MUSHROOM BODY INFLUENCE ON LOCOMOTOR ACTIVITY AND CIRCADIAN RHYTHMS IN DROSOPHILA MELANOGASTER

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Whether or not mechanisms underlying circadian locomotor rhythms and learning are related anatomically through the mushroom bodies (MBs) was investigated by monitoring behavioral rhythmicity in flies with MB lesions induced by chemical ablation and by mutations in five

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different genes. All flies tested were later examined histologically to assess (1) MB neuroanatomy, and (2) the condition of the putative pacemaker cells — the ventral Lateral Neurons (LN,s) and their terminals that project to the vicinity of the MB calyces. All groups of flies had normal rhythms except for mushroom body miniature (mbm; only in a wild-type Berlin genetic background) and mushroom body defect (mud). MB ablation had no effect on the gender-specific differences in the rhythmic activity profile that are typical of wild-type flies. However, ablated males had a slightly longer period than untreated males and were more active under constant dark conditions. LN,s and their arborization patterns appeared normal in MB-ablated and in most mutant flies. Activity defects of mbm flies were attributed to genetic background rather than to the mutation alone. Misrouted LN,v projections and ~14% arrhythmia of mud flies were uncorrelated and attributed to pleiotropy rather than to specific effects of MB lesions. Our results imply that MBs are not involved in circadian activity rhythms but that they do have an inhibitory effect on activity levels of male flies.

Keywords: corpora pedunculata; hydroxyurea; period gene; mushroom body mutants; pigment-dispersing hormone; immunohistochemistry

INTRODUCTION

The mechanism of circadian rhythms has been revealed to a large extent through genetic, molecular, and neuroanatomical studies of the period (per) clock gene in Drosophila (reviews Hall, 1998a; 1998b). Together with timeless (tim), clock (Clk), cycle (cyc), and doubletime (dbt), per is critically involved in the generation of cell autonomous circadian cyclings which take place in nearly all clock gene-expressing cells (Plautz et al., 1997; reviews: Dunlap, 1999; Blau, 2001). Behavioral rhythmicity is controlled by clock gene-expressing neurons in the brain which are best characterized anatomically by antisera against PER (Zerr et al., 1990; Ewer et al., 1992; Frisch et al., 1994). Two different groups of neurons express per (see Figure 1B,D). Cell bodies of the dorsal neurons (DNs) lie in the posterior dorsal cortex of the central brain close to the calyces of the mushroom bodies (MBs), neuronal assemblies mediating odor recognition, and learning in flies (Heisenberg et al., 1985; de Belle & Heisenberg, 1994; Connolly et al., 1996; Zars et al., 2000; Dubnau et al., 2001; reviews: Heisenberg, 1998; de Belle & Kanzaki, 1999). The cell bodies of the lateral neurons (LNs) lie in the lateral brain close to the
optic lobes. A subgroup of these, the small ventral LNs (sLN\textsubscript{s}), project into the posterior dorsal cortex of the brain where they terminate close to the MB calyces (Helfrich-Förster & Homberg 1993; Helfrich-Förster, 1995; 1997). Evidence points to sLN\textsubscript{s} as the site of the circadian pacemaker in \textit{Drosophila} (reviews: Helfrich-Förster, 1996; Kaneko, 1998; Hall, 1998a). Arborization of these neurons close to the calyces is critical for the transfer of rhythmic signals to the central brain (Helfrich-Förster, 1998; Helfrich-Förster et al., 2000). The proximity of the MBs to both the

\textbf{FIGURE 1} Brains of control (left) and HU-treated (right) flies photographed at the level of the calyces (Ca). Scale bars = 50 µm. (A,B) Fluorescence images of \textit{Canton-S} frontal paraffin sections (7 µm). Perikarya and neuropil appear yellow and green, respectively. (C,D) Differential interference contrast (DIC) images of CS wholemounts stained with anti-PDH to reveal terminals of the small ventral Lateral Neurons in the central brain (sLN\textsubscript{v}; \nabla). sLN\textsubscript{v} terminal shape was similar in all groups of flies. (E,F) DIC images of \textit{BG6a} wholemount brains expressing the β-galactosidase reporter of \textit{per} gene activity. Three groups of Dorsal Neurons (DN1, DN2, DN3) were stained in all groups of flies. (see Color Plate at back of issue).
sLNv, terminals and the DNs suggests that they might be involved in the output pathway of the circadian clock.

A link between rhythmic phenomena and learning was proposed a long time ago for vertebrates (Jenkins & Dallenbach, 1924; review: Smith, 1985). In one study, involving conditioned behavior and rhythms, rats learned to associate air puffs to the eye with light (Amir & Stewart, 1996). After training, air puffs alone delivered during the dark phase of the cycle were sufficient to induce phase shifts of the clock. Two caveats were that (1) Zeitgeber and unconditioned stimulus (US) light must be distinct from one another, and (2) US light must induce a phase shift during training (Arvanitogiannis & Amir, 1999; Arvanitogiannis et al., 2000; Rusak & de Groot, 2000). These findings suggest a fundamental overlap in mechanisms underlying rhythms and associative learning. The idea receives further support from work with invertebrates. Honey bees have a remarkable “Zeitgedächtnis” (time memory), since they can be trained to several odors presented at different times of the day (von Frisch, 1965; Menzel, 1990). This Zeitgedächtnis requires an internal clock that must somehow be integrated with the learning and memory machinery. In Drosophila, some observations indicate that the control of rhythms and learning share underlying genetic factors and biochemical signaling pathways. Several mutants that are impaired in the cyclic AMP (cAMP) signal transduction pathway show deficits in learning and memory (Dudai et al., 1976; Livingstone et al., 1984; Skoulakis et al., 1993; Li et al., 1996; Goodwin et al., 1997; reviews: Davis et al., 1995; Dubnau & Tully, 1998). Some of these mutants also have altered rhythmic behavior (Levine et al., 1994; Majercak et al., 1997; Park et al., 2000b). Similarly, the cAMP response element binding protein (CREB), which is involved in memory consolidation (Bourchuladze et al., 1994, Yin et al., 1994; 1995; reviews: Yin & Tully, 1996; Silva et al., 1998), is also important for cycling of cellular oscillators that underlie rhythms in both flies and mice (Ginty et al., 1993; Foulkes et al., 1996; Ding et al., 1997; Belvin et al., 1999).

The behavioral, anatomical, biochemical, and genetic evidence for a common cellular mechanism underlying rhythms and learning have inspired the current study. Here, we address the possibility that rhythms and learning are related anatomically through sLNv fiber projections toward the MB calyces by conducting neurohistochemical and behavioral studies with chemically ablated and structurally mutant flies. The rhythmic behavior of the MB mutant, mushroom body deranged (mud), had been investigated in a previous study (Vosshall and Young, 1995), but few flies were tested and structural defects in the brains of these animals were not evaluated. This is important because mud and other brain structure mutants often show considerable variation from one individual to the next (de Belle & Heisenberg, 1996; J.S. de Belle, unpublished observations).
Besides their participation in olfactory learning and memory, *Drosophila* MBs are also implicated in sex-specific behaviors. Early mosaic analyses suggested that MBs are important for the initiation of male courtship (Hall, 1977; 1979). In more recent studies, male courtship and conditioned male courtship both appeared to be unaffected by MB ablation (McBride et al., 1999; O’Dell et al., 1999). However, the consolidation of conditioned courtship memory was found to be MB-dependent. In females, a region in the dorsal anterior brain that might correspond to the MB α-lobes was implicated as a hypothetical processing center for the response to male courtship (Tompkins & Hall, 1983; review: Siegel et al., 1984). Interestingly, when portions of the MBs are “feminized” in transgenic flies, it appears as if males cannot discriminate between the sexes during courtship (Ferveur et al., 1995; O’Dell et al., 1995). If MBs do support some aspect of sex-specific behavior during courtship, this might correlate with a sexual dimorphism in MB anatomy or gene expression. Indeed, Kenyon cell fiber number is sexually dimorphic (Technau, 1984), and becomes highly exaggerated in mushroom body miniature (mbm) mutants (Heisenberg et al., 1985; de Belle & Heisenberg, 1996).

A sexual dimorphism was also recently described for *Drosophila* activity rhythms (Helfrich-Förster, 2000)—specifically in the phase relationship of morning activity with the onset of the light cycle (lights-on). Males showed an earlier morning activity than females. This may be of adaptive significance for locating their female mates before they become active. The underlying neuronal control of this sexual dimorphism is unknown, but it could depend on a differential influence of the MBs on activity (see below).

The *Drosophila* MB is a paired neuropil structure each consisting of about 2500 neurons called Kenyon cells (Kenyon, 1896; Hinke, 1961; Technau, 1984). Their cell bodies lie in the dorso-posterior cortex of the protocerebrum and send parallel fibers inward to form the MB neuropil. All Kenyon fibers in each MB are derived from four neuroblasts that are mitotically active at the time of larval hatching (Truman & Bate, 1988; Ito & Hotta, 1992). Each MB neuroblast autonomously generates all types of Kenyon cells and the entire repertoire of adult MB substructures (Ito et al., 1997; Armstrong et al., 1998). Three types of Kenyon cells can be distinguished in adults based on their time of birth and projection patterns (Tettamanti et al., 1997; Armstrong et al., 1998; Lee et al., 1999). Those born in the embryo and early larval instars form the γ-lobe, those born in later larval development contribute to the α’- and β’-lobes, while Kenyon cells born in the pupa form the α- and β-lobes.

The DNA-synthesis inhibitor hydroxyurea (HU) fed to newly hatched larvae selectively deletes the MB neuroblasts, resulting in complete, precise ablation of all postembryonically-derived MB structures in adult flies (de Belle & Heisenberg, 1994; Armstrong et al., 1998). Here we
generated MB-ablated flies by this technique and tested them for circadian locomotor activity rhythms. Subsequently, these flies were scored for the effectiveness of MB ablation and for the presence and appearance of sLN_v terminals. DNs were also examined in a subset of MB-ablated flies. Furthermore, we measured rhythmicity in five different MB structural mutants \( [mmbm^1], \text{mushroom bodies reduced (}mbm^1\text{)}, \text{small mushroom bodies (}smu^1\text{)}, \text{central brain deranged (}ceb^1\text{)}, \text{and} mud^4 \) (Heisenberg et al., 1985; Strauss and Heisenberg, 1993; de Belle and Heisenberg, 1996). We later examined each fly histologically for neuroanatomical defects, including the condition of sLN_v terminals. All mutations were outcrossed to the wild-type Canton Special (Canton-S) strain to control for effects of genetic background (de Belle & Heisenberg, 1996). For comparison, \( mmbm^1 \) was also studied in a wild-type Berlin genetic background and in a partially outcrossed genetic background (see de Belle & Heisenberg, 1996, for details).

HU-treated flies and all mutants, except \( mmbm^1 \) in the Berlin genetic background and \( mud^4 \), had normal rhythms and normal sLN_v arborization patterns. The DNs of MB-ablated flies were also unaffected by HU treatment, indicating that they are probably not Kenyon cells derived from MB neuroblasts. Minor differences were found in period, activity level, and activity profile between mutants and Canton-S control flies. In HU-treated males, mean activity level and period were slightly increased relative to untreated controls. The rhythm of \( mmbm^1 \) mutants dampened rapidly under constant conditions. However, this phenotype disappeared after outcrossing with Canton-S wild-type, indicating that it was a genetic background effect. In \( mud^4 \), \( \approx 14\% \) of the flies were arrhythmic. We discuss the likelihood that arrhythmic behavior of these \( mud^4 \) flies is due to pleiotropic effects of the mutation on other parts of the central nervous system, rather than MB-specific lesions. Overall, our results show that MBs are not involved in the control of circadian activity rhythms.

**MATERIALS AND METHODS**

**Fly Strains**

We used wild-type Canton Special (Canton-S, derived from a stock in Würzburg, Germany) as the standard control strain in all anatomical and behavioral analyses. MB structural mutants and their anatomical phenotypes are described elsewhere (Heisenberg et al., 1985; Strauss & Heisenberg, 1993; de Belle & Heisenberg, 1996). Briefly, the MBs of \( mmbm^1, mbm^1, \text{and} smu^1 \) are reduced in size, and in \( mmbm^1 \) the reduction is more pronounced in females than in males. MBs in \( ceb^1 \) and \( mud^4 \) have enlarged calyces but reduced pedunculi and lobes. All mutant strains were outcrossed with Canton-S to control for background effects.
[designated by (CS); de Belle & Heisenberg, 1996]. mbm^1 was also tested in its original genetic background (Berlin) and in a partially outcrossed background consisting of an intact mutant second chromosome substituted into Canton-S (designated as mbm^1; CS; de Belle and Heisenberg, 1996).

To visualize per-expressing DNs, we used a transgenic line (BG6a), which carries a per-lacZ fusion gene (Stanewsky et al., 1997). In this line β-galactosidase expression mimics the spatial and temporal pattern of natural per in adults and in larvae and therefore is a faithful reporter for per (Kaneko et al., 1997; Stanewsky et al., 1997). HU treatment of BG6a larvae was performed to determine whether DNs are affected by MB ablation. Furthermore, DNs were checked in mud^4/Y males by crossing mud^4(CS) females with BG6a males and staining for β-galactosidase activity.

All strains were raised on standard cornmeal medium under a light regimen of 12 h light and 12 h dark (LD 12:12).

**Mushroom Body Ablation**

HU treatment of Canton-S and BG6a flies was performed as described elsewhere (de Belle & Heisenberg, 1994; Sweeney et al., 2000). Briefly, larvae were collected between 0–1 h post-hatch and incubated in an HU and heat-killed yeast suspension (50 mg/ml) for 4 h. They were then washed in distilled water and transferred to normal medium and standard conditions. Control larvae were treated similarly, except that HU was omitted.

**Locomotor Activity Rhythms**

*Recording.* Locomotor activity of individual flies was recorded photoelectrically as described previously (Helfrich-Förster, 1998; 2000). Briefly, activity was monitored during consecutive 4-min intervals. Any activity within an interval resulted in a scan value of one, while no activity resulted in a value of zero. Male and female flies (ratio 1:1) were recorded at 20 ± 1°C. They were first monitored for about 5 days in a light-dark cycle of 12 h light and 12 h darkness (LD 12:12). Light intensity during the light phase was 1000 lux. Locomotor activity was recorded for 15 to 20 days under constant DD conditions. Afterward, each fly was sacrificed for pigment-dispersing-hormone (PDH)-immunohistology (see below).

*Analysis.* The raw data were first displayed as actograms and later analyzed for rhythmicity as described below. During LD, the phase relationship of peak activity to light on was calculated as described in
Helfrich-Förster (2000). Phases of the peaks were given in Zeitgeber Time (ZT), where the beginning of a 12:12 LD cycle (lights-on) is ZT₁₀ and lights-off is ZT₁₂. Periodogram analysis combined with a χ² test with 5% significance level (Sokolove & Bushell, 1978) served for calculation of period and rhythm power under DD conditions. Power of rhythmicity was given in percent of variance (see Helfrich-Förster, 1998). Rhythmicity was classed as simple, complex, or arrhythmic. Simply rhythmic flies showed a single significant peak in the periodogram, complex rhythmic flies had more than one significant peak, while arrhythmic flies had no significant peaks. Simply rhythmic flies were either classed as strongly rhythmic when power was > 20% of the variance or weakly rhythmic when power was ≤ 20% of the variance (see Helfrich-Förster, 2000). Data were arranged in contingency tables for χ² analyses (Zar, 1984). Mean period and mean power were determined for the simple rhythmic flies of all groups and compared to Canton-S.

To compare the activity pattern of the flies in LD and DD, an average day was calculated and plotted as a histogram for each fly. The calculation of an average day out of the average days of several flies served as a tool to compare the LD and DD behavior of the different fly groups (Helfrich-Förster, 2000). Mean ± standard error (SE) activity levels during LD and DD were also calculated for each fly group. To compare the activity levels throughout the recording interval (in LD and DD), daily activity levels were calculated for each fly. Activity curves of individual flies were then compiled to give average activity level curves for all treatments and genotypes.

All measured parameters were tested for significant influences of genotype and gender using two-way analyses of variance (ANOVA). In a few cases, the data were not normally distributed. Here, the level of significance (α) was adjusted according to Glaser (1978). Values were regarded as significantly different at $p \leq 0.05$. Comparisons between means for more than two groups were made using the Student-Newman-Keuls (SNK) multiple range test (Zar, 1984).

**Histology**

*PDH Immunohistochemistry.* After locomotor activity was recorded, flies were exposed to light for about 1 to 2 h to reset the circadian clock to a phase close to lights-on at which time PDH-immunohistochemistry was found to be optimal (Park et al., 2000a). Flies were then anaesthetized on a cold plate, placed in mass histology "collars" (Heisenberg & Böhl, 1979), and fixed for 4 h in a mixture of glutaraldehyde, picric acid, and acetic acid (Boer et al., 1979). They were embedded in paraffin, cut frontally in 7 μm sections, and adhered to microscope slides. After removal of the paraffin and subsequent
rehydration, the slides were transferred to 0.1 M TRIS HCl/0.3 M NaCl (pH 7.4) containing 0.1% Triton X-100. They were subsequently subjected to PDH-immunohistochemistry using the peroxidase-antiperoxidase method (Helfrich-Förster & Homberg, 1993). Crustacean PDH was used to generate the anti-PDH serum (Dirksken et al., 1987) which we applied at a dilution of 1:2000 in our experiments. This antiserum reveals reliably the LN, s and their arborization patterns that are part of the neuronal network of the circadian clock (Helfrich-Förster & Homberg, 1993; Helfrich-Förster, 1995). The preparations were judged blind for the presence of PDH-immunostained projections in the calyx region. Data were arranged in contingency tables for $\chi^2$ analyses (Zar, 1984) to test for a correlation between sLN, arborizations and MB lesions. Staining intensity of these terminals was scored as described in Kaneko et al. (1997).

Brains sampled from each group of HU-treated flies and MB mutants (the siblings of those tested behaviorally) were also subjected to PDH-immunostaining as whole-mounts, following a procedure described previously (Helfrich-Förster, 1997). Reconstructions of the PDH-immunoreactive neurons were made from these brains with the aid of a Zeiss microscope equipped with a camera lucida attachment.

$\beta$-galactosidase Histochemistry. X-gal histochemistry provided a means to visualize the per (therefore also lacZ)-expressing cells in BG6a flies, in HU-treated BG6a flies, and in mud$^d$/Y; +/BG6a heterozygotes. In BG6a, the PER-$\beta$-galactosidase-fusion product cycles with the peak at ZT$_{21}$ (Stanewsky et al., 1997). Therefore, dissections were done at ZT$_0$ when the level of the PER-$\beta$-galactosidase-fusion product is still high and a minimal effect of light during dissection is expected (Zeng et al., 1994; Kaneko et al., 1997; Stanewsky et al., 1997). In order to see whether PER is still cycling in the DN$s$ of HU-treated flies, dissections were also made at ZT$_{12}$ when the level of PER-$\beta$-galactosidase-fusion product is lowest in wild-type flies (Stanewsky et al., 1997). The dissected brains were fixed as whole-mounts for 30 min in 1% glutaraldehyde, subsequently rinsed in phosphate buffer (PB, pH 7.2) for 3–5 min, and incubated at 37°C for 5 to 12 h in staining solution containing 20 μl of 10% 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside (X-gal) in dimethyl sulfoxide (DMSO)/ml staining buffer (Simon et al., 1985). After incubation they were washed again in PB for 3–5 min and fixed for 3 h in 6.25% glutaraldehyde.

Autofluorescence. To visualize gross MB morphology, flies were placed in mass histology collars, fixed in Carnoy’s solution, and embedded in paraaffin (Heisenberg & Böhl, 1979). Heads were cut in 7 μm serial frontal sections and inspected under a fluorescence microscope (de Belle & Heisenberg, 1994).
RESULTS

Mushroom Body Ablation

Histology. In wild-type flies, MBs are prominent structures in the dorso-posterior brain. Under a fluorescence microscope their neuropils appear contrasted against the mass of perikarya surrounding them dorsally (Figure 1A). The fibers of the _per_-expressing LN₅s that seem to transfer circadian rhythmicity to the central brain terminate among these perikarya – slightly dorso-frontally to the MB calyx neuropil (Figure 1C). In our experiments, these neurons were stained by anti-PDH in ca. 91% of the control flies (52/57; Table I; males and females pooled). The _per_-expressing DN₅s are also situated among these perikarya, as revealed in a _per-lacZ_ reporter strain (Figure 1E). In this reporter strain, levels of β-galactosidase expression reflect faithfully the amount of PER produced (Kaneko et al., 1997; Stanewsky et al., 1997). We found that the DN₅s were strongly stained at ZT₀ (in each of the 10 brains studied), but only weakly (in three brains) or not at all stained (in seven brains) at ZT₁₂. This phase of PER cycling in DN₅s mirrors that described recently elsewhere (Kaneko et al., 1997; Stanewsky et al., 1997).

HU treatment appeared to result in complete MB ablation in all flies (Figure 1B), except one (out of 58) that had a remnant MB in one brain hemisphere. This fly was discarded from the analysis. Although embryonically-derived Kenyon cells with projections to the γ-lobe must have been present in all HU-treated animals (Ito et al., 1997; Tettamanti et al., 1997; Armstrong et al., 1998), they are below the resolution of auto-fluorescence microscopy. The PDH-immunoreactive terminals of the sLN₅s were stained in 93% MB-ablated flies (53/57; Table I; males and females pooled), and appeared morphologically normal (Figure 1D). As observed in untreated flies, the _per-lacZ_ reporter strain revealed DN₅s faithfully in MB-ablated flies at ZT₀, but not at ZT₁₂ (Figure 1F). We saw no obvious effect of HU treatment on both the number of DN₅s stained and the intensity of staining using X-Gal histochemistry (data not shown). Similarly, we saw no obvious effect of HU treatment on the number of LN₅s stained using PDH-immunohistochemistry.

PDH-staining intensity in the LN₅ terminals was influenced by gender, with males having significantly stronger staining than females (cf. Park & Hall 1998; Table I; ANOVA: \( F_{[1,110]} = 5.54; \ p = 0.020 \)). We found no significant effects of either HU treatment (\( F_{[1,110]} = 1.96; \ p = 0.164 \)) or interaction (\( F_{[1,110]} = 0.74; \ p = 0.391 \)) on staining intensity.

Locomotor Activity Rhythms. The rhythmic behavior of control and HU-treated flies was strikingly similar, as can be seen in the actograms (Figure 2), and in the average days plotted separately for LD (Figure 3A,B) and DD (Figure 3C,D). All flies in the two groups were found to...
### TABLE I Effects of Mushroom Body Ablation on sLNv Histology and Behavior

<table>
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<th>Treatment</th>
<th>Gender</th>
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<th>Morning Peak$^1$</th>
<th>Rhythmicity$^2$</th>
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<td>n</td>
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<td>♂</td>
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Control and HU-treated flies were first measured for locomotor activity rhythms and then subjected to PDH-immunohistochemistry to assess the condition of LNv.s.

*Flies with sLNv terminals stained by anti-PDH.

$^1$Number of days with morning activity peaks under LD conditions.

$^2$Classification of rhythmicity under DD conditions.

$^3$Flies with sLNv terminals stained by anti-PDH.

$^4$Number of days with morning activity peaks under LD conditions.

$^5$Classification of rhythmicity under DD conditions.
be synchronized under LD cycles and to be simply rhythmic under DD (Table I).

In LD, both control and HU-treated flies showed bimodal activity patterns with a first activity peak around lights-on (morning peak) and a second more pronounced activity peak around lights-off (evening peak) (Figures 3A,B; 4A). Whereas the evening peak was present in each fly and on all 5 days during LD, the morning peak was sometimes not visible (Table I). This difference between treated and control flies was not significant (genders pooled; $\chi^2_{[1]} = 2.33; p = 0.059$). As described previously (Helfrich-Förster, 2000), male flies of both groups showed an earlier morning peak than females, whereas the evening peak occurred at similar times in both sexes (Figures 3A,B; 4A). ANOVA revealed a highly significant effect of gender on the early phase of the morning peak.
FIGURE 3 Mean ± SE activity of control (left) and HU-treated (right) Canton-S ♀ s (top) and ♂ s (bottom). (A,B) Average day in LD was characterized by morning peaks occurring later in ♀ s than in ♀ s and evening peaks that were the same for all flies. —— = lights on, ——— = lights off; ZT = Zeitgeber time. (C,D) Average day in DD was unimodal except for a small morning peak in HU-treated ♀ s (↓), which were also more active than controls. —— = lights off; CT = circadian time. (E,F) Total activity increased after shifting to DD for HU-treated ♀ s. —— = LD, ——— = DD.
but no effects of HU treatment ($F_{[1,110]} = 2.56; p = 0.113$), nor of interaction between gender and HU treatment ($F_{[1,110]} = 0.27; p = 0.607$). Evening peak variation was not significant ($F_{[3,110]} = 1.24; p = 0.312$). The second sex-specific difference revealed under LD conditions was activity level (Figure 4B). As observed for morning peak phases, females were more active than males ($F_{[1,110]} = 28.31; p < 0.0001$), but effects of HU treatment ($F_{[1,110]} = 0.49; p = 0.484$) and interaction ($F_{[1,110]} = 0.11; p = 0.747$) were both not significant.

The only observed behavioral differences between control and HU-treated flies were found under DD conditions. Here, the majority of flies in both groups showed strong rhythms, with only a few classified as weakly rhythmic (Table I). This variation was independent of MB condition ($\chi^2_{[1]} = 0.48; p = 0.164$). Rhythm period (Figure 4C) was $\sim 10$ min longer in ablated flies ($F_{[1,110]} = 7.03; p = 0.009$) and $\sim 19$ min longer in females ($F_{[1,110]} = 27.10; p < 0.0001$). Interaction between ablation and gender had no significant influence on period ($F_{[1,110]} = 1.08; p = 0.300$). Rhythm power (Figure 4D) was stronger in females ($F_{[1,110]} = 4.82; p = 0.030$) but was not influenced by HU treatment ($F_{[1,110]} = 1.37; p = 0.244$) nor by interaction ($F_{[1,110]} = 0.01; p = 0.941$). MB ablation also increased activity of males by $\sim 16\%$ but not females (Figures 3C,D; 4E). ANOVA revealed a highly significant effect of HU treatment ($F_{[1,110]} = 17.41; p < 0.0001$), no effect of gender ($F_{[1,110]} = 1.39; p = 0.241$), and a significant effect of interaction ($F_{[1,110]} = 6.29; p = 0.023$). This increased activity level of MB-ablated males under DD conditions is clearly visible in the plots of daily activity level during the course of the experiment (Figure 3E,F). In untreated flies activity levels dropped to lower values immediately after transfer from LD to DD and either remained at that lower level throughout recording time (in males) or slowly increased to nearly LD levels (in females). MB-ablated males showed no initial decrease after transfer to DD and steadily increased until the end of recording, when they were more than twice as active as normal males. Activity of MB-ablated females was indistinguishable from that of their untreated sisters.

In summary, MB ablation had no effect on the appearance of either the LN$_v$s and their arborizations, or the DN$_s$. However, we observed a small effect of MB ablation on PDH-staining intensity in the LN$_v$ terminals. We also observed no effect of MB ablation on either the strength of circadian rhythmicity or the sexual dimorphism in the rhythmic activity pattern that is mainly characterized by an early morning peak of male flies. Nevertheless, MB ablation resulted in lengthened period in both genders and increased activity levels in male flies.

*Period and Activity Correlation.* MB-ablated males had a longer period and were more active than control males, suggesting a relationship between these two parameters of behavior. As described in mammals
FIGURE 4 Mean ± SE locomotor activity of control and HU-treated Canton-S ♀ s (■) and ♂ s (○). (A) Phase of morning peaks (m) in LD was sexually dimorphic but not influenced by HU treatment, while evening peaks (e) were not different. ZT = Zeitgeber time; lights-on at ZT₀, lights-off at ZT₁₂. (B) Activity level in LD was also sexually dimorphic but not influenced by HU treatment. (C) Rhythm period in DD was longer in HU-treated flies and in ♂ s. (D) Rhythm power in DD was sexually dimorphic but not influenced by HU treatment. (E) Activity level in DD was elevated by HU treatment in ♀ s only.
(Mrosovsky, 1996), elevated activity might feed back on the internal clock and lead to a longer period. To test this possibility, we plotted mean activity level during DD against period for individual flies (Figure 5). Correlation coefficients were not significant for control males (Figure 5A; \( r_{[1,26]} = 0.125; \ p = 0.535 \)), control females (Figure 5A; \( r_{[1,29]} = 0.005; \ p = 0.977 \)), nor for MB-ablated females (Figure 5B; \( r_{[1,27]} = 0.014; \ p = 0.945 \)). However, the correlation was significant for MB-ablated males (\( r_{[1,28]} = 0.497; \ p = 0.006 \)), suggesting that longer period in these flies may be due to feed back of higher activity on the circadian clock.

**mushroom body miniature**

**Histology.** We scored calyces to represent MB neuropil condition. As described previously (Heisenberg et al., 1985; de Belle & Heisenberg, 1996), we found a prominent sexual dimorphism in calyx size in the original mbm\(^1\) mutants. Whereas females had very small calyces, those of males were only slightly smaller than wild-type. Furthermore, calyx size in females was different in the two experimental runs we performed. In the first experiment, practically all females had strongly reduced calyces, whereas in the second experiment (~half a year later), calyces were nearly normal in size. As observed previously (de Belle & Heisenberg, 1996) calyces of the outercrossed lines [mbm\(^1\); CS and mbm\(^1\)(CS)] were not distinguishable from wild-type. In all mbm\(^1\) stocks, the LN\(_s\) and their arborizations revealed by PDH-staining appeared unchanged, irrespective of MB condition.

**Locomotor Activity Rhythms.** In LD, locomotor behavior of all mbm\(^1\) stocks (except males in the original Berlin genetic background) was synchronized and similar to that of Canton-S (Figure 6; Table II). The morning activity peak was suppressed in four of 17 mbm\(^1\) males (\( \cong 24\% \)). The sexual dimorphism observed in wild-type Canton-S behavior (Figures 3, 4) was also found in mbm\(^1\). Males had an earlier morning peak under LD conditions and were less active than females under LD and DD conditions (data not shown).

After transfer to DD the activity of mbm\(^1\) males dropped to almost zero, but then increased steadily and was finally higher than that of Canton-S (data not shown). Some rhythmicity was often seen after these flies resumed activity, but usually this dampened rapidly to the point of complete arrhythmia (Figure 6). Periodogram analyses of the entire recording period during DD revealed simple weak rhythms with low power in most mbm\(^1\) flies (29/41 \( \cong 71\% \), both genders; Table II). Three females exhibited complex rhythms (\( \cong 7\% \)) and eight mbm\(^1\) flies were completely arrhythmic (\( \cong 20\% \), both genders). Arrhythmic flies were equally prevalent among mbm\(^1\) males and females despite the sexual dimorphism in
FIGURE 5 Scatter and regression plots showing the relationship between period and activity level of (A) control and (B) HU-treated Canton-S \( \delta \) s (\( \square \), −) and \( \Phi \) s (\( \bigcirc \), −). We found a weak influence of activity on period for HU-treated \( \delta \) s only.
MB size (Table II; $\chi^2_{[1]} = 0.02; p = 0.303$). Furthermore, arrhythmic flies were equally prevalent among $mbm^1$ females in the two experiments despite differences in MB size (Table II; $\chi^2_{[1]} = 0.18; p = 0.385$). These observations suggest that rhythmicity was correlated more with genetic background than with MB anatomy.

Interestingly, mutant behavior associated with $mbm^1$ was rescued by placing the mutant allele into an all or part Canton-S genetic background [$mbm^1;CS$ and $mbm^1(CS)$; Table II; Figure 6]. In LD, the morning activity peak was suppressed in only one of the 72 flies (both genotypes combined). The bimodal activity peaks of $mbm(CS)$ were not distinguishable from those of Canton-S control flies (Figures 8A,B; 9A). However, male activity levels were slightly elevated (Figure 9B).

In DD, all flies of both outcrossed $mbm^1$ genotypes were rhythmic. Frequencies of strong, weak, and complex rhythmic $mbm^1;CS$ flies were intermediate between those of $mbm^1$ and $mbm^1(CS)$, suggesting that some of the behavioral phenotypes associated with $mbm^1$ were derived from the original genetic background rather than from the mutant allele itself (Table II). A majority of $mbm^1;CS$ males had strong rhythms, while

**FIGURE 6** Typical actograms of (A) $mbm^1$, (B) $mbm^1;CS$ and (C) $mbm^1(CS)$ $\delta$s (top) and $\varphi$s (bottom). See Figure 2 for Canton-S control actograms. $\square =$ lights on, $\square =$ lights off. Flies were entrained in LD and typically had bimodal activity patterns. In DD $mbm^1$ were almost arrhythmic and $mbm^1;CS$ were weakly rhythmic. $mbm^1(CS)$ were similar to Canton-S but $mbm^1(CS)$ $\delta$s were more active.
TABLE II  Effects of Mushroom Body Mutations of sLNv Histology and Behavior

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gender</th>
<th>Histology*</th>
<th>Morning Peak†</th>
<th>Rhythmicity‡</th>
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<td></td>
<td></td>
<td>n</td>
<td>sLNv</td>
<td>n</td>
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<td>22</td>
<td>30</td>
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<tr>
<td></td>
<td>♀</td>
<td>26</td>
<td>23</td>
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<tr>
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<td>12</td>
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<tr>
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<td>4</td>
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<tr>
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<td></td>
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<tr>
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<tr>
<td><strong>mudI(CS)</strong></td>
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</table>

Control and MB mutant flies were first measured for locomotor activity rhythms and then subjected to PDH-immunohistochemistry to assess the condition of LNv.

*Flies with sLNv terminals stained by anti-PDH.
†Number of days with morning activity peaks under LD conditions.
‡Classification of rhythmicity under DD conditions.
§Data for mbrI from experiments I and II pooled.
*Eight smuI(CS) ♂s had no morning peaks.
§One mudI(CS) ♂ had no morning peaks.
in contrast most females had weak rhythms. This difference was highly significant ($\chi^2_{[1]} = 11.16; p = 0.0003$). In $mbm^l(CS)$ both genders were strongly rhythmic and not significantly different from each other (Table II; Figure 9C; $\chi^2_{[1]} = 0.43; p = 0.511$). Rhythmicity of $mbm^l(CS)$ was also not significantly different from the Canton-S control (genders pooled; Table II; $\chi^2_{[1]} = 0.12; p = 0.265$).

For all $mbm$ strains, ANOVA revealed a significant influence of genotype on period ($F_{[3,153]} = 3.99; p = 0.009$). While gender had no effect ($F_{[1,153]} = 0.00; p = 0.972$), genotype by gender interaction was highly significant ($F_{[3,153]} = 8.84; p < 0.0001$). A SNK test on period length revealed the relationship: $[\text{Canton-S} = mbm^l(CS) = mbm^l; CS = mbm^l \delta] > mbm^l \varphi$ ($p \leq 0.05$). Rhythm power was also highly influenced by genotype ($F_{[3,153]} = 24.33; p < 0.0001$), but not by gender ($F_{[1,153]} = 0.45; p = 0.503$). Interaction effects were significant ($F_{[3,153]} = 4.54; p = 0.004$). For power, the relationship among $mbm$ genotypes was: $mbm^l(CS) \delta > [\text{Canton-S} = mbm^l(CS) \varphi] > [mbm^l; CS = mbm^l]$ (SNK; $p \leq 0.05$). $mbm^l(CS)$ males were more active than both Canton-S and $mbm^l(CS)$ females in DD (Figures 8G,H; 9E) and throughout the entire experiment (Figure 8M,N), but correlation between activity level and period in $mbm^l(CS)$ was not significant (data not shown).

**mushroom bodies reduced, small mushroom bodies and central brain deranged**

**Histology.** The sLN$_v$ terminals revealed by PDH-staining in $mbr^l(CS)$, $smu^l(CS)$ and $ceb^l(CS)$ appeared normal (Table II), in spite of the fact that MB calyces were strongly reduced in the first two mutants and enlarged in the third (data not shown). $mbr^l(CS)$ and $smu^l(CS)$ females could not be reliably obtained and were not used in these experiments (see MATERIALS AND METHODS).

**Locomotor Activity Rhythms.** Under LD conditions, most $mbr^l(CS)$ flies demonstrated bimodal activity patterns with morning peaks (12/15 = 80%; Table II; Figures 7A; 8C; 9A) but with relatively low levels of activity (Figures 8C; 9B). Under DD conditions, $mbr^l(CS)$ was strongly rhythmic (13/15 = 87%; Table II) but had a significantly shorter period than Canton-S controls (Figure 9C; SNK; $p \leq 0.05$). However, power of rhythmicity did not differ from that of Canton-S (Figure 9D; SNK; $p > 0.05$). Daily activity levels of $mbr^l(CS)$ steadily increased during the course of the experiment (Figures 8I,O; 9E).

Most $smu^l(CS)$ flies had weak or no morning activity peaks (21/24 = 88%; Table II; Figures 7B; 8D; 9A) and were even less active than $mbr^l(CS)$ in LD (Figures 8D; 9B). Although $smu^l(CS)$ flies were
rhythmic in DD, only half of them had strong rhythms (11/24 = 46% Table II). smu\(^1\)(CS) had a significantly shorter period than the Canton-S control (Figure 9C; SNK; \(p \leq 0.05\)). Power of rhythmicity was also significantly reduced (Figure 9D; SNK; \(p \leq 0.05\)), likely because of low activity levels throughout the experiment (Figures 8J,P; 9E).

ceb\(^1\)(CS) showed a prominent bimodality in activity in LD, and all flies exhibited morning activity peaks (Table II; Figures 7C; 8E; 9A). In males, amplitudes of both morning and evening peaks were equivalent, and morning peaks showed a much more pronounced anticipation of lights-on than those of all other strains in this report. The characteristic sexual dimorphism in the advancement of morning peak phase was not disrupted by calyx enlargement in ceb\(^1\)(CS). Unlike in Canton-S, ceb\(^1\)(CS) males were as active as females (Figure 9B). In DD, most mutant flies were strongly rhythmic (39/43 = 91%; Table II). While period was not significantly different from that of Canton-S (Figure 9C; SNK; \(p > 0.05\)), rhythm power was greater (Figure 9D; SNK; \(p \leq 0.05\)). Shifting from LD to DD did not reduce ceb\(^1\)(CS) activity levels, and the morning peak usually persisted throughout the experiment in both sexes (Figures 8K,Q; 9E).

**FIGURE 7** Typical actograms of (A) mbr\(^1\)(CS), (B) smu\(^1\)(CS) and (C) ceb\(^1\)(CS) \(\delta\)'s (top) and \(\varphi\)'s (bottom). See Figure 2 for Canton-S control actograms. ––– = lights on, —— = lights off. Flies were entrained in LD, with mbr\(^1\)(CS) and ceb\(^1\)(CS) showing bimodal activity patterns. smu\(^1\)(CS) lacked morning peaks and showed a very low activity level throughout the experiment. In contrast, mbr\(^1\)(CS) and ceb\(^1\)(CS) were very active with especially pronounced morning activity.
FIGURE 8 Mean ± SE activity of MB mutant ♀s (top) and ♂s (bottom) in the Canton-S genetic background. (A–F) Average day in LD was characterized by morning and evening peaks except in smu¹(CS) which had no morning peaks. ■ = lights on, □ = lights off; ZT = Zeitgeber time. (G–L) Average day in DD was generally unimodal, except that morning peaks persisted in mbm¹(CS), mbr¹(CS) and ceb¹(CS) (↑). □ = lights off; CT = circadian time. (M–R) Total activity remained high in most mutants after transfer into DD (▼), except that mbm¹(CS) ♀s were extremely active and smu¹(CS) ♀s were very inactive. □ = LD, ■ = DD.
mushroom body defect

Histology. MB calyces were grossly enlarged and LNv, arborization patterns were highly variable in all mud^4(CS) flies (Figure 10). While LNv terminals in the central brain were generally present, sLNv terminals were sometimes displaced to more anterior or lateral positions by the enlarged calyces. In contrast, all DNs were stained in each of the 18 BG6a; disco flies examined and maintained their usual positions dorsal to the calyces in the posterior brain (data not shown). Therefore, the DN and sLNv terminals—normally in close proximity to each other in wild-type flies—were sometimes separated in mud^4(CS) mutants.

Locomotor Activity Rhythms. In LD, the characteristic sexual dimorphism in the phase of the morning peak was conserved in mud^4(CS) (Table II; Figures 8F; 9A). Activity levels were slightly lower than control flies (Figures 8F; 9B). In DD, the majority of mud^4(CS) flies were rhythmic, but with a high degree of variability (Table II). Only four of 28 flies were arrhythmic (≈14%). Both period length and power of rhythmicity in mud^4(CS) were not significantly different from the Canton-S control (Figure 9C, D; SNK, \( p > 0.05 \)). Activity levels in DD tended to be slightly higher than in control flies (Figures 8L; 9E). As in ceb^4(CS), shifting from LD to DD did not reduce activity levels in either gender of mud^4(CS) (Figure 8R).

sLNv Terminal Displacement and Arrhythmic Locomotor Activity. To determine whether arrhythmicity in mud^4(CS) might have been caused by displacement of the sLNv terminals, we looked for a correlation between arrhythmic behavior and the degree of displacement. In wild-type flies, sLNv terminals are in the same plane of frontal section as the great commissure and posterior to the central complex (CC). We deemed the sLNv terminals to be “strongly displaced” when they were found at the level of (or more anterior to) the ellipsoid body. Based on this criterion we found strong displacement of sLNv terminals in nine of 24 flies. Five of these were strongly rhythmic (Figure 10A), two were complex/weakly rhythmic (Figure 10B,C), and two were arrhythmic (Figure 10D,E). Thus, mud^4(CS) arrhythmicity is not derived from displacement of sLNv terminals. Interestingly, these flies not only show abnormalities in MB anatomy but in other brain regions as well (some of which have not been described previously). Three of the four arrhythmic flies had “semi-split” brains (i.e., the dorsal furrow was extended ventrally; Figure 10D,E). In two of these cases, the CC was completely divided. In the third arrhythmic fly, the CC was intact but misshapen. Although we did not look thoroughly at other brain regions, some additional features of mud^4(CS) brains were apparent. The ocellar tract and/or the optic lobes were occasionally misshapen, the antennal lobes were often enlarged, and
FIGURE 9 Mean ± SE locomotor activity of MB mutant $\delta$s (■) and $\varphi$s (○) in the Canton-S genetic background. (A) Phases of morning peaks (m) were sexually dimorphic while evening peaks (e) were not different. ZT = zeitgeber time; lights-on at
sometimes the whole central brain appeared larger than in Canton-S. Moreover, the number of LN$_s$ in mud$^4$(CS) was often either fewer (Figure 10B,C,E) or greater (Figure 10D) than in Canton-S, and their arborizations were often irregular (Figure 10A,E). These abnormalities indicate that multiple brain regions are affected by the mud$^4$(CS) mutation and that the aberrant rhythmic behavior of some of these flies was likely not attributed to enlarged calyces and misrouted sLN$_v$ projections alone.

**DISCUSSION**

The aim of the present study was to investigate whether *Drosophila* MBs are involved in (1) the circadian clock output pathway to locomotor rhythms, and (2) gender-specific differences known to be associated with that behavior (Helfrich–Förster, 2000). Our results show clearly that MBs are not important for either the entrainment or maintenance of diurnal motor activity rhythms and are not responsible for gender-specific differences in aspects of motor activity. HU-treated flies and most MB mutants—except mbm$^l$ in the original *Berlin* genetic background and mud$^4$(CS)—were normally rhythmic.

Under LD conditions, males of these strains showed an earlier morning peak than females. Sex-specific differences in rhythmic behavior might be due to different cellular activity of LN$_s$. Park and Hall (1998) found that the *pdf* mRNA is detected at a higher level in male flies compared with females. We also observed higher PDH–immunoreactivity of the LN$_s$ terminals in males. These differences were not affected by MB ablation and were found in most MB mutants. We suggest that PDF may be secreted at different levels into the dorsal protocerebrum and this may influence downstream neurons differently in female and male flies. Interestingly, a small cluster of neurons in the pars intercerebralis (PI) was recently implicated in gender-specific differences in motor activity patterns of *Drosophila* (Gatti et al., 2000). In larger insects, such PI neurons have arborizations in the medial and lateral dorsal proto-

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ZT$_0$, lights-off at ZT$_{12}$. (B) Activity level in LD of ♀s tended to be higher than ♀♀, except for mbm$^l$(CS). (C) Rhythm period in DD had the relationship: mbm$^l$(CS) ♀ > [Canton-S = ceb$^l$(CS) = mbm$^l$(CS) ♂ = mbr$^l$(CS) ♂] > [mud$^4$(CS) = smu$^l$(CS) ♀] (SNK; *P* ≤ 0.05). (D) Rhythm power in DD had the relationship: mbm$^l$(CS) ♀ > [Canton-S = ceb$^l$(CS) = mbm$^l$(CS) ♂ = mbr$^l$(CS) ♂ = mud$^4$(CS) ♀] > smu$^l$(CS) ♂ (SNK; *P* ≤ 0.05). (E) Activity level in DD tended to be higher in mutants and ♀♀, except for the wild-type activity of mbm$^l$(CS) ♀♀ and the reduced activity of smu$^l$(CS).
FIGURE 10 Brain anatomy and behavior of typical mud⁴(CS) mutants. Camera lucida drawings of wholemount brains stained with anti-PDH (left), corresponding actograms (center) and periodograms (right). Despite enlarged calyces and strong displacement of sLNᵥ terminals in these flies, rhythm strength was variable (▼) and classed as (A) strong, (B) complex, (C) weak, and (D,E) arrhythmic. sLNᵥ terminal displacement cannot be the underlying reason for arrhythmicity in mud⁴(CS).
cerebrum (reviewed by Homberg, 1994). If this is also true for *Drosophila*, PI neurons may be influenced by, or even postsynaptic to, the LNv terminals in that region of the brain.

In mbm<sup>1</sup> strains, the arrhythmic activity pattern of the original mutant was rescued completely by outcrossing with *Canton-S* wild-type. This demonstrated that abnormal circadian behavior of mbm<sup>1</sup> was caused by genetic background effects rather than by the mbm gene itself. As described previously, the reduced calyces observed in this mutant are due to interaction between mbm and the genetic background, since outcrossed flies had MBs of normal size (de Belle & Heisenberg, 1996). Nevertheless, mbm<sup>1</sup>(CS) retains an olfactory conditioning deficit and is regarded as learning mutant (de Belle & Heisenberg, 1996). In the present study, locomotor behavior of mbm<sup>1</sup>(CS) males was also different from that of *Canton-S* wild-type controls (see below). One might initially conclude that there is a good correlation between MB anatomy and behavior among mbm mutant stocks with different genetic backgrounds. While MB anatomy was vastly different among the original mbm<sup>1</sup> mutants examined ~ half a year apart, behavior was not.

In mud<sup>4</sup>(CS), the higher-than-normal percentage of weakly rhythmic and arrhythmic flies is most probably due to damage in a variety of brain regions rather than to MB-specific lesions. Besides enlarged calyces, we found several additional brain structure abnormalities in mud<sup>4</sup>(CS) mutants. Furthermore, numbers of LNv cells were variable. Several mud alleles have been induced in screens for structural brain mutants (Heisenberg, 1980; Fischbach et al., 1987). Such mutations give rise to increased numbers of Kenyon cells, leading to enlarged calyces at the expense of pedunculi and lobes. This defect results from proliferation of an excess number of MB neuroblasts (Prokop & Technau, 1994). mud also affects proliferation patterns of neuroblasts in other regions of the developing central nervous system (Prokop & Technau, 1994). In a subset of lateral brain neuroblasts, mud mutations suppress mitotic arrest and lead to excessive proliferation of neurons. LNv<sub>s</sub> are located in the lateral brain, and the supernumerary LNv<sub>s</sub> we found in some brains might be caused by prolonged mitotic activity of precursor neuroblasts. In cases where we found fewer LNv<sub>s</sub> than in wild-type flies, we propose that some may have been lost during metamorphosis in mud mutants (Heisenberg, 1980; Technau & Heisenberg, 1982; Fischbach et al., 1987). Interestingly, DN<sub>s</sub> appeared completely normal in all mud<sup>4</sup>(CS) brains we examined. This suggests that DN<sub>s</sub> arise from neuroblasts that do not contribute to Kenyon cells and that DN-specific neuroblasts are not affected by the mud<sup>4</sup> mutation.

DN neuroblasts were also not affected by HU treatment. HU fed to early first instar larvae deletes the four MB neuroblasts and one additional lateral neuroblast contributing to specific antennal lobe interneurons (Ito & Hotta, 1992; de Belle & Heisenberg, 1994; Stocker et al., 1997). Only a small bundle of fibers survive HU ablation, representing
embryonically-derived Kenyon cells (Tettamanti et al., 1997; Armstrong et al., 1998) and perhaps also extrinsic MB tracts (Heisenberg, 1980; Ito et al., 1998). Not only do DNs survive HU treatment, but they also showed normal PER-cycling. We found high β-galactosidase activity (reporting per gene expression) in the early morning when levels of natural PER are high, and very little or no activity in the evening when levels of PER are low (e.g., Zerr et al., 1990). HU treatment did not affect PER cycling. Similarly, PER cycling in LN₅s was normal (as far as can be judged from two opposite-phase time points). Furthermore, sLN₅ terminals in the central brain revealed by anti-PDH appeared morphologically unchanged. This indicates that they are not derived from MB neuroblasts, nor are they dependent on normal MB development. Consequently, Kenyon cells are also likely not the direct targets of sLN₅s. At present, it is unknown whether HU treatment influences sLN₅ connectivity in the dorso-lateral protocerebrum. PDF neuropeptide appears to be critical for the rhythmic output pathways of the sLN₅s since rhythmic circadian behavior is severely disturbed in null pdf mutants (Renn et al., 1999) and by ectopic PDF expression in the dorso-lateral brain (Helfrich-Förster et al., 2000). However, behavioral rhythmicity is not affected when synaptic transmission is blocked by expression of tetanus toxin light chain targeted to the LN₅s (Kaneko et al., 2000). This indicates that PDF secretion from sLN₅ terminals is independent of normal synaptic transmission. An earlier study in which rarely encountered disconnected mutant flies with strongly displaced sLN₅ terminals had normal rhythmic behavior led to the same conclusion (Helfrich-Förster, 1998). In our study, we also found that rhythmicity was not dependent on sLN₅ terminal displacement by enlarged calyces in mud⁴(CS) mutants. PDF may act as a locally secreted neuromodulator, providing a circadian signal to neurons in the superior protocerebrum. MB Kenyon cells may be among these neurons, but we show here that they are not important for transferring rhythmic signals to motor centers in the thoracic nervous system.

Although MBs are not critical for general rhythmicity, they do appear to influence overall activity levels under continuous darkness in male flies. In DD, HU-treated or MB-mutant males were generally more active than control males with intact MBs. Males of two additional mutants not reported in this study showed similar patterns (calyx bulging [cxb¹(CS)] and mushroom bodies deranged [mbd¹(CS)]; C. Helfrich-Förster and J.S. de Belle, unpublished observations). These results indicate that higher activity in males is a specific effect of MB damage.

A general inhibitory effect of MBs on activity in crickets was reported by Huber (1960). More recently, a similar MB influence was described in Drosophila (Martin & Heisenberg, 1998). HU-treated flies, MB mutants (mbm¹ and mbd¹), and MB-targeted tetanus toxin transgene-expressing flies showed higher spontaneous walking activity than normal flies. These
findings are consistent with our results and suggest that MBs might have some role in the general process of organizing the temporal and spatial patterns of locomotion. However, there are several differences between the studies of Martin and colleagues and our own. First, we found higher activity levels in males only [HU-treated flies and mbm<sup>1</sup>(CS), mbr<sup>1</sup>(CS) and ceb<sup>1</sup>(CS) mutants], whereas activity levels of females appeared unaltered. Martin and Heisenberg (1998) reported increased activity after MB ablation in female flies (they did not look at males). Second, they did not find significant differences in activity between male and female wild-type flies (Martin et al., 1999). In our study, females were significantly more active than males, under both LD and DD conditions. We suggest that the latter discrepancy may be due to methodological differences in the recording of activity. Martin and colleagues measured spontaneous locomotor activity with high temporal resolution for several hours after each fly was placed into a new environment (Martin & Heisenberg, 1998; Martin et al., 1999). We recorded activity with low temporal resolution but for several weeks and did not measure the absolute amount of activity, since we did not count the number of times a fly interrupted the infrared light beam. Instead, total time was divided into 4-min intervals and a fly would be scored as active whether it tripped the light gate once or several times during this interval. Consequently, a fly that walked a lot in alternative 4-min intervals and paused completely during intervening intervals throughout the day would be assessed with a mean activity level of 0.5. On the other hand, a fly that was less active overall but managed to pass the light beam only once in every 4-min interval throughout the day would have a mean activity level of 1.0. In reality, such extreme examples probably never happen, but the temporal organization of locomotor activity in high frequency patterns does influence the measured level of activity by other recording systems. Martin and Heisenberg (1998) found that typical activity of <i>D. melanogaster</i> is organized in bouts lasting ca. 3 min separated by ca. 2-min pauses. The mean duration of bouts and pauses was similar in males and females but the frequency of light beam interruptions per bout was significantly higher in males. This suggests that males either walk faster or with fewer pauses than females. However, it was also found that males actually interrupt their activity more often and with longer pauses than females. Therefore, males likely achieve total activity equivalent to that of females by walking faster but in fewer, shorter, and more dispersed bouts (Martin et al., 1999). In our study, we suggest that males may appear less active than females because these high activity bouts could not be revealed by our recording system.

Gender-specific differences were not altered by MB ablation, at least not in LD conditions. In LD, control and HU-treated females appeared more active than both groups of males. Furthermore, darkness had a strong inhibitory effect on activity for most flies—both immediately after the lights were turned off and throughout DD (see DISCUSSION in
Helfrich-Förster, 2000). Darkness was not inhibitory for activity of MB-less males. Instead, they showed increased daily activity throughout DD. MB-less females were not different from their intact sisters, showing lower activity after being shifted from LD to DD. By contrast, Martin and Heisenberg (1998) found elevated activity levels in the dark for both genders of HU-treated flies. Their observation was attributed to an increase in the duration of activity bouts, and therefore could have been detected in our system. We suggest that the high activity of MB-less females reported by Martin and Heisenberg (1998) was due to arousal provoked by transfer to a novel environment (the recording chamber). Control flies showed similar arousal patterns, but they reduced their activity in the first hours of the experiment (Martin & Heisenberg, 1998; Martin et al., 1999). In our studies, we recorded for several weeks so flies were well acclimatized to their environment. Therefore, the two studies are probably assessing completely different mechanisms of activity stimulation. This explanation receives support from observations by Cobb et al. (1987) that levels of locomotor behavior could be elevated by transferring flies to new containers.

A previous study suggested that males may be active in the early morning before females to confer a selective advantage in courtship (Helfrich-Förster, 2000). We suggest that the earlier morning peak observed in males under LD conditions might reflect this activity. MB-less and MB-mutant males retain the “predictive” morning peak, indicating that MBs do not mediate this gender-specific behavior. However, MBs could be necessary for repressing mate-search behavior in unfavorable conditions such as continuous darkness.

In control flies the morning peak is reduced after transfer into DD conditions (Helfrich-Förster, 2000). In the present study, morning peaks were barely detected in the average days of control males under DD conditions. This was not the case for mbr¹(CS) and ceb¹(CS) males, which had very pronounced morning peaks in DD. HU-treated and mbr¹(CS) males also had morning peaks under DD conditions, although not as pronounced. We suggest that this might prevent males with disrupted MBs from down-regulating courtship-related locomotor behavior in “unfavorable” (= dark) conditions. One explanation might be that such down-regulation of activity involves olfactory-based learning, a process that is dependent on normal MB function. Interestingly, Drosophila males show courtship suppression after being exposed to unreceptive females (Siegel & Hall, 1979, Gailey et al. 1984). This experience-dependent courtship modification is impaired in several learning mutants (Gailey et al., 1985; O’Dell et al., 1999), although MBs are apparently not involved in this type of learning (Wolf et al., 1998). However, MBs are required for visual context during learning (Liu et al., 1999). Flies deprived of functional MBs are not able to retrieve spatial memory when experimental lighting conditions are changed. Conse-
quently, such males may be incapable of learning that searching for mates in the dark is maladaptive and they may increase activity in their attempts to reorient in the dark environment. In other words, MBs may be of some adaptive value in suppressing courtship under suboptimal conditions.

In HU-treated flies, the only other affected parameter besides the activity level was behavioral cycle duration. This was slightly lengthened (mainly in males). All other rhythmic parameters were unaltered by HU treatment. Period lengthening in HU-treated males was correlated with the activity increase and might be due to a feedback of activity on the circadian clock (Mrosovsky 1996). We thus considered the possibility that period lengthening might be a secondary effect of elevated activity rather than a direct effect of MB ablation. However, we did not find a significant correlation between activity level and period in any of the MB mutants. Furthermore, not all MB mutants showed a longer period than wild-type flies. Similarly, other rhythm parameters such as power and phases of activity peaks relative to lights-on varied in the MB mutants. In one case, power was similar to the wild-type [ceeb(CS)], in one case even higher [mbm(CS)] and in the remaining mutants lower than in the wild-type. Only in mbm(CS) were phases of morning and evening peaks similar to wild-type. In all other MB mutants, one or both activity peak were shifted in phase. Since these parameters are not influenced by MB ablation alone, and since they are affected in a variety of ways in the various MB-mutants, it is unlikely that they are solely caused by MB damage. Indeed, most of the MB mutants tested have pleiotropic effects on other brain structures, especially on the CC (de Belle & Heisenberg, 1996). The CC is a higher coordination center for motor behavior in insects (Strauss & Heisenberg, 1993). It might also be a delay station for circadian signals coming from the LN, via still unknown interneurons. In some insects PDH-immunoreactive fibers from the pacemaker neurons connect directly to the CC (Homberg, 1994).

$smu^1(CS)$ is the only MB mutant that has no obvious CC structural defects (de Belle & Heisenberg, 1996). Nevertheless, $smu^1(CS)$ behavior was exceptional in many respects. Despite having strongly reduced MBs, $smu^1(CS)$ males were very inactive. Further, the pronounced morning activity peak in MB-ablated males and in other MB mutants was virtually absent in $smu^1(CS)$. We suggest that $smu^1$ has pleiotropic defects in the brain that remain to be described. Alternatively, $smu^1$ may affect different subsets of Kenyon cells than do other MB mutations. Kenyon cells were once considered functionally equivalent isomorphic arrays. MB subcompartments exhibit different patterns of gene expression, suggesting that parallel channels may sub-serve different functions (de Belle, 1995; Yang et al., 1995; Connolly et al., 1996; Zars et al., 2000; Dubnau et al., 2001). $smu$ mutants may prove to be important for evaluating MB function in the generation and modification of behavior.
In summary, we found that (1) MBs are not involved in the output pathway from LN,s to locomotor activity rhythms in Drosophila, and (2) they are not necessary for mediating gender-specific differences in the rhythmic activity patterns. However, they may be involved in suppression of locomotor activity under certain conditions. Our study does not support a common genetic and cellular basis of rhythms and learning. We do not rule out the possibility that MB functions are under rhythmic control of the sLN,s. In this way, flies may be able to associate specific odors with other stimuli in a circadian manner.

REFERENCES


