. In the following figures, perievent histograms were constructed around the times (10-ms accuracy) for specific stages of the step cycle that correlate with the firing rate of individual cerebellar neurons. Videotape analysis of the gait patterns of 5 rats examined across the estrous cycle (*bottom panel*) revealed that the ET and LT stages of the step cycle of the hindlimb were significantly shorter ($P < 0.05$) when assessed on the night of BE than on the night of diestrus.

tested on diestrus. In contrast to this finding, a slight decrease in discharge was noted on BE compared with diestrous values when the rat was run at the slower treadmill speed.

When the population data were evaluated, maximal discharge from 90% of the Purkinje cells recorded correlated with the stance phase of the step cycle. Of the cells recorded, 60% exhibited discharge correlated with the ipsilateral hindlimb and 40% with the ipsilateral forelimb. The averaged data are presented to illustrate differences in discharge rate recorded during stance and swing phases of the step cycle and compared across circadian and estrous cycles of rats run at two treadmill speeds (Fig. 7, *A* and *B*). When tested at the faster treadmill speed (Fig. 7*B*), stance-correlated Purkinje cell discharge was seen to increase significantly ($P < 0.02$) on BE [estrus (E), 28.3 ± 2.1 Hz, DARK], compared with values obtained on diestrus (D, 15.8 ± 2.4 Hz, DARK), an effect also observed during the light phase of the circadian cycle. In addition, discharge during the swing phase was either lower or not significantly different on estrus than comparable discharge recorded on diestrus. This differential effect resulted in a greater step cycle modulation associated with an increase in circulating E_2: the step cycle modulation was fivefold greater on estrus (LIGHT) and twofold greater on estrus (DARK) versus comparable diestrus data. (The step cycle modulation of discharge exhibited significant differences at a confidence level <0.05 for all groups except for the diestrus-light data.)

In addition, the temporal relationship between increases in Purkinje cell discharge with the onset of the correlated behavior was altered by the estrous cycle: correlated discharge tended to precede the step cycle correlate (by a mean of 130 ± 25.2 ms) when recorded on estrus (dark) compared with diestrus ($P < 0.05$, data not shown).

The slower treadmill speed (Fig. 7*A*) was associated with significant decreases in stance-correlated discharge as well as less significant step cycle modulation for estrous rats compared with data obtained at the faster speed ($P < 0.0002$). At the slower pace, only estrous rats run in the dark exhibited significant step cycle modulation ($P < 0.02$, stance- vs. swing-correlated discharge). The estrous-dark stance-correlated discharge (16.4 ± 4.2 Hz) was signficantly greater than diestrous-dark data (10.1 ± 2.3 Hz) by a mean of 62.4% ($P < 0.02$); it was also twofold greater than comparable data recorded in the light (8.0 ± 3.1, Hz, $P < 0.02$). However, rats run in the light at the slower treadmill speed did not exhibit significant differences across the estrous cycle. Thus estrous and circadian cycles are associated with changes in step cycle modulation of Purkinje cell discharge, but this effect is signficantly influenced by the treadmill speed.

Actions of E_2 on Purkinje cell responses to forepaw stimulation

Enhanced neuronal responsiveness to stimulation of peripheral afferents (by an average of 150 ± 8.5%) was observed 35 min after a 30-ng intraperitoneal injection of E_2. This dose of E_2 has been shown to result in circulating physiological levels by 15 min poststeroid (Di Paolo et al. 1985). In Fig. 8, Purkinje cell responses to forepaw stimulation are depicted before and after E_2 administration to a urethan-anesthetized rat in proestrus. Here, E_2 administration produced a 35% increase in excitatory responses of this neuron to forepaw stimulation 12 min after injection. A peak response was observed (167% increase above control levels) by 35 min poststeroid. Steroid-induced enhancement of forepaw responses persisted for as long as 6 h poststeroid, the duration of the recording, and responses were not observed to recover to control levels. These results are representative of 12 cells tested in 12 rats.

DISCUSSION

The present results suggest that locomotor-correlated simple spike discharge, recorded from ensembles of Purkinje cells within the paravermal cerebellum, is not static but fluctuates consistently across the 4-day hormone cycle. This behaviorally correlated discharge reached maximal values during the night of BE, following elevations in both E_2 and progesterone, at a time when improved sensorimotor perfor-

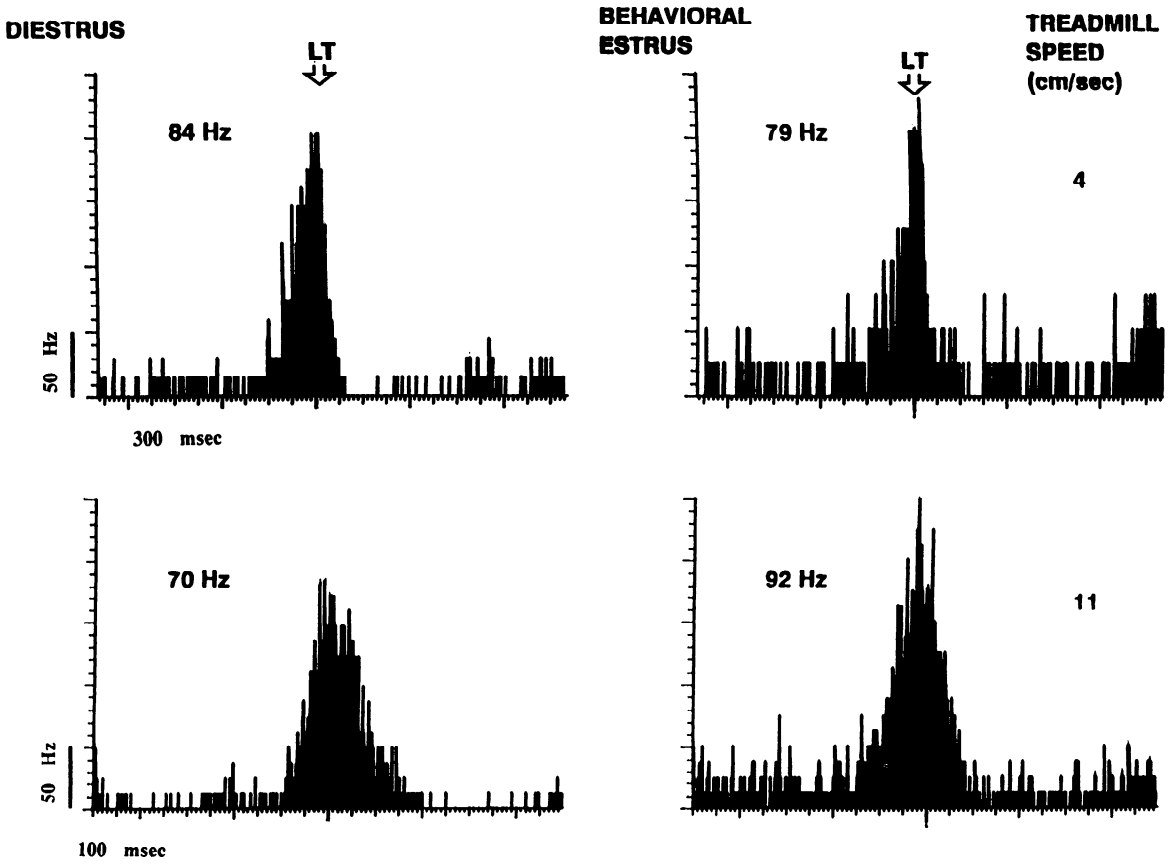

FIG. 6. Estrous-cycle-related changes in step-cycle-correlated discharge of a cerebellar Purkinje cell. In this figure, perievent histograms constructed around the time of the late thrust extensor phase (LT) of the ipsilateral hindlimb depict changes related to both estrous cycle stage and treadmill speed. At the faster speed, the discharge correlated with movement was significantly greater and the onset of discharge was advanced when tested on BE compared with diestrus. At the slower treadmill speed, no changes were observed across the estrous cycle. These results are representative of 12 cells exhibiting discharge correlated with either footfall or the LT stage of the step cycle.

mance has been reported. In addition, the onset of step-cycle-correlated discharge was advanced by 130 ms when assessed on the night of BE compared with values assessed on diestrus, when hormone levels are low. These results suggest that fluctuations in the circulating level of endogenous hormones alter both the magnitude and the timing of behaviorally correlated discharge patterns of neurons in this motor area.

Maximal levels of movement-correlated Purkinje cell discharge occurred on the night of BE, coinciding with the period of enhanced limb coordination (Beatty 1979; Becker et al. 1987; Smith 1994) observed at this time. In addition to simple increases in locomotor activity, estrous rats exhibit improvements in the rapid coordinated movements of the limbs necessitated by changes in terrain (i.e., changes in treadmill speed, hurdles, etc.; Smith 1994). During BE, rats are able to run on a balance beam (Becker et al. 1989) and negotiate hurdles (Broverman et al. 1968) more quickly and accurately than at other times of the cycle. In human studies, improved performance on the peg-and-board paradigm, a test of manual dexterity, is seen at the midcycle peak in reproductive hormones (Hampson and Kimura 1988), as is facilitation of finger-tapping frequency (Becker et al. 1982). Changes in cerebellar discharge associated with the estrous cycle observed in the present study may play some role in mediating estrous-cycle-associated improvements in limb coordination. It should be noted, however, that estrous-associated improvements in performance are specific for rapid limb movements (Broverman et al. 1968), because increases in circulating estradiol have not been found to be associated with improvements in cognitive or visuospatial tasks requiring a response delay; neither is selective attention improved under these conditions (Broverman et al. 1968; Hampson and Kimura 1988). Therefore the performance-enhancing effect of E_2 appears to be specific for tasks requiring rapid, coordinated movement. This type of behavioral change is more likely to be due to hormone effects on cerebellar output rather than nonspecific hormone effects on arousal or motivation.

The paravermal cerebellum (intermediate) is intrinsic to sensorimotor coordination (for reviews see Brooks and Thach 1981; Ebner and Bloedel 1987; Eccles et al. 1967; Houk and Gibson 1987). Lesions of this area produce ataxia (Ebner and Bloedel 1987; Houk and Gibson 1987), as do genetically produced deficiencies in climbing fiber–Purkinje cell synaptic contacts (Heckroth et al. 1990). Further, lesions of the nucleus interpositus, the output neuron for the paravermal cerebellum, result in dysmetria and slower onset and termination of distal limb movements (Marchetti-Gauthier et al. 1990). Therefore the paravermal cerebellum is a

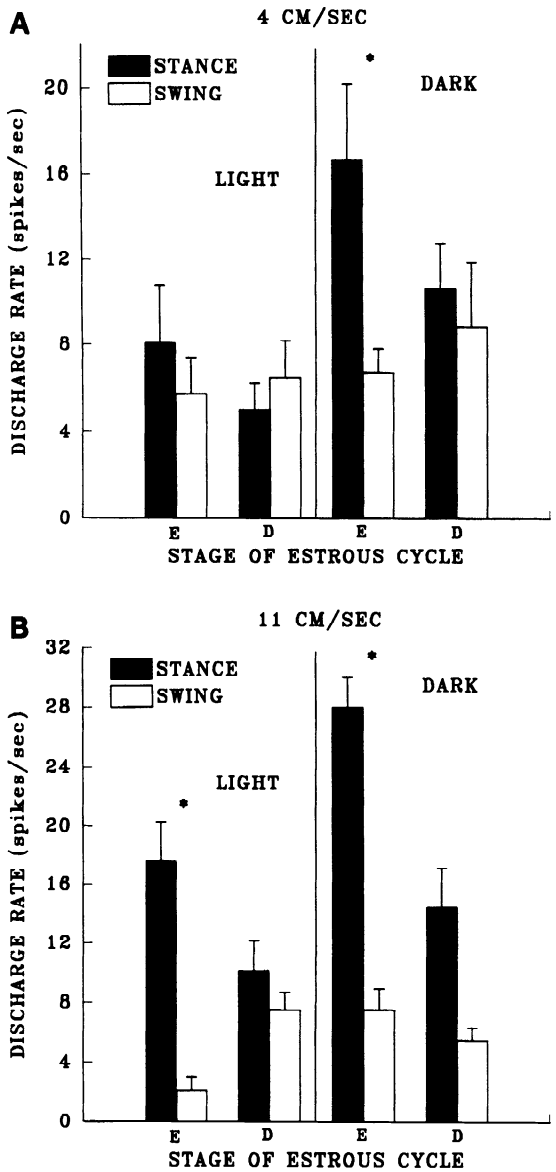

FIG. 7. Estrous-cycle-related changes in step-cycle-correlated cerebellar discharge: summary data. These graphs summarize the average Purkinje cell discharge correlated with the stance phase (filled bar) or swing phase (open bar) of the step cycle assessed across both estrous [estrus (E); diestrus (D)] and circadian cycles. Rats were tested at treadmill speeds of either 4 cm/s (*A*) or 11 cm/s (*B*). Discharge was correlated with either the ipsilateral hindlimb (60%) or forelimb (40%). Stance-correlated discharge was signficantly altered by hormone state, assessed during the dark at either treadmill speed. The circadian cycle produced significant changes in stance-correlated discharge only when tested at the slower treadmill speed. A greater motor modulation of discharge was observed for all groups tested at the faster vs. the slower treadmill speed. Stance- vs. swing-correlated discharge was significantly different ($P < 0.05$) for estrus-dark (*A*) and for all groups except diestrus-light (*B*), when assessed at the slower and faster speeds, respectively. Data were obtained from 10–17 Purkinje neurons per group averaged over 50–100 step cycles. * $P < 0.05$ vs. diestrous values.

particularly likely candidate as one CNS substrate for estrous hormone actions that could produce improvements in rapid coordinated movement of the limbs.

Discharge levels of paravermal Purkinje neurons are synchronized with the step cycle, and increase maximally at the stance phase, as has been demonstrated in the dog (Orlovsky 1972), cat (Armstrong and Edgley 1984; Ebner and Bloedel 1981; Edgley and Lidierth 1988), and rat (Smith et al. 1989a). A smaller percentage of Purkinje cells (10% from the present study) were found to increase simple spike discharge at the late thrust or flexion phase of the step cycle, a finding in agreement with that reported by Apps and Lidierth (1989). It has been suggested that Purkinje neurons assess the difference between planned and actual movement by comparing convergent input from proprioceptors and motor structures via mossy and parallel fiber systems (Ebner and Bloedel 1987; Houk and Gibson 1987). This system can then function as a variable pattern generator to change ongoing limb trajectory as appropriate for changes in terrain. Several studies have utilized chronic recording techniques to assess Purkinje cell activity during relevant behaviors (Armstrong and Edgley 1984; Ebner and Bloedel 1981); a number (Llinas and Sasaki 1989; McDevitt et al. 1987; Sasaki et al. 1989) have also employed systems to record multiple Purkinje cells in order to describe the temporospatial distribution of activity in response to sensorimotor stimuli. Transient increases in Purkinje cell activity accompany increases in the gain of reflexive movements such as limb stepping (Lou and Blodel 1988, 1992) as well as vestibuloocular (Stone and Lisberger 1986) and eye blink (Evinger et al. 1989) reflexes. Increases in Purkinje cell discharge correlated with the stance phase of the step cycle, as observed in the present study across the estrous cycle, might be expected to accompany an increase in the gain of limb stepping to result in more accurate limb placement during unexpected changes in terrain.

The estrous cycle is associated not only with improvements in limb coordination; results from the present study suggest that changes in gait are also observed on the night of BE. In particular, the duration of the extensor thrust or flexion stage (both early and late thrust, as depicted in Fig. 5) is shortened considerably when evaluated on BE. Electrophysiologically, discharge correlated with this movement preceded the behavior by 130 ms, when tested on BE, compared with diestrus 1. In contrast, neither the swing nor stance phases were signficantly altered across the estrous cycle. The enhanced stance-correlated discharge observed on estrus may set the behavioral state (i.e., muscle tone, gamma-efferent activity, etc.) that permits significant shortening of the flexion phase that follows the stance phase.

The mechanism for estrous-cycle-associated changes in movement-correlated cerebellar discharge cannot be determined conclusively with this paradigm. Actions of E_2 at peripheral or central synapses are difficult to distinguish with an in vivo, behavioral paradigm such as employed here. However, several findings suggest that direct steroid effects at the level of the Purkinje cell may contribute to the observed cyclic changes in the physiology of this system. Previous findings from this laboratory have determined that local application of E_2 in the vicinity of a Purkinje cell significantly enhances responses of this neuron to iontophoretically applied excitatory amino acids, an effect specific for the QUIS receptor (Smith 1989a; Smith et al. 1988), the predominant excitatory amino acid receptor subtype on this neuronal population (Olsen et al. 1987). Mossy fiber input, bringing in sensorimotor information, is relayed to the Purkinje cell via QUIS receptors at the parallel fiber synapse

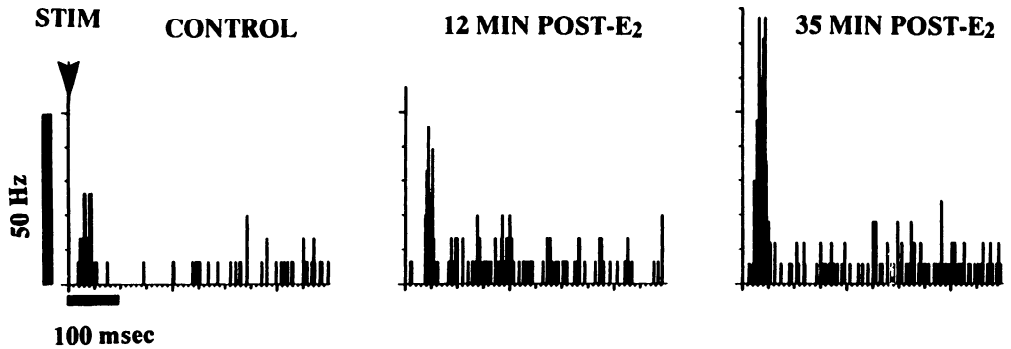

FIG. 8. Purkinje cell responses to forepaw stimulation are enhanced after systemic administration of E_2. Poststimulus histograms indicate Purkinje cell responses to forepaw stimulation before (CONTROL, *left*) and 12 and 35 min after (POST-E_2, *middle* and *right*, respectively) systemic injection of E_2 (30 ng iv in 0.01% propylene glycol–saline) to a urethan-anesthetized rat in proestrus. E_2 administration increased Purkinje cell responses 3-fold above control responses. Vehicle alone had no effect on Purkinje cell responses. These results are representative of 12 cells tested in 12 rats.

(Audinat et al. 1990; Llano et al. 1991). E_2-induced increases in QUIS responses at this synapse would be expected to enhance responses to afferent input, as observed in the present study. This effect was also seen after systemic application of the steroid (Smith et al. 1987b) at physiological doses, suggesting that during the estrous cycle direct effects of circulating E_2 might increase the sensitivity of cerebellar Purkinje cells to afferent input. These observed modulatory effects of E_2 persisted for up to 8–12 h after exposure of the neuron to the steroid, a finding that may explain why, in the present study, maximal increases in stance-correlated discharge were observed on the night of BE 7–10 h after the peak of E_2 on the day of proestrus.

Recent studies by Wong and Moss (1992) have also noted potentiating effects of E_2 on QUIS responses of CA1 neurons recorded intracellularly using the hippocampal slice preparation. In both cases, the observed neuromodulatory actions of E_2 were fast, occurring within seconds to minutes, suggesting that these are novel, nongenomic actions of the steroid. The nonclassical nature of the present effects is further suggested by the fact that Purkinje cells do not contain cytosolic/nuclear receptors for E_2 (Simerly et al. 1990).

In the present study, the ability of E_2 to enhance Purkinje cell responsiveness to QUIS was found to vary across estrous-cycle-associated fluctuations in endogenous steroids. This parameter was maximal on BE, suggesting that increases in the background steroid milieu on the preceding day of proestrus can exert a permissive effect on E_2 enhancement of the QUIS response by altering a substrate (receptor, enzyme, etc.) for subsequent steroid action.

Other possible cellular mechanisms include potential inhibitory effects of E_2 on glutamate degradative enzyme activity (Michel et al. 1978), as has been demonstrated in other tissue, and stimulatory actions of this steroid on *N*-methyl-D-aspartate receptor binding within the granule cell population (Wieland 1992) or glutamate release (Fleischmann et al. 1990), which have been shown to occur in hippocampus and hypothalamus, respectively. In addition, recent reports have localized mRNA for classical E_2 receptors within the deep cerebellar nuclei (Simerly et al. 1990). Classical receptor-mediated genomic actions of the steroid could account for the observed estrous cycle effect on Purkinje cell physiology through indirect connections from the deep cerebellar nuclei via the inferior olivary complex or via collaterals that project to the cerebellar cortex.

That E_2 is capable of enhancing responses to sensory input from the periphery is also strongly suggested by the present study. Systemic administration of a physiological dose of E_2 markedly enhanced Purkinje cell responses to forepaw stimulation by 12 min poststeroid. A peripheral action of the steroid cannot be ruled out in this case. However, these results clearly indicate a similar physiological outcome, i.e., E_2 enhances discharge of Purkinje cells either evoked by electrical or pharmacological stimuli or associated with a behavioral paradigm.

Both E_2 and progesterone are elevated before the night of BE. Unlike E_2, progesterone has been shown to enhance responses of cerebellar Purkinje cells to inhibitory amino acids (Smith 1989b; Smith et al. 1987a). This effect is due to a specific metabolite of the steroid that acts directly to potentiate the function of γ-aminobutyric acid-A receptors (Majewska et al. 1986; Smith et al. 1987c), which are densely localized on Purkinje cell dendrites (Olsen and Tobin 1990). Specific binding of progesterone to cerebellar membranes has also been reported (Tischkau and Ramirez 1993) and occurs at a physiological dissociation constant (K_d). Therefore the present study is important for verifying the net effect of cyclic increases in these two steroids with opposite effects on neuronal excitability. The finding that movement-correlated excitatory discharge is enhanced on BE suggests that the activating effects of E_2 on neuronal responses predominate over the primarily inhibitory actions of progesterone. This finding is consistent with a previous report from this laboratory demonstrating that progesterone produces no change in movement-correlated increases in Purkinje cell discharge when tested on proestrus (Smith et al. 1989), during elevations in circulating E_2.

Estrous-associated improvements in motor performance may, however, result from hormonally enhanced neuronal responsiveness at multiple, distributed sites. Other work from this laboratory has demonstrated that E_2 enhances sensory responses recorded from the rostral dorsal accessory olivary nucleus and the principal trigeminal nucleus (Kennedy et al. 1994), suggesting that E_2 can potentiate sensorimotor areas other than the paravermal cerebellum. [Decreases in locomotor-correlated discharge from prefrontal cortex have also been

observed after administration of E_2, however, suggesting that the potentiating effect of this steroid is not nonspecific (Smith and Bergqvist 1989).] Other potential sites of steroid action include basal ganglia (Becker 1990; Dluzen and Ramirez 1990) and sensory cortex (Bereiter and Barker 1984).

On estrus, the dark part of the circadian cycle was also associated with increases in movement-correlated Purkinje cell discharge. Because the rat is nocturnal, general activity level is markedly increased for this rodent during the dark compared with the light phase of the light:dark cycle. In addition, limb coordination is also improved during the dark (unpublished findings). Both factors contributed to maximize stance-correlated cerebellar discharge. A purely hormonal effect would be reflected by maximal modulation on the day of proestrus, when circulating levels of E_2 peak; this was not observed. A purely circadian effect would be expected to maximize modulation of cerebellar discharge in the dark, regardless of estrous cycle stage. Instead, the dark period following peak levels of circulating E_2, i.e., the night of BE, was associated with maximal cerebellar modulation, and is the time when limb coordination is improved. However, stance-correlated discharge was somewhat higher when assessed in the dark on diestrus, compared with the light period of the same (low) hormone state. Thus these data further suggest that increases in the movement-correlated discharge of the cerebellar Purkinje cell accompany general dark-cycle-related increases in activity, as well as hormone-associated improvements in the speed and accuracy of limb trajectory during the dark phase.

The degree of step cycle modulation of cerebellar discharge was also altered by the initial training and eventually habituated such that locomotor-correlated discharge was initially increased and then, over the long term, exhibited a gradual decline in magnitude. Estrous cycle modulation of cerebellar discharge was observed after the initial training period, but was masked by a lengthy habituation session. Whether the gradual decline in correlated discharge with habituation represents a training effect or a change in arousal, or is merely the result of a more efficient treadmill locomotor gait, is unclear. However, similar kinds of changes have been reported to accompany behaviorally correlated discharge recorded from the deep cerebellar nuclei during a reaching task (Bloedel et al. 1993). Although habituation could mask estrous-cycle-induced changes in stance-correlated discharge, alterations in gait due to environmental challenges might limit habituation; thus estrous cycle modulation of cerebellar discharge would be more likely to occur under natural conditions.

In sum, behaviorally correlated discharge recorded from the cerebellum appears to be dynamically altered across the hormone cycle as well as by training and the circadian cycle. Potentiation of cerebellar activity on estrus may be a result of reported actions of estrous hormones on the sensitivity of the system to excitatory amino acids. Increases in the gain of locomotor-correlated discharge of cerebellar Purkinje cells may then result in improvements in the speed and accuracy of coordinated limb trajectory, as has been oberved on estrus.

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