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Central complex substructures are required for the maintenance of locomotor activity in *Drosophila melanogaster*

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Abstract In *Drosophila melanogaster*, former studies based on structural brain mutants have suggested that the central complex is a higher control center of locomotor behavior. Continuing this investigation we studied the effect of the central complex on the temporal structure of spontaneous locomotor activity in the time domain of a few hours. In an attempt to dissect the internal circuitry of the central complex we perturbed a putative local neuronal network connecting the four neuropil regions of the central complex, the protocerebral bridge, the fan-shape body, the noduli and the ellipsoid body. Two independent and non-invasive methods were applied: mutations affecting the neuroarchitecture of the protocerebral bridge, and the targeted expression of tetanus toxin in small subsets of central complex neurons using the binary enhancer trap P[GAL4] system. All groups of flies with a disturbed component of this network exhibited a common phenotype: a drastic decrease in locomotor activity. While locomotor activity was still clustered in bouts and these were initiated at the normal rate, their duration was reduced. This finding suggests that the bridge and some of its neural connections to the other neuropil regions of the central complex are required for the maintenance but not the initiation of walking.

Key words Motivational control · Protocerebral bridge · Fan-shape body · Noduli
Spontaneous walking

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Abbreviations *CC* central complex · *eb* ellipsoid body · *fb* fan-shaped body · *ICI* inter-count interval · *MBs* mushroom bodies · *no* noduli · *pb* protocerebral bridge

Introduction

Locomotion plays a major role in animal behavior. Measuring locomotion, one cannot but notice the large spontaneous fluctuations in the animal's readiness to walk. Yet, except in chronobiology, little attention has been paid to the temporal pattern of these changes. It is well established that the thoracic and abdominal ganglia of insects, like the spinal cord of vertebrates, harbor the basic motor programs of walking (Bässler 1983; Graham 1985) and that these are controlled by the head ganglia. For example, Graham (1979) has demonstrated that a stick insect with cut circumoesophageal connectives can still walk, whereas cutting the neck connectives largely suppresses walking activity. Kien (1983) was able to evoke various types of walking in tethered locusts by delivering systematic electrical microstimulation to the circumoesophageal and neck connectives, yet hardly anything is known about how the brain generates the endogenous fluctuations in walking activity.

In an early attempt to analyze central brain function in grasshoppers and crickets, two structures, the central complex (CC) and the mushroom bodies (MBs), were implicated in the regulation of behavioral activity (including walking). Small lesions and focal electrical stimulation assigned an activity-reducing effect to the MBs and a behavior-initiating or maintaining function to the CC (Huber 1955, 1960, 1963, 1965; Otto 1971; Wadepuhl and Huber 1979; Wadepuhl 1983; reviewed in Homberg 1987).

In *Drosophila*, genetic dissection has provided independent and more detailed information about roles of the CC in the control of walking. Strauss and Heisenberg (1993) found mutants with defects in the CC to be affected in a variety of walking parameters, like walking

speed, straightness of walking, time-course of walking activity, leg coordination during turns and start-stop (Leng and Strauss 1998). In one of the mutant strains the flies from time to time fell into a comatose state during which they could not walk but still stood up and performed occasional erratic leg movements. Using genetic mosaics, this and several of the other defects could be assigned to the head and were highly correlated with the structural defects in the CC (Strauss et al. 1992; Strauss and Heisenberg 1993; Leng and Strauss 1997). In addition, CC mutants have defects in olfactory and visual learning tasks (Heisenberg et al. 1985), and in various properties of visual flight control (Ilius et al. 1994). Finally, also activity labeling with deoxyglucose has suggested the CC to be involved in locomotor control (Bausenwein et al. 1994).

We have started to investigate spontaneous walking in the fly *Drosophila melanogaster* (Martin et al. 1999). Genetically or chemically ablating the intrinsic neurons of the MBs, or blocking them by the targeted expression of tetanus toxin (enhancer-trap P[Gal4] technique; Brand and Perrimon 1993; Sweeney et al. 1995) increases walking activity (Martin et al. 1998), as had been reported for grasshoppers and crickets (see above). In the present study we show that genetically blocking or eliminating parts of the CC has the opposite effect, it reduces locomotor activity. This again parallels the findings in orthopterans nearly 50 years ago.

Like surgical lesions and drugs, genetic manipulations also have uncontrolled and undesirable side effects which need to be distinguished from the effects of the intended impairment. Preferentially, therefore, one would like to use several independent methods for disturbing the structures of interest hoping that the side effects would differ. Here we use eight genetically distinct lines of flies with defects in closely related components of CC circuitry. In all of them the temporal structure of walking activity is altered in the same way, suggesting that the same circuitry is affected and that this circuitry is involved in the control of walking activity.

Materials and methods

Flies

Stocks (*D. melanogaster*) were maintained at 25 °C on a standard cornmeal/molasses medium in a 16 h light/8 h dark cycle at 60% relative humidity. Wild-type strains Canton-S (CS) and the mutants *no-bridge*^{1} (*nob*^{1}), *central-complex*^{1} (*cex*^{1}) and *ocelliless*^{1} (*oc*^{1}) were used. The *nob* and *cex* mutations were isolated by mass histology (Heisenberg and Böhl 1979) following ethylmethane sulfonate mutagenesis and have been extensively back-crossed to CS (de Belle and Heisenberg 1996). The *oc*^{1} mutant is a viable and hypomorphic allele of the *orthodenticle* (*otd*) gene which is essential for proper brain segmentation in early embryonic development (Hirth et al. 1995). The mutant is a chromosomal inversion disrupting the non-coding region of the gene (Finkelstein et al. 1990).

The P[GAL4] enhancer-trap lines P[GAL4] C5, P[GAL4] C584, P[GAL4] 78Y, and P[GAL4] 7Y were kindly provided by K. Kaiser (Yang et al. 1995). The line P[GAL4] 205 has been generated by H. Pfister (1997). We used the gene for tetanus toxin light chain inserted on the second chromosome in a CS genetic background, downstream of four UAS_{GAL4} enhancer elements (P[UAS_{GAL4}-tetanus toxin]). This stock, called Cnt-E, was kindly provided by C. O'Kane (Sweeney et al. 1995). The five enhancer-trap lines are homozygous viable. Since GAL4-directed tetanus toxin expression was tested in flies heterozygous for both the P[GAL4] and P[UAS_{GAL4}-tetanus toxin] constructs, the corresponding heterozygous P[GAL4]/Canton-S flies were tested as controls.

Measuring walking activity

The apparatus was a transparent rectangular chamber ($40 \times 3 \times 3$ mm^3) with an infra-red light gate situated in the middle. The light gate was sampled once every second. Any number of interruptions of the light gate during the preceding second were counted as a single event [count(1)/no count(0)]. In the chamber, a single fly could walk freely during a time period of 4 1/2 hours (16 200 s). A moist filter paper permitted the fly to drink water. Single mated females at the age of 3 days were tested in complete darkness. All experiments were performed at 25 °C, at 50–60% relative humidity and at the same time of day starting at 1800 hours to avoid circadian variations (for a detailed description of the apparatus see Martin et al. 1999). All recordings were performed on three successive days at two different periods of 1998 (May and August). Data were pooled since no significant differences were found between flies tested on successive days and between different months of the year.

Data processing

Data recording and analysis were programmed in C++ (Microsoft Visual C++). For statistical tests Statistica (StatSoft) was used. Total activity was approximated as the total number of counts for each fly during the recorded time. For the time-course of activity, counts were summed for successive 10-min periods. Time intervals between consecutive counts (inter-count intervals; ICIs) were calculated as the sum of the zero events between counts. To reveal the clustering (bout structure) in the time traces, we calculated for each fly a 'minimum pause' which is the shortest ICI separating bouts. To obtain the minimum pause we plotted the cumulative frequency of ICIs (on a logarithmic scale) for increasing ICIs ('log-survivorship curve', Machlis 1977; Slater and Lester 1982; Sibly et al. 1990). The visual estimate of the location of the break in the curve closely corresponds to the intersection of the two approximations of the non-linear function. This curve provided an unambiguous criterion for the minimum pause for each fly. To obtain the number and duration of bouts as well as the duration of the pauses between bouts, onset and end of each bout were determined in the time traces using the individual minimum pauses as a criterion. As expected, since a linear time axis is unilateral and therefore not compatible with a Gaussian distribution, the parameters extracted were not normally distributed. We therefore subjected the data in Figs. 2C–E, 4C–E, 6C–E to a log transformation (Sachs 1992) to approximate a normal distribution and then calculated the means and standard errors of the mean, which were weighted to the number of observations for each fly, as a conservative statistical estimate (Sachs 1992). For a more reliable representation of the bout structure, all bouts consisting of a single count were removed together with the subsequent pause. The results are not qualitatively affected by this processing step. Finally, to reveal that the temporal distribution of activity has a fractal structure, we plotted the decreasing cumulative frequencies of ICIs (%) as a function of increasing ICIs, both on a log scale (for a more detailed description of this analysis see Martin et al. 1999).

Immunohistochemistry

Immunohistochemistry methods of Buchner et al. (1988) were used. Briefly, *Drosophila* adult heads were fixed for 4 h in buffered 4% paraformaldehyde and washed overnight in 25% sucrose

solution. Frontal and horizontal sections were cut on a cryostat microtome at 10 μm thickness and incubated overnight at 4 °C with a monoclonal anti-tetanus toxin antibody (1:10 000). Antibody and unpublished information were kindly provided by J. Thierer and H. Niemann, Hannover. Biotine-streptavidine coupled to peroxydase (ABC Kit, Vectastain) using diaminobenzidine as the chromogen was used to visualize the primary antibody.

Results

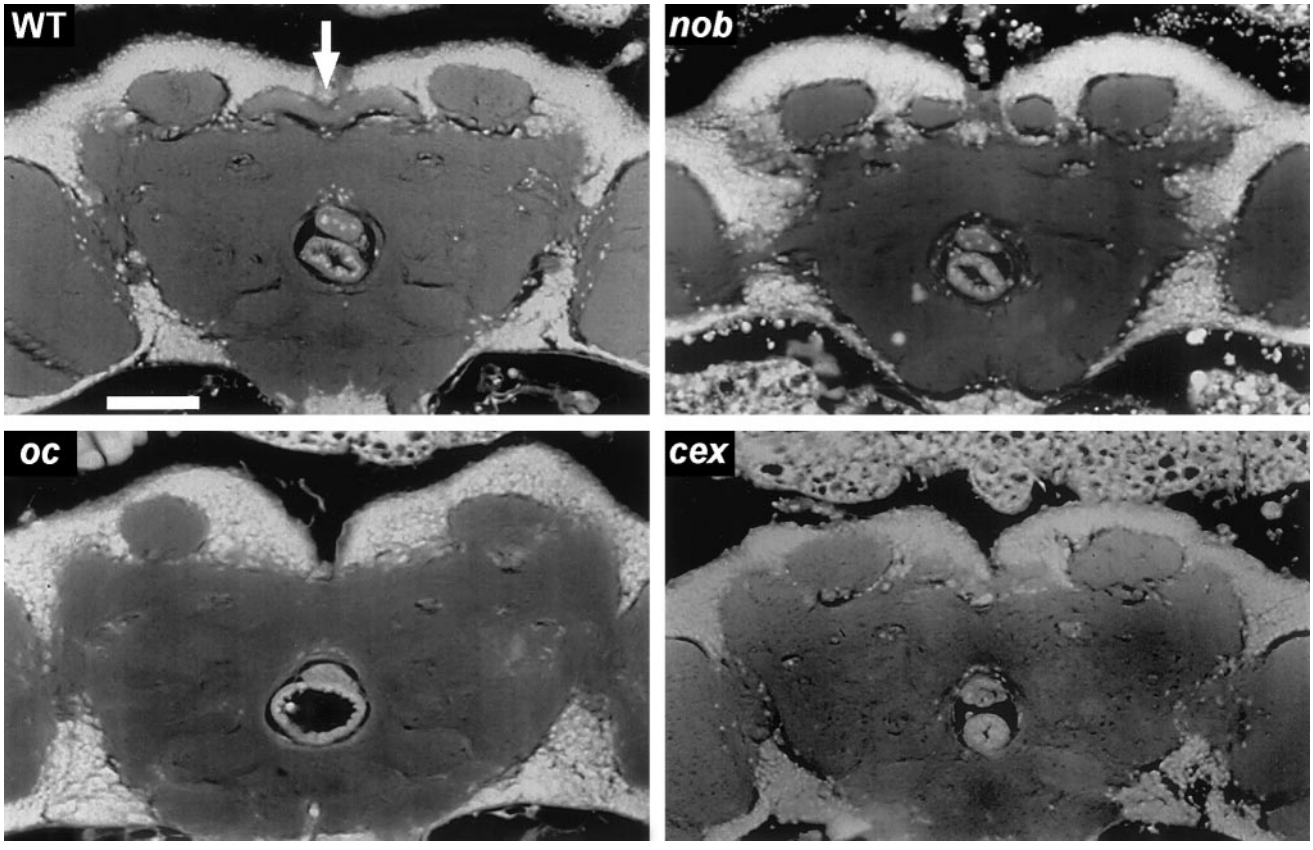
Methods of intervention in the central complex

Two kinds of intervention were used to generate flies with disturbed CC substructures. In one approach three mutants were studied in which the development of the protocerebral bridge (pb) was altered leading to the absence or malformation of the pb in the imago. In the second approach, we used the P[GAL4] enhancer-trap system together with a UAS_{GAL4} tetanus toxin light chain construct (Cnt-E; Sweeney et al. 1995). These authors have demonstrated that tetanus toxin expression in larval motorneurons blocks synaptic transmission at the neuromuscular junction (and see expression in MBs above; Martin et al. 1998). Five lines expressing the transcription factor GAL4 in different subsets of neurons in the CC were chosen to drive the expression of the effector gene. Besides the expression pattern, two prerequisites for selecting those lines were first, that flies had to survive to adulthood despite the GAL4-dependent toxin expression, and second, that those flies had the general appearance of wild-type flies regarding their postural and/or walking behavior. Three of the lines showed expression in very similar subsets of neurons linking one tangential layer of the fan-shape body (fb) to a particular region of the so-called unstructured neuropil of the central brain (Fig. 3). In the two other lines similar or identical subsets of intrinsic (columnar) CC neurons were marked connecting the pb, noduli (no) and ellipsoid body (eb) (Fig. 5). In all five P[GAL4] lines few other neurons unrelated to the CC were marked in addition but between the five lines very little overlap occurred with respect to these neurons.

The protocerebral bridge is required for maintenance of locomotor activity

Three mutants (*no-bridge*^{1} [*nob*^{1}], *central-complex*^{1} [*cex*^{1}] and *ocelliless*^{1} [*oc*^{1}]) with defects in the neuroarchitecture of the adult pb have been described (Fig. 1). The bridge consists of eight glomeruli on either side of the midline

Fig. 1 Frontal 7-μm paraffin sections of adult brains at the level of the protocerebral bridge (pb) and the calyces of the mushroom bodies (MBs). In wild-type (*WT*), the pb (*arrow*), located between the two calyces is easily recognizable. In *nob*^{1}only the middle part of the pb is missing, while in *oc*^{1} the pb is missing entirely. In *cex*^{1}, only parts of the pb lie in the plane of section. Its architecture is disturbed as it look more 'glomerular' than in wild type. Scale bar = 50 μm



harboring arborizations of columnar elements that project to other parts of the CC. The 16 glomeruli are connected by a scaffold of transverse fibers running across the midline and linking the pb to regions outside the CC. In *nob*^{1}, a mutant that has been already intensely studied, the three most medial glomeruli on either side are missing (Strauss et al. 1992; Strauss and Heisenberg 1993). In addition, silver-impregnated brain sections have revealed that fiber systems connecting the pb to the fb (Hanesch et al. 1989) are also disturbed in the fb (Leng and Strauss 1998). In adult *oc*^{1} flies, the protocerebral bridge is entirely missing (Hirth et al. 1995). However, in contrast to *nob*^{1}, all the tracts normally linking the pb to the fb are still present as revealed by silver-impregnated serial sections (Leng and Strauss 1997). In the third mutant, *cex*^{1}, the pb is still present but its neuroarchitecture is disturbed: it is thinner and the individual glomeruli are more apparent. Probably, transverse elements of the scaffold are missing (for a more complete description see Strauss and Heisenberg 1993).

Former behavioral studies of these mutants showed the pb to be involved in optimizing walking speed. Moreover, ethograms suggested that in *nob*^{1} and *oc*^{1} flies total behavioral activity was low. Interestingly, in *nob*^{1} and *oc*^{1}, genetic mosaic analysis yielded a full correlation of the behavioral defects with the state of the pb (Strauss and Heisenberg 1993; Leng and Strauss 1997), while in *cex*^{1} some of the behavioral defects were associated with a mutant thorax (Strauss and Heisenberg 1993).

As already reported (Martin et al. 1999), the locomotor activity of single flies walking in small chambers was continuously recorded for 4 1/2 h. The apparatus registered walking only when the fly passed the light gate in the middle of the tube's long axis. Pilot experiments using a linear array of 1000 light gates showed that flies tended to patrol the whole length of the chamber. For the 2×2 cm from the midpoint of the chamber to the end and back they needed 5–10 s. If they took longer they either had a rest period, or walked around at the end of the tube as if trying to escape. In the present apparatus in which rest periods could not be recorded directly, ICIs instead were evaluated assuming that most ICIs much longer than 10 s contained rest periods. Direct inspection of the behavior in these tubes suggested that periods of continuous walking were clustered in time (bouts). In order to quantify this clustering we separated the ICIs into two classes: frequent short ones and rare long ones (Martin et al. 1999; see also Materials and methods). For a large majority of individual flies, the critical ICI duration separating the two classes (minimum pause) could easily be determined. The short ICIs were said to occur within bouts, the long ones were taken to separate bouts and were called 'pauses' (although they too were longer than the real pauses that were not recorded). The minimum pause was used to mark bouts in the original recordings of light gate passages (counts). We remind the reader here of this recording situation to emphasize that bouts and pauses are not synonyms for walking and rest periods. Rather, bouts are clusters of walking phases. Likewise, pauses may be clusters of briefly interrupted rest periods. Bouts and pauses probably represent two motivational states in which the probability of walking is different (for a more detailed discussion see Martin et al. 1999).

In the mutants *nob*^{1}, *oc*^{1} and *cex*^{1} spontaneous locomotor activity was affected similarly: their total walking activity was significantly decreased in comparison to CS flies (Fig. 2A). Although the mutant *oc*^{1} has an unknown genetic background, its defects in locomotor activity resemble those of the other two mutants. They lie outside the range of the various wild-type lines already reported (e.g. the difference between Wild-type Berlin and CS in Martin et al. 1999).

To measure the time-course of activity it was determined separately for each successive 10-min period. Wild-type flies showed an initial decrease of activity interpreted as a decaying response to the handling or the new conditions of the chamber (exploratory behavior). Subsequently their activity reached a plateau. In *nob*^{1} the decay of the responsive phase was only slightly faster than in wild type, in *oc*^{1} this phase was very short and in *cex*^{1} flies no reactive component was observed. Activity stayed at a very low level throughout the experiment (Fig. 2B). Note that *nob*^{1} flies were less severely impaired structurally as well as functionally compared to *oc*^{1}. In *nob*^{1} about 60% of the pb was still present and flies exhibited about 60% of normal locomotor activity while in *oc*^{1} no remnants of the pb were left and locomotor activity was down to 30%. Mutant *cex*^{1} flies do not follow this trend; they exhibited the strongest defect in locomotor activity while the pb seemed the least affected. As already mentioned, it is known from mosaic studies that the defects in this mutant are not entirely confined to the CC (Strauss and Heisenberg 1993).

As in wild-type flies, locomotor activity also occurred clustered in bouts in the mutants. We determined the minimum pause for each individual fly and calculated the number of bouts and the intrinsic bout parameters. For *nob*^{1}and *oc*^{1}, the mean of the minimum pause was not significantly different from wild type (data not shown). Moreover, for all three mutants the number of bouts was normal (compare mean period in Fig. 2E). The intrinsic bout structure of the three mutants also showed that the activity during bouts was normal and that the decrease in locomotor activity was due to a shortening of the bouts (Fig. 2C). Indeed, mean bout duration was reduced to about 50%. Correspondingly, pauses were almost twice as long as in wild-type flies (Fig. 2D). To summarize, in three different lines of pb-disturbed flies episodes of high walking probability occurred as frequently as in normal flies but these episodes were of much shorter duration. This suggests that neurons in the pb are involved in the maintenance of a state of high walking probability (restlessness).

Finally, as a more subtle parameter of the temporal pattern of locomotor activity, we evaluated the frequency distribution of the ICIs. If the cumulative ICI frequency is plotted on a double-logarithmic scale as a function of

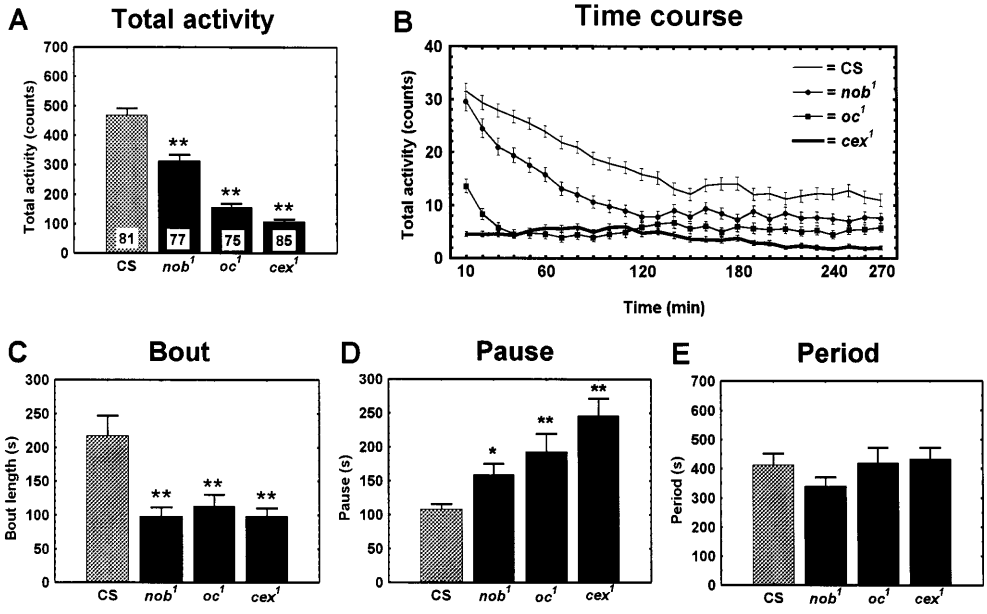


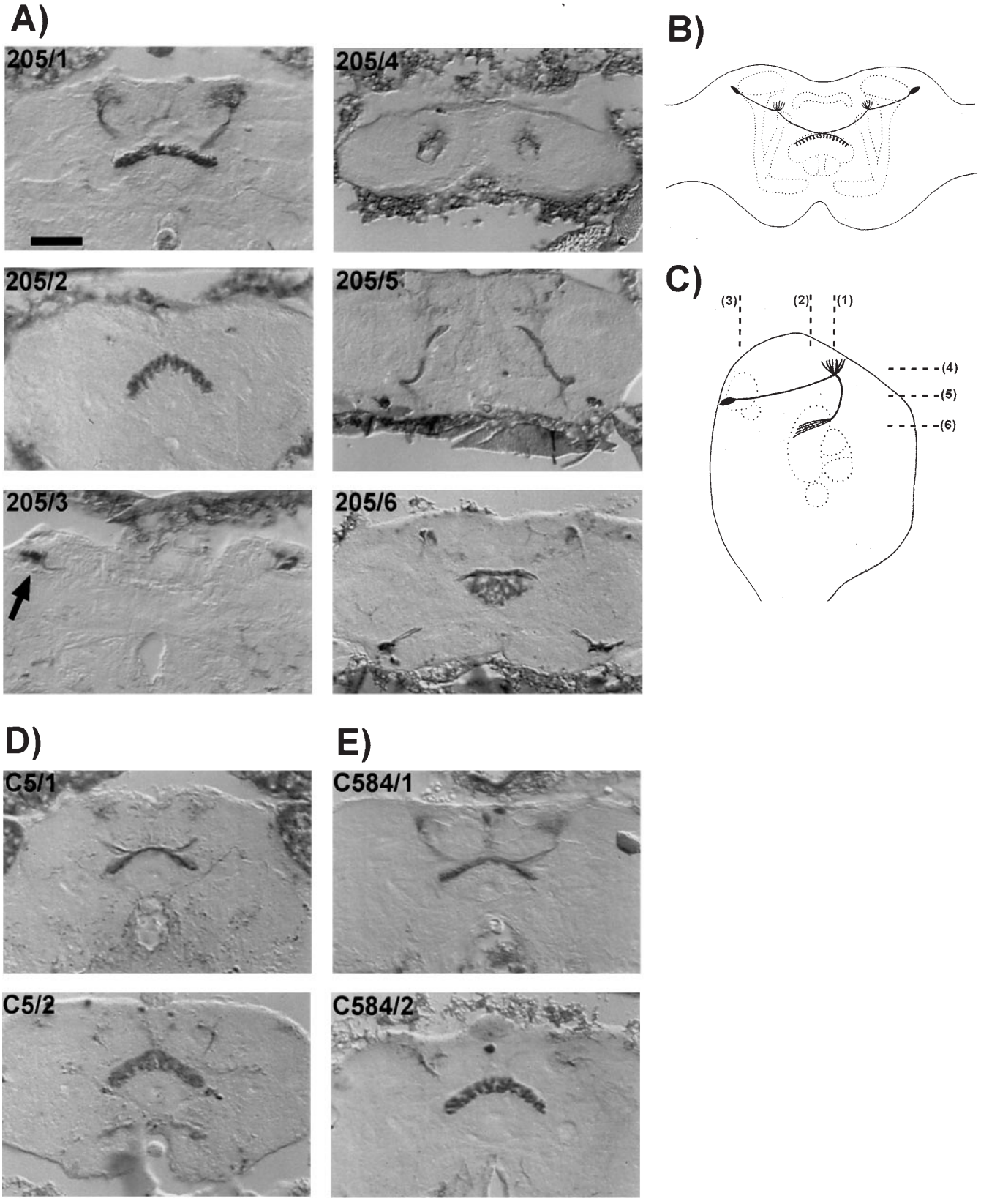
Fig. 2A–E Bout structure of locomotor activity is disturbed in three mutants affecting the pb. **A** Total walking activity for each group of mutant flies compared to the control, wild type Canton-S (*CS*). Total activity is represented by the mean (±SEM) of the total counts/fly during the recording period of 4 1/2 h. Number in each column indicates number of flies for the respective group. For each group, a one-way analysis of variance (ANOVA) was performed. All groups are compared to Canton-S. To reveal significant differences, the *P*-value is represented by one ($0.05 > P < 0.001$) or two ($P < 0.001$) *asterisks*. **B** Time-course of walking activity for each group of flies. Same flies as in **A**. Curves represent the mean (±SEM) of total counts for successive 10-min periods. See legend in graph. **C–E** Three calculated parameters of the intrinsic bout structure. As expected for time series events, data are not normally distributed but have a negative exponential distribution. Therefore, for statistical analysis the data were transformed to log-scale and the means and standard errors of the means, weighted to the number of observation, were calculated. For each group, a one-way ANOVA was performed. All groups were compared to WT CS flies [the *P*-value is represented by one ($0.05 > P < 0.001$) or two ($P < 0.001$) *asterisks*]. For presentation, the data are re-transformed to normal values. Note that the SEM is not symmetrically spread on both side of the mean. However, as the differences are too small to be visible in the figure only positive SEMs are shown. Data are the same as in **A**. **C** duration of bouts; **D** duration of pauses; **E** duration of periods. Note, that due to the transformation and re-transformation, periods are not the algebraic sum of bouts and pauses

increasing ICI duration one observes in wild-type flies a linear relation over one to two decades suggesting a fractal structure (Martin et al. 1999). This linearity implies a strict relationship between the frequencies of ICIs of different duration (and hence of longer and shorter rest periods). Interestingly, although the total activity of the three kinds of mutant flies was severely decreased, the log-log plot still showed a straight line for each of them (Fig. 7A). The fact that the three mutants kept this putative fractal structure in their temporal pattern of locomotor activity shows that the level of activity as expressed in the duration of bouts can be changed without destroying the particular frequency distribution of ICIs observed in fractality. Moreover, the pb seems not to be involved in generating this distribution.

Blocking large-field tangential neurons of the fan-shaped body decreases locomotor activity

According to anatomical data (Hanesch et al. 1989), the CC is a three dimensional matrix of small-field columnar elements and large-field tangential neurons forming strata perpendicular to the columns. At least two sets of columnar fibers, the vertical (VFS) and the horizontal fiber system (HFS) have been well identified (Hanesch et al. 1989). They project from the pb through the fb where they may contact tangential neurons, and terminate in the noduli (VFS) or the ventral body (HFS), one of the two accessory areas and the main output region of the CC. Large-field neurons in the fb, the pb (see above the transverse neurons connecting the 16 glomeruli) and the eb (ring neurons) are thought to provide the main input to the CC (Hanesch et al. 1989). The tangential neurons of the fb with their conspicuous stratified arborization in this neuropil, are a heterogeneous population. Some of them have been shown to contain neuropeptides or biogenic amines in *Drosophila* (Hanesch et al. 1989; Nässel and Elekes 1992) as well as in others insects (Vitzthum et al. 1996; Wegerhoff et al. 1996; Vitzthum and Homberg 1998; and for review see Homberg 1994). A full account of this type of neuron is still required. We have studied three enhancer trap lines (P[GAL4] 205; P[GAL4] C5; P[GAL4] C584) driving GAL4 expression in a new subset of large-field tangential neurons and have blocked these neurons using the tetanus toxin transgene.

The expression patterns of the three lines are depicted in Fig. 3. As they share a characteristic small cluster of large-field tangential neurons, one line, P[GAL4] 205 is described in detail. The cell bodies of the tangential neurons, about five or six on either side of the brain, are located in the dorso-caudal cellular cortex just lateral to the calyx of the MB (Fig. 3). The axons run in antero-medial direction horizontally or slightly upward, and


A)
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C584/2

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Fig. 3 Cryostat sections (10 μm) of the adult brain of three groups of P[GAL4]/Cnt-E flies expressing the tetanus toxin in the large-field tangential neurons of the fan-shape body (**A**, **D**, **E**). Tetanus toxin expression is revealed by anti-tetanus toxin antibody. The expression pattern correlates well with that of other reporter genes (e.g. β-galactosidase; data not shown). **B**, **C** Schematic drawings of frontal (**B**) and sagittal (**C**) projections of the tangential neurons, with dotted outlines of the central complex (CC) and MBs. In **C** the six planes of section (*dotted lines*) of Fig. 3A are indicated. **A** Line P[GAL4] 205. On the left (1–3) frontal sections of the same brain, from anterior to posterior, are shown. In (1) the dorsal arborization, the descending axons toward the fan-shaped body and the frontal margin of the layer in the fan-shaped body (fb) can be recognized. Section (2) shows the same tangential layer more caudally in the fb. In (3), lateral to the calyces of the MBs, the bilateral clusters of five to six cell bodies (*arrow*) of the tangential neurons are found. On the right (*4–6*): horizontal sections from dorsal to ventral, rostral on top (for position of corresponding planes of section see **C**. In (4) the dorsal arborization, in (5) the almost horizontal projections of the neurites are shown. Section (6): the dorsal-most part of the tangential layer in the fb and the two cell body clusters. **D** Line P[GAL4] C5. Two frontal sections (1, 2) corresponding to the planes of section (1) and (2) of **C**. The two sections reveal labeled neurons with similar characteristics as the corresponding neurons in P[GAL4] 205. Differences are most likely due to slightly different inclination of plane of section. **E** Line P[GAL4] C584. Two frontal sections (1, 2) corresponding to the planes of section of **C**. The labeled neurons again resemble those of lines P[GAL4] 205 et P[GAL4] C5. Scale bar = 50 μm

form a spiny lobe-like arborization in the rostro-dorsal protocerebrum, in the vicinity of the tip of the α′-lobe of the MB (Fig. 3). At this point, axons turn sharply downward and run medio-ventrally toward the fb (Fig. 3). They enter the fb from the front and spread in a quasi-horizontal stratum (probably layer 2 of Hanesch et al. 1989), with the lateral parts slightly bent downward. The arborizations show a weak tendency to segregate into the eight segments of the fb where they form bleb-like (presumably presynaptic) terminals.

The new group of neurons resembles the Fm1-neurons of Hanesch et al. (1989, see Fig. 2C), excepted that they do not enter the fb through the ellipsoid body canal. Whether this difference constitutes variability between wild-type lines or whether these are, indeed, different subsets of cells remains open. Figures 3B and 3C show, respectively, a schematic frontal and sagittal view of the projection of these neurons. The neurons labeled in the lines P[GAL4] C5 (Fig. 3D) and P[GAL4] C584 (Fig. 3E) exhibit similar projection patterns suggesting that they belong to the same cluster of neurons. However, in each of them, probably different subsets of neurons are labeled, as they differ in the density of spiny arborizations in the dorsal protocerebrum. Moreover, the three lines differ by their staining intensity and pattern outside the fb. The line P[GAL4] 205 shows staining in the lamina of the optic lobe, in the ocelli and in the antennal nerve. The line P[GAL4] C584 also exhibits a staining in the antennal nerve, in a few neurons of the antennal lobe and a faint staining at the periphery of the anterior part of the ellipsoid body. In addition, three or four neurons of the pars intercerebralis, with projection in the anterior median bundle are stained. The line P[GAL4] C5 exhibits a faint staining in the MBs. The three lines are independent isolates and have their P-element insertions at different positions in the genome.

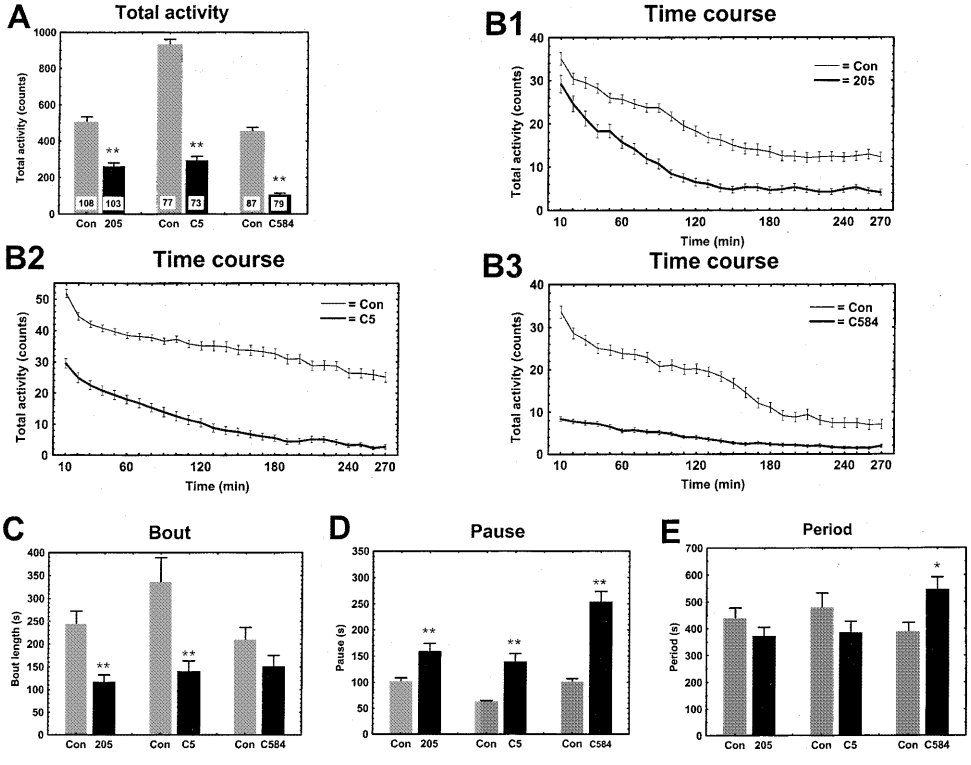

Fig. 4A–E Bout structure of spontaneous locomotor activity of the three lines in which similar sets of large-field tangential neurons in the fb are blocked. **A** Total walking activity for each group of flies. *Con* Control = respective P[GAL4]/CS flies. Data processing and statistics are explained in legend to Fig. 2

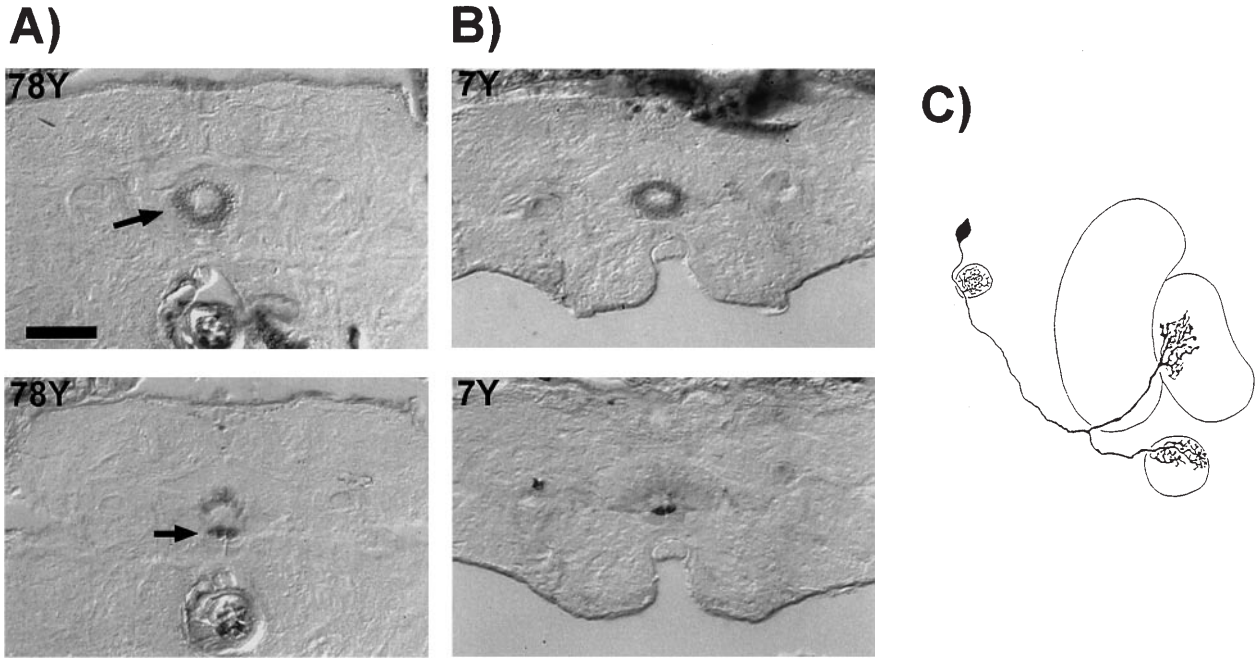

Fig. 5A–C Two frontal cryostat sections (10 μm) of the adult brain of the two groups of the P[GAL4]/Cnt-E flies expressing the tetanus toxin in pb-eb-no neurons. The tetanus toxin expression is revealed by anti-tetanus toxin antibody. Expression pattern is largely the same as that obtained with β-galactosidase as reporter (data not shown). **A** P[GAL4] 78Y line. Section (1) reveals staining in the ellipsoid body (*arrow*), section (2) shows staining in the upper part of the noduli (*arrow*). **B** P[GAL4] 7Y line. Same planes of section as in **A**. **C** Camera lucida drawing of a sagittal view of a pb-eb-no neuron (adapted from Hanesch et al. 1989; Fig. 12d). A complete set of pb-eb-no neurons is stained in lines P[GAL4] 78Y and P[GAL4] 7Y. Note that Hanesch et al. (1989) suggested but did not demonstrate that these neurons constitute a complete set. Interestingly, the neurons of this set superimpose their arborizations in the ipsilateral nodulus but juxtapose their arborizations in the pb and ellipsoid body (eb). Scale bar = 50 μm

With the tetanus toxin transgene expressed in the labeled neurons, all three lines exhibited a pronounced reduction of total locomotor activity, compared with their appropriate controls (Fig. 4A). Note that different controls were used for the three groups. The respective P[GAL4]/Cnt-E flies were compared with controls that were heterozygous for the same P[GAL4] transposon. Apparently, for P[GAL4] C5/CS flies, the insertion of the transposon itself caused a substantial increase in locomotor activity (Fig. 4A). As revealed by the time-course of activity, in the three groups of flies the reduction of activity occurred throughout the recording time (Fig. 4B1–3). Determination of the intrinsic bout structure showed that the decrease of locomotor activity was due to a shortening of the bouts as had been observed for the pb-disturbed flies. For P[GAL4] C205 and P[GAL4] C5 the duration of bouts was shorter, the pause between bouts longer, while the period stayed constant (Fig. 4C–E). Interestingly, although the total activity of the P[GAL4] C5/CS control flies was increased, blocking these neurons led to a level of activity similar to the one of the P[GAL4] 205/Cnt-E flies, as if the transposon in P[GAL4] C5/CS flies would stimulate the activity of these neurons.

Although the bout structure in P[GAL4] C584/Cnt-E flies seemed to be modified in the same direction as in the other two lines the changes were less clear-cut and the extremely low activity exhibited by these flies required further explanation. It could be due to the particular subset of tangential neurons in the fb expressing the transgene, or to tetanus toxin expression in some other parts of the brain, such as the neurons in the pars intercerebralis and median bundle.

Nevertheless, in three independent P[GAL4] enhancer trap lines in which the only common neurons expressing the transgene are the tangential neurons of the fb, blocking the marked neurons led to a common phenotype. Moreover, this phenotype was similar to the phenotype generated by the three mutations affecting the pb. Finally, as for the pb-disturbed flies, blocking the tangential neurons of the fb also seemed not to affect the fine tuning of the temporal pattern of locomotor activity. For P[GAL4] C205 and P[GAL4] C5 the log-log plots of the cumulative frequencies of the ICIs as function of ICI duration showed extensive linear domains, suggesting that the putative fractal organization is preserved (Fig. 7B–C) . The finding that in the P[GAL4] C584/Cnt-E flies linearity of the log-log plot was lost (Fig. 7D), will be discussed below.

Sets of columnar neurons in the central complex participate in the control of locomotor activity

Two further lines with specific expression in CC neurons were included in this study. In the P[GAL4] 78Y line,

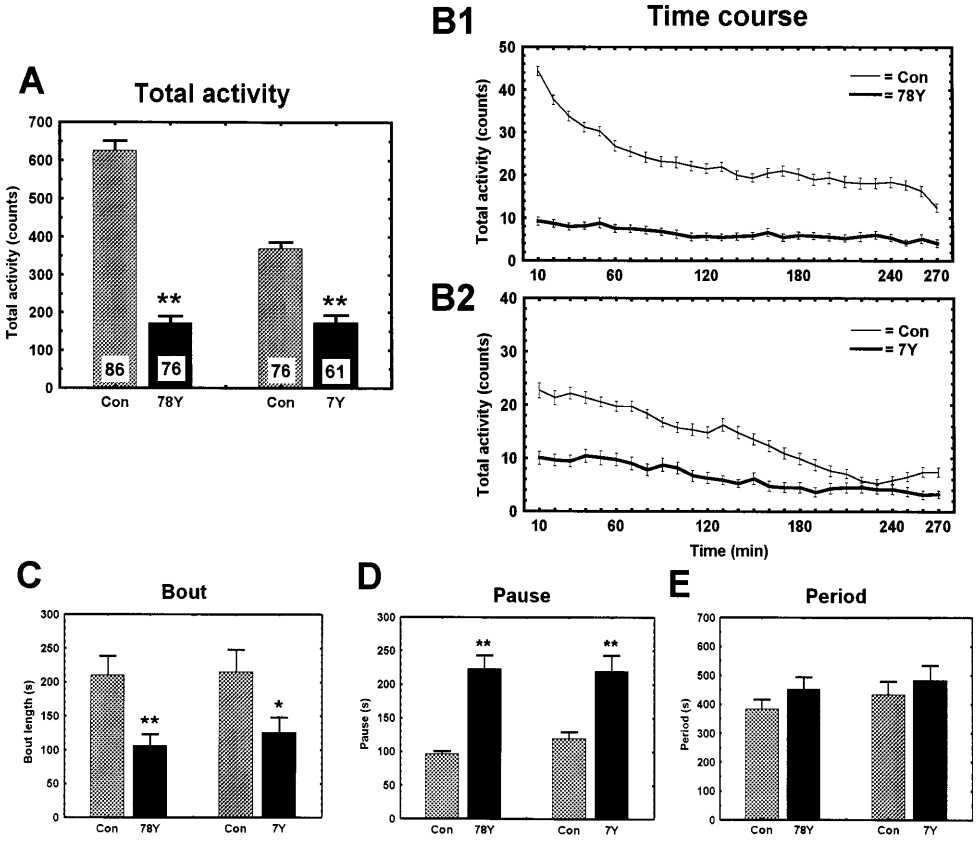

Fig. 6A–E Bout structure of locomotor activity of the two P[GAL4] lines with blocked pb-eb-no neurons. **A** Total walking activity for each group of flies. *Con* Control = respective P[GAL4]/CS flies. For data presentation and statistical treatment in **A–E** see legend to Fig. 2

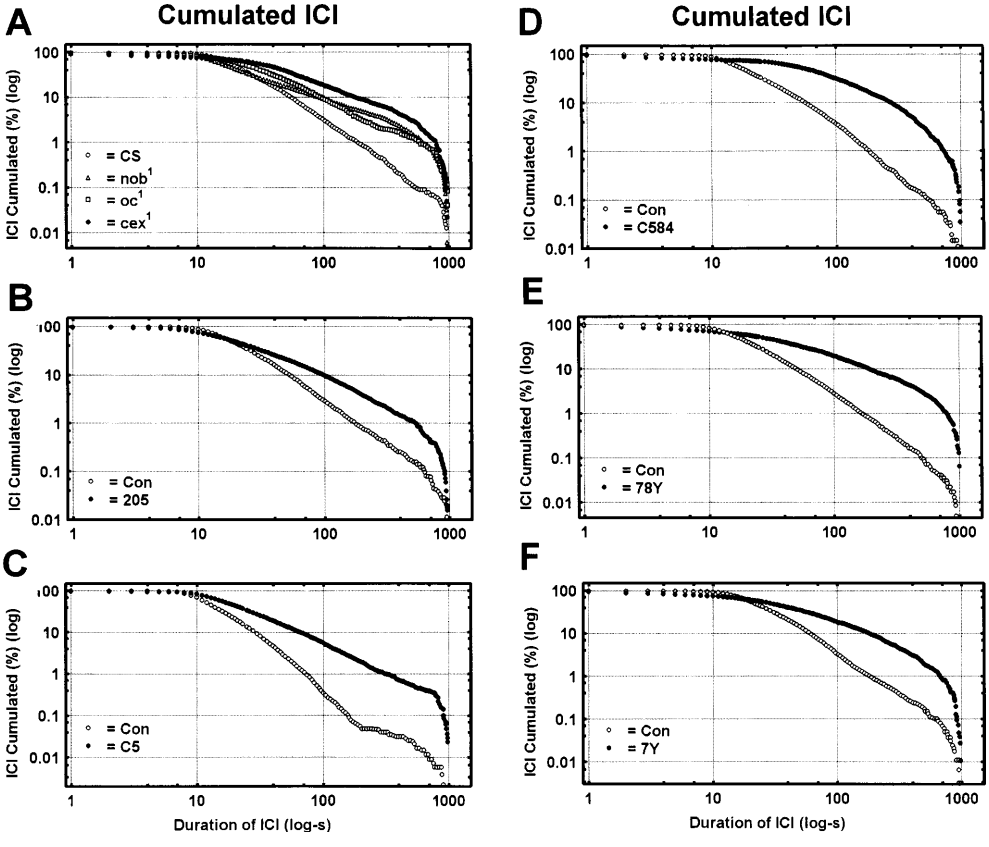

Fig. 7A–F Log-log plots of mean cumulative frequencies of inter-count intervals (ICIs) as functions of ICI duration. Same raw data as used in previous figures. Genotypes of flies and names of GAL4 lines are indicated in each graph. *CS* Canton-S; *Con* controls (P[GAL4]/CS). For CS flies in **A**, and control flies in **B**, **C**, **D**, **E**, and **F** functions are linear for more than one decade of ICI durations (with a regression coefficient $r^2 > 0.99$) (Coughlin et al. 1992; Martin et al. 1999). Linearity is destroyed in the lines P[GAL4] C584/Cnt-E (**D**), P[GAL4] 78Y/Cnt-E (**E**) and P[GAL4] 7Y/Cnt-E (**F**), i.e. in the lines expressing tetanus-toxin in the ellipsoid-body

transgene expression was observed in the posterior part of the ellipsoid body and in the noduli (Fig. 5). A faint staining was also detected in the protocerebral bridge. The cell bodies of these neurons lie in the pars intercerebralis; there are at least four of them on each side, possibly eight. They project first to the pb where they arborize in one or two glomeruli. From there the fibers run ventrally along the caudal surface of the fb, grow underneath it and arborize in the eb (Fig. 5A, top). At the most ventral point, the fibers send a side branch to the dorsal or dorso-rostral half of the ipsilateral nodulus where they form a very dense arborization (Fig. 5A, bottom). The projection pattern of these neurons (Fig. 5C) strongly suggests that they could correspond to the pb-eb-no neurons described by Hanesch et al. (1989, see Fig. 12d). The P[GAL4] 7Y line exhibits an expression pattern similar to the P[GAL4] 78Y line. The line P[GAL4] 78Y revealed no staining outside the CC. The P[GAL4] 7Y line, in contrast, shows staining in a few other neurons: two neurons sending projections into the anterior and medial protocerebrum, with cell bodies located in the lateral protocerebrum, posterior to the cluster of ring-neurons; and two or three neurons located in the pars intercerebralis with a projection in the median bundle.

Blocking the pb-eb-no neurons had a strikingly similar effect on locomotor activity, both in the total activity (Fig. 6A) and time-course (Fig. 6B), as destroying the bridge or blocking the tangential neurons of the fb. This impression was reinforced by the analysis of the bout structure. As for the previous groups of flies, the number of bouts was roughly the same between the P[GAL4]/Cnt-E flies and their controls. The decrease of activity occurred within the bouts (Fig. 6C–E). Their duration was cut by half, while the pauses between bouts more than doubled, keeping once again the period constant. As with the defects in the pb and fb, the number of bouts was not affected suggesting that the initiation of activity was normal, and that their decrease was due to a perturbation in the maintenance of the activity.

One striking difference between the previous groups of flies and the two lines expressing the toxin in the pb-eb-no neurons was the frequency distribution of the ICIs. In both cases the log-log plot had no linear part, suggesting that the temporal pattern had lost its fractal structure (Fig. 7E–F). Interestingly, preliminary data show that two other P[GAL4] enhancer trap lines with tetanus toxin expression restricted to the ellipsoid body have also lost the fractal structure of their locomotor activity (J.R. Martin, unpublished observations). In these two lines the overall level of locomotor activity and bout duration are in a normal range suggesting that the fine tuning of the temporal pattern of locomotor activity may be specially sensitive to disturbances in the ellipsoid body. This assumption also could explain the loss of fractality in the P[GAL4] C584/Cnt-E flies (Fig. 7D). As described above, they have a weak expression of toxin in unidentified neurons of the eb.

Discussion

Previous studies have shown that the integrity of the pb is essential for the fine tuning of leg coordination during walking and also for a high level of behavioral activity. Quantifying the temporal pattern of spontaneous walking activity (Martin et al. 1999) we show here that eight genetically distinct *Drosophila* lines have as a common behavioral mutant phenotype drastically shortened episodes of high readiness for locomotion (restlessness). In all eight cases the defect can be traced to neuronal substructures in the same region of the CC. We therefore propose that these substructures are part of a neural circuit which is required for the maintenance of these episodes. In the present study, two elements of this circuit have been identified, a set of columnar neurons linking the pb, eb and no (pb-eb-no neurons), and a small cluster of large-field tangential neurons which form a narrow stratum in the upper part of the fb and have a second field of arborizations outside the CC, in the vicinity of the α'-lobe of the MB. Based on the morphology of the terminal arborizations, the putative input-output properties of these neurons can be assessed (Hanesch et al. 1989). On this basis, the fb tangential neurons could receive input in the region of the MB, possibly from MB output neurons (Ito et al. 1998) and be presynaptic in the fb. The columnar neurons have spiny (postsynaptic) endings in the pb and may be pre- as well as postsynaptic in the eb and no. The connectivity can now be worked out more definitively using the novel *UAS-neuronal synaptobrevin-green fluorescent protein* (*n-syb-GFP*) construct as a reporter (Ito et al. 1998). If the two types of neurons described here indeed form a common circuit, columnar neurons linking the fb tangential neurons to the pb, as well as neurons downstream of the pb-eb-no neurons need to be identified. Conceivably, maintaining a high readiness for locomotion requires persistent cycling of neuronal activity. A more complete structural analysis of the circuit postulated here may eventually reveal recurrent connections within the CC or involving other parts of the central brain.

Our behavioral paradigm differs from previous ones (e.g. Strauss and Heisenberg 1993) in that flies are left completely undisturbed for 4 1/2 h, with a minimum of sensory stimulation (no light, no wind, continuous background odour, no or little sounds, no food, constant temperature, humidity, etc.). In this way, the endogenous pattern of locomotor activity should be more apparent than in the presence of additional uncontrolled stimuli or during shorter experiments in which the reactive component due to setting up the measuring situation still prevails.

Three quantifiable temporal patterns are extracted from the time traces. One is the time-course of mean locomotor activity, the second its clustering (bout structure), and the third is the log-log plot, which suggest a fractal organization in the frequency distribution of ICIs. Only the bout structure is affected consistently

in all eight lines. The initial decline of activity reflecting a waning response to the installation of the fly in the chamber is still observable in six or seven of the lines. The putative fractal structure, suggested by the linearity of the log-log plot is preserved in five of them. We presently pursue the hypothesis that defects in the eb are responsible for disturbing fractality (see Results). If this is true one has to assume that in the pb mutants the pb-eb-no neurons are still present although, at least in oc^1 their input region in the pb must be missing, suggesting that it is only the connection between the no and eb that is required for fractality. The presence and structure of the pb-eb-no neurons in oc^1 and other mutants need to be studied. Also, we do not know for any of the P[GAL4] lines where in the central nervous system (CNS) tetanus toxin is expressed during earlier stages of the life cycle, possibly leaving subtle developmental defects behind. However, while these open questions may be very important for a better understanding of the relation between CC structures and the control of walking, they do not weaken our main conclusion that a specific circuitry of tangential and columnar neurons in the pb and fb is required for the maintenance of high walking motivation. This is based on the finding that eight independent genetic lines with defects in a common target area of the CC share a specific behavioral impairment.

The bout structure is a second-order pattern. The primary pattern is that of the phases of uninterrupted walking and rest. With the present apparatus we cannot reliably measure this first level since slow uninterrupted walks are indistinguishable from walks with short rest periods. In a new version of the apparatus (in which the light gate is replaced by a bar code reader) the position of the fly in the chamber can be recorded continuously. It will be important to directly measure the alternations between walking and rest as well as the clustering of the walking phases. This will allow us to determine, for instance, whether within bouts longer and shorter rest periods are randomly distributed or whether these get longer towards the end of the bout.

The CC-defective lines have a remarkably well-preserved overall bout structure. Their minimum pause is about as long and as easily scorable as in the controls. The number of bouts and the counts per time during bouts are also normal. All these properties are not dependent upon the special CC substructure described above. We were specially interested in the initiation of bouts as well as walking phases. The endogenous initiation of behavior patterns and of motivational states seems to be of central importance for an animal. This property, however, is surprisingly robust. In none of our genetically manipulated lines, so far, is it significantly affected.

The only parameter of the bout structure we have been able to systematically alter, either by mutations or targeted toxin expression, is the duration of bouts (and pauses). Moreover, in a previous study in which we blocked or eliminated the MBs it was also the duration of bouts which was changed but in the opposite direction. In MB-defective flies bouts lasted longer and pauses correspondingly shorter. Thus, the antagonism of the MBs and CC in the control of behavioral activity postulated by Huber and coworkers in the 1950s and 60s (see Introduction for references) does in fact exist for locomotion in *Drosophila* but, surprisingly, the only property in which this antagonism is exerted is bout duration. A specific neuronal circuit comprising parts of the MBs and CC may underlie this control. The fb tangential neurons which arborize in a MB output region, might link these structures.

The above experiments show that blocking small numbers of identified neurons in the central brain of *Drosophila* can lead to well-defined behavioral alterations. We provide the first preliminary evidence that a putative central brain circuit involving parts of the CC and MBs controls one particular property of the temporal pattern of endogenous locomotor activity. This property can best be described as the maintenance of a state of high probability of walking (restlessness).

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