A protein related to p21-activated kinase (PAK) that is involved in neurogenesis in the *Drosophila* adult central nervous system

Jörg Melzig*, Karl-Heinz Rein*, Ulrich Schäfer†, Heiko Pfister*, Herbert Jäckle†, Martin Heisenberg* and Thomas Raabe*

Brains are organized by the developmental processes generating them. The embryonic neurogenic phase of *Drosophila melanogaster* has been studied in detail at the genetic, cellular and molecular level [1–3]. In contrast, much of what is known of postembryonic brain development has been gathered by neuroanatomical and gene expression studies. The molecular mechanisms underlying cellular diversity and structural organisation in the adult brain, such as the establishment of the correct neuroblast number, the spatial and temporal control of neuroblast proliferation, cell fate determination, and the generation of the precise pattern of neuronal connectivity, are largely unknown. In a screen for viable mutations affecting adult central brain structures, we isolated the *mushroom bodies tiny* (*mbt*) gene of *Drosophila*, which encodes a protein related to p21-activated kinase (PAK). We show that mutations in *mbt* primarily interfere with the generation or survival of the intrinsic cells (Kenyon cells) of the mushroom body, a paired neuropil structure in the adult brain involved in learning and memory [4–6].

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Results and discusssion

Identification of *mbt* mutations

A collection of 2000 viable P-element insertion lines on the X chromosome were established and histologically screened for obvious adult brain abnormalities (see Supplementary material published with this paper on the internet). Two non-complementing mutant lines were isolated that show a prominent reduction in the mushroom body neuropil size in the hemizygous or homozygous state (Figure 1b–g) and were therefore named *mushroom bodies tiny* (mbt^{P1} and mbt^{P2}). In *mbt* mutant flies, the calyx (Figure 1c), the peduncle (Figure 1e) and the lobe system (Figure 1g) of the mushroom bodies are reduced. The Kenyon cell body layer above and behind the calyces is thinner than in wild-type flies. The subdivision of the lobe system is still maintained, however, and no Kenyon cell fiber misrouting is observed (see also later results). Other neuropil structures in *mbt* central brains are also reduced in size, albeit to a much lesser degree (see Supplementary material). Additionally, *mbt* flies have a rough eye phenotype and bristle malformations.

Molecular characterization of *mbt*

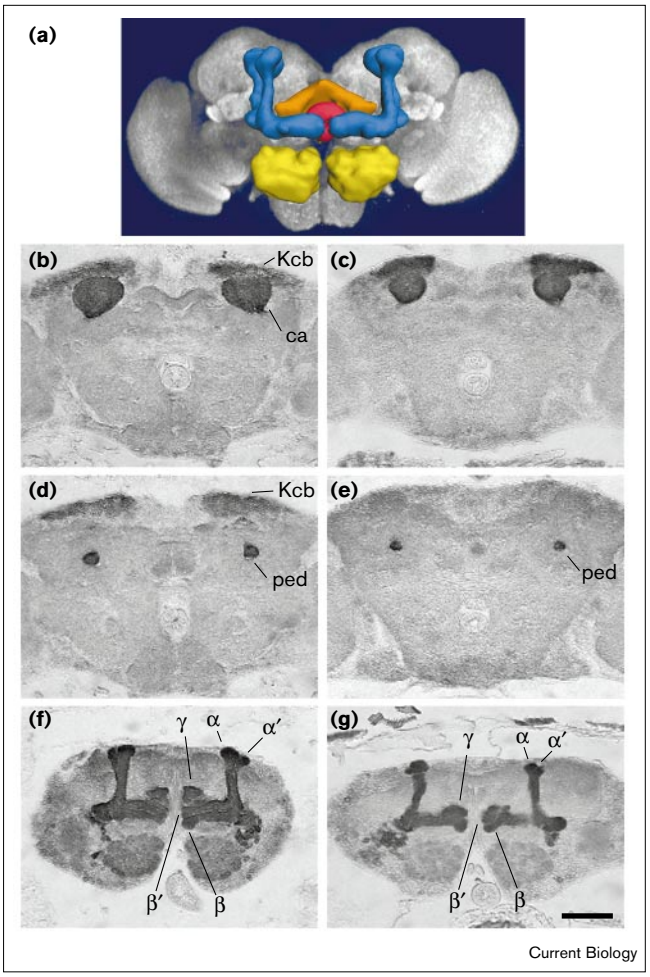
Both *mbt* alleles contain a P-element insertion at cytological position 14E on the X chromosome. Genomic fragments flanking the *mbt* P-insertion sites were recovered and used to identify cDNA clones. All cDNAs belonged to the same transcription unit (Figure 2a). The longest *mbt* cDNA (3262 bp, EMBL accession number AJ011578) contained a single open-reading frame of 1917 bp with in-frame stop codons preceding the first initiation codon at position 457, potentially encoding a 639 amino acid polypeptide with a predicted molecular weight of 69 kDa (see Supplementary material)

Several lines of evidence suggest that the observed mutant phenotypes are due to mutations in the isolated gene. First, the P-insertion sites of mbt^{P1} and mbt^{P2} were mapped to the first exon within the *mbt* coding sequence (Figure 2a). Second, northern blot analyses revealed a single 3.3 kb transcript that is expressed in wild-type animals at different developmental stages but is absent in mbt^{P1} and altered in mbt^{P2} (see Supplementary material). Third, the *mbt* mutant phenotypes could be reverted by precise excision of the P elements. New *mbt* alleles created by imprecise excision of the mbt^{P1} and mbt^{P2} P elements did not show an enhanced mushroom body or eye phenotype when transheterozygous with mbt^{P1}. From these data we conclude that mbt^{P1} represents a loss-of-function allele. The synthesis of an aberrant transcript could explain the slightly weaker mushroom body phenotype observed in mbt^{P2} (see Supplementary material). Finally, expression of the *mbt* cDNA in transgenic flies during mushroom body development using the Gal4/UAS system [7] rescued the mbt^{P1} and mbt^{P2} mutant phenotypes (see Supplementary material).

The *mbt* gene encodes a PAK-related protein

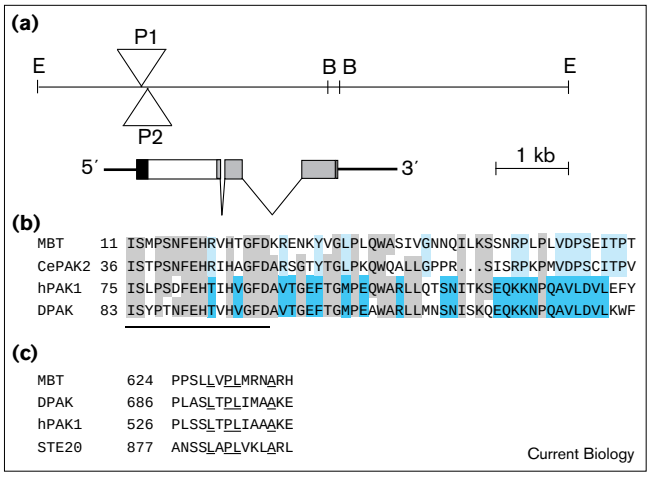
The MBT protein sequence revealed significant homology with the PAK family of serine/threonine kinases [8].

Figure 1



A genetic screen for structural brain mutants. **(a)** Surface rendering of prominent neuropil structures in the adult *Drosophila* central brain after neuropil staining and three-dimensional reconstruction of whole-mount preparations in front of a maximum intensity projection that outlines the whole brain neuropil. Antennal lobes are shown in yellow; ellipsoid body, red; fan-shaped body, orange; mushroom bodies, blue. The noduli are not visible in this frontal view and are located behind the ellipsoid body. **(b–g)** Frontal sections (7 µm) from paraffin-embedded heads of (b,d,f) wild-type and (c,e,g) mbt^{P1} adult brains at the level of the (b,c) calyces, (d,e) peduncle and (f,g) lobe system stained with an antibody against the 14-3-3 protein which is preferentially expressed in the mushroom bodies and the ellipsoid body [19]. The Kenyon cell bodies (Kcbs) of the mushroom bodies are located in the dorsal–posterior cortex. Kenyon cell dendrites and extrinsic input fibers constitute the calyx (ca). The Kenyon cell fibers form the peduncle (ped) which projects anterior–ventrally where it divides into the dorsally projecting α and α′ lobes and the medially projecting β, β′ and γ lobes [6,20]. The *mbt* phenotype is characterized by a general reduction of the mushroom body neuropil and Kenyon cell body volumes; the overall architecture of the mushroom bodies is not disturbed, however. Scale bar represents 50 µm.

Figure 2



The *mbt* gene encodes a PAK-related protein. **(a)** Genomic organization of the *mbt* locus. The horizontal line represents genomic DNA. Restriction sites for *Bam*HI (B) and *Eco*RI (E) are indicated. A diagram of the *mbt* mRNA is shown below. Within the coding region (boxes), sequences encoding the amino-terminal p21-binding domain (PBD) and the carboxy-terminal G-protein β-subunit binding site are represented by black boxes, and the kinase domain is shown in grey. The P-element insertion sites for mbt^{P1} (P1) and mbt^{P2} (P2) are located within the first exon, 39 bp and 181 bp downstream of the predicted translation start site, respectively. **(b)** Alignment of the putative PBD of MBT with the corresponding binding domains of human PAK1 (EMBL accession number U51120) and *Drosophila* PAK (accession number U49446) as representative members of the PAK family and a *C. elegans* PAK-related protein (CePAK2) encoded by cosmid C45B11 (accession number Z74029). Highly conserved amino acids are indicated in grey, and amino acids conserved between MBT/CePAK2 and hPAK1/DPAK are shown in light blue and dark blue, respectively. The 16 amino acid core sequence of the PBD is underlined [8,12]. **(c)** Alignment of the G-protein β-subunit binding site of the *Saccharomyces cerevisiae* PAK homologue STE20 with the corresponding MBT, DPAK and hPAK1 sequences. A fragment containing residues 877–890 of STE20 is necessary and sufficient to bind the G-protein β-subunit STE4 [11] and contains a number of highly conserved residues (underlined).

Several distinct PAK proteins were identified in different vertebrate species including human, mouse and rat [9]. Thus far, only one *Drosophila* member of this family (DPAK) has been described [10]. PAK proteins contain an amino-terminal binding domain for the Rho subfamily of Ras-related small GTPases (p21 proteins), a carboxy-terminal kinase domain, and a heterotrimeric G-protein β-subunit binding site at the carboxyl terminus (Figure 2c) [11]. There are some differences between MBT and other PAK family members, however. The p21-binding domain of PAK is limited to 57 amino acids, with an amino-terminal 16 amino acid core critical for binding to the Rho-type GTPases Cdc42 and Rac *in vitro* [8,12]. The core sequence of the MBT p21-binding domain is well conserved with other PAK proteins, but the homology is less significant in the carboxy-terminal half of the complete putative MBT p21-binding domain (Figure 2b). Interestingly, a *Caenorhabditis elegans* PAK-related protein (referred to here as CePAK2), predicted from an open-reading frame analysis of cosmid C45B11, shares extensive

homology with the complete MBT p21-binding domain (Figure 2b). A second difference between the PAK proteins so far described and MBT or CePAK2 is that MBT and CePAK2 lack an amino-terminal proline-rich Src homology 3 (SH3) domain binding motif. In hPAK1, this sequence mediates binding to the SH3 domain of the NCK adaptor protein [13]. The structural features of MBT and CePAK2 indicate that these proteins may form a new subclass of the PAK family.

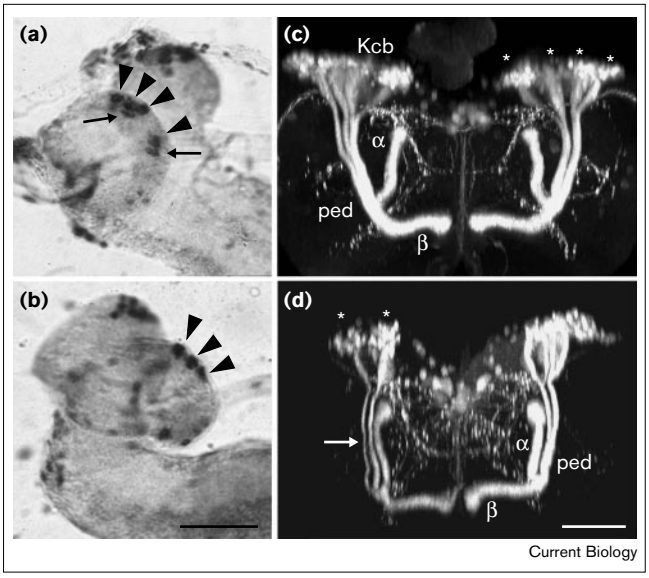
Phenotypic analysis of *mbt*

Although PAK proteins were discovered by their ability to bind Rac and Cdc42 [8], the biological function of PAK proteins in mediating GTPase functions such as cytoskeleton reorganization, neurite outgrowth, cell cycle progression and activation of the Jun N-terminal kinase (JNK) signaling cascade is not well understood [9,14,15]. The isolation of mutations in the *mbt* gene allowed us to investigate the putative role of this PAK-related protein *in vivo*. The dramatic reduction in mushroom body neuropil volume in *mbt* correlates with a reduction in the Kenyon cell body layer volume (see Supplementary material). Compared to wild-type flies, neither Kenyon cell body size nor packaging density are altered in *mbt* flies (8–10 Kenyon cell bodies per 100 μm^2). We concluded that the Kenyon cell number is reduced in *mbt* flies. Thus, the *mbt* phenotype could be caused by a loss of mushroom body neuroblasts, a defect in mushroom body neuroblast or ganglion mother cell proliferation, or increased cell death among Kenyon cells.

Labeling mitotically active mushroom body neuroblasts at early larval stages with bromodeoxyuridine (BrdU) [16] showed that most *mbt* larvae contained the wild-type number of four mushroom body neuroblasts in each brain hemisphere. Very few *mbt* larvae had a reduced number of labeled mushroom body neuroblasts (Figure 3a,b). In wild-type animals, the four mushroom body neuroblasts in each brain hemisphere proliferate throughout development and contribute equally to the entire adult mushroom body structure [16,17]. The resulting four-fold clustering within the cell body layer and the fiber projections can be visualized in three-dimensional reconstructions of Gal4 enhancer trap lines labeling subsets of Kenyon cells in each of the four cell clusters (Figure 3c) [17,18]. In most adult *mbt* brains, the four-fold clustering of the mushroom bodies was preserved whereas a dramatic decrease in the number of labeled cells in each cluster and in the fiber tract diameter was observed (Figure 3d). Occasionally, *mbt* mushroom bodies showed only two or three clusters indicating a loss of mushroom body neuroblasts or a complete failure of some neuroblasts to proliferate (Figure 3d). No Kenyon cell fiber misrouting was observed in *mbt* mutant flies, however.

In addition to the defects in the central brain, *mbt* flies have a rough eye phenotype. Tangential sections revealed that a variable number of photoreceptor cells in many ommatidia were missing (Figure 4a,b). At least in the case of the R7 and the R8 photoreceptor axons, the innervation pattern in the medulla appeared to be normal (Figure 4c,d).

Figure 3



Phenotypic analysis of *mbt*. **(a,b)** Dorso-lateral view of BrdU-labeled mushroom body neuroblasts [16] at 1–8 h after larval hatching. In (a) wild type, four neuroblasts (arrowheads) are visible; associated ganglion mother cells are occasionally also seen (arrows). In (b) mbt^{P1}, the number of labeled mushroom body neuroblasts is sometimes reduced. **(c,d)** Frontal view of three-dimensional reconstructions of adult (c) wild-type and (d) mbt^{P1} mushroom bodies. Gal4 line 17d labels a subset of cells in each of the four clonal units (asterisks) that form the inner part of the peduncle (ped) and the α/β lobes. The main defect in *mbt* is a marked decrease in mushroom body cell number. (d) Only small cell clusters (asterisks) and thin fiber bundles (arrow) can be detected. In addition, the four-fold symmetry in the left mushroom body in (d) is disturbed. Fibers that form the outer shell of the peduncle, the α′, β′ and γ lobes of the mushroom bodies [6,20], are present in *mbt* (Figure 1g), but are not labeled with line 17d. Kcb indicates Kenyon cell body; scale bars represent 50 μm.

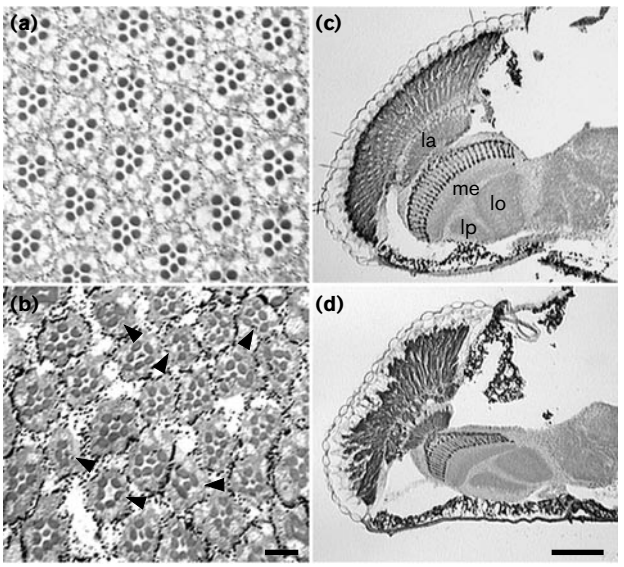
In summary, our experiments indicate that the MBT protein could be part of a more general mechanism regulating cell number in a variety of neuronal tissues. Utilizing both genetic approaches and *in vitro* assays will allow us to identify components of the MBT signaling pathway and to analyse their function during neuronal development *in vivo*.

Materials and methods

Confocal microscopy

For confocal images, intact adult brains from mbt^{P1} flies or control animals coexpressing the *UAS-lacZ* reporter construct [7] and mushroom-body-specific Gal4 enhancer trap lines [18] (H.P. and T.R., unpublished observations) were dissected and fixed for 1 h in 2% paraformaldehyde. A rabbit anti-β-galactosidase primary antibody

Figure 4



The *mbt* gene affects cell number but not axonal projections in the adult eye. Tangential sections of adult **(a)** wild-type and **(b)** mbt^{P1} eyes are shown. Each ommatidium comprises eight photoreceptor cells (R1–R8) and 12 accessory cells. (a) R1–R6 have large rhabdomeres, the central R7 rhabdomere is small. The rhabdomere of the R8 cell is not visible in this apical section. (b) In *mbt* mutant ommatidia, a variable number of photoreceptor cells are missing (arrowheads). Scale bar represents 10 μm. **(c,d)** Cryostat sections of adult wild-type and mbt^{P1} heads stained with the photoreceptor-cell-specific antibody nb181. In (c) wild type, axons from photoreceptors R1–R6 terminate in the lamina (la). Because of the high density of R cell fibers in the lamina, individual fibers cannot be distinguished. The axons from photoreceptor cells R7 and R8 terminate in distinct layers in the medulla (me). (d) Despite the frequent loss of photoreceptor cells in *mbt* mutant eyes, no misrouting of the R7 and R8 cell fibers in the medulla was observed. The label lo indicates lobula; lp, lobula plate; scale bar represents 50 μm.

(Cappel) and a Cy3-conjugated secondary antibody (Jackson Immuno Research) were used to reveal the expression of the reporter construct. Optical sections were recorded with a step size of 0.8 μm using a LeicaTCS laser confocal microscope. For three-dimensional reconstructions, the AMIRA program (Zuse Institut für Informationstechnologie) was used. The staining and data recording for Figure 1a essentially followed the same procedure with the exception that a neuropil-specific primary antibody was used.

Supplementary material

Four figures showing the effect of the mbt^{P1} mutation on different central brain structures, the MBT protein sequence, northern blot analysis and transgenic rescue of the *mbt* phenotype, and additional methodological details are published with this paper on the internet.

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References

1. Campos-Ortega CA: **Early neurogenesis in *Drosophila melanogaster.*** In *The Development of Drosophila melanogaster.* Edited by Bate M, Martinez-Arias A. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1993:1091-1129.
2. Goodman CS, Doe CQ: **Embryonic development of the *Drosophila* central nervous system.** In *The Development of Drosophila melanogaster.* Edited by Bate M, Martinez-Arias A. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1993:1131-1206.
3. Reichert H, Boyan G: **Building a brain: developmental insights in insects.** *Trends Neurosci* 1997, **20:**258-264.
4. Menzel R, Erber J, Masuhr T: **Learning and memory in the honeybee.** In *Experimental Analysis of Insect Behavior.* Edited by Brown LB. Berlin: Springer; 1974:195-217.
5. Davis RL, Han KA: **Mushrooming mushroom bodies.** *Curr Biol* 1996, **6:**146-148.
6. Heisenberg M: **What do the mushroom bodies do for the insect brain? An introduction.** *Learn Mem* 1998, **5:**1-10.
7. Brand AH, Perrimon N: **Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.** *Development* 1993, **118:**401-415.
8. Manser E, Leung T, Salihuddin H, Zhao Z, Lim L: **A brain serine/threonine protein kinase activated by Cdc42 and Rac1.** *Nature* 1994, **367:**40-46.
9. Sells MA, Chernoff J: **Emerging from the PAK: the p21-activated protein kinase family.** *Trends Cell Biol* 1997, **7:**162-167.
10. Harden N, Lee J, Loh H-J, Ong Y-M, Tan I, Leung T, *et al.*: **A *Drosophila* homolog of the Rac- and Cdc42-activated serine/threonine kinase PAK is a potential focal adhesion and focal complex protein that colocalizes with dynamic actin structures.** *Mol Cell Biol* 1996, **16:**1896-1908.
11. Leeuw T, Wu C, Schrag JD, Whiteway M, Thomas DY, Leberer E: **Interaction of a G-protein β-subunit with a conserved sequence in STE20/PAK family protein kinases.** *Nature* 1998, **391:**191-195.
12. Burbelo PD, Drechsel D, Hall A: **A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases.** *J Biol Chem* 1995, **270:**29071-29074.
13. Bokoch GM, Wang Y, Bohl BP, Sells MA, Quilliam LA, Knaus UG: **Interaction of the Nck adapter protein with p21-activated kinase (PAK1).** *J Biol Chem* 1996, **271:**25746-25749.
14. Van Aelst L, D'Souza-Schorey C: **Rho GTPases and signaling networks.** *Genes Dev* 1997, **11:**2295-2322.
15. Noselli S: **JNK signaling and morphogenesis in *Drosophila.*** *Trends Genet* 1998, **14:**33-38.
16. Ito K, Hotta Y: **Proliferation pattern of postembryonic neuroblasts in the brain of *Drosophila melanogaster.*** *Dev Biol* 1992, **149:**134-148.
17. Ito K, Awano W, Suzuki K, Hiromi Y, Yamamoto D: **The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells.** *Development* 1997, **124:**761-771.
18. Yang MJ, Armstrong JD, Vilinsky I, Strausfeld NJ, Kaiser K: **Subdivision of the *Drosophila* mushroom bodies by enhancer-trap expression patterns.** *Neuron* 1995, **15:**45-54.
19. Skoulakis EMC, Davis RL: **Olfactory learning deficits in mutants for leonardo, a *Drosophila* gene encoding a 14-3-3 protein.** *Neuron* 1996, **17:**931-944.
20. Crittenden JR, Skoulakis EMC, Han KA, Kalderon D, Davis RL: **Tripartite mushroom body architecture revealed by antigenic markers.** *Learn Mem* 1998, **5:**38-51.

Supplementary material

A protein related to p21-activated kinase (PAK) that is involved in neurogenesis in the *Drosophila* adult central nervous system

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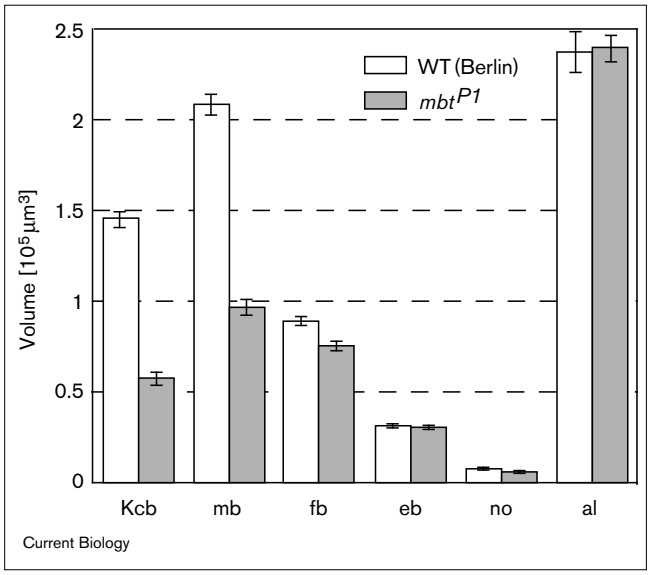
Figure S1



The *mbt* gene affects central brain structures to a different degree. Quantification of the volumes of neuropil structures in the central brain of wild-type (WT, Berlin) and *mbt*^{P1} flies. The mean neuropil volumes were calculated from at least nine individuals. In addition, the mean Kenyon cell body layer volume (Kcb, $n = 10$) was determined. Mushroom bodies are indicated by mb; fan-shaped body, fb; ellipsoid body, eb; noduli, no; antennal lobes, al.

Figure S2

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1    MFSKKKKKPL ISMPSNFEHR VHTGFDKREN KYVGLPLQWA SIVGNNQILK
51   SSNRPLPLVD PSEITPTEIL DLKTIVRPHH NNNKADTTSL NSSSTMMMGS
101  MAPMNPMAPG AHPMMSHGPG MMMPPETGGI VLPKTSHVAR SNSLRSSSPP
151  RVRRVANVPP SVPEEEGPPA AGTPGVGGAS SGGFKPPGAH PSLLYNSQHA
201  HANGATGPLA VRTDQTNLQQ YRSNLAPPSG GSMPQQQQTS PVGSVASGTR
251  SNHSHTNNGN SGGSYPPMYP TSHQQQQQQQ QQAKQGGDQN QNPLHPHAHP
301  HPHHHQHLAK SASRASSSSG GASSAAQQAS GASGGAAGQP KQDQRLTHEQ
351  FRAALQMVVS AGDPRENLDH FNKIGEGSTG TVCIATDKST GRQVAVKKMD
401  LRKQQRRELL FNEVVIMRDY HHPNIVETYS SFLVNDELWV VMEYLEGGAL
451  TDIVTHSRMD EEQIATVCKQ CLKALAYLHS QGVIHRDIKS DSILLAADGR
501  VKLSDFGFCA QVSQELPKRK SLVGTPYWMS PEVISRLPYG PEVDIWSLGI
551  MVIEMVDGEP PFFNEPPLQA MRRIRDMQPP NLKNAHKVSP RLQSFLDRML
601  VRDPAQRATA AELLAHPFLR QAG PPSLLVP LMRNARH HP*
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Predicted amino acid sequence of MBT (EMBL accession number AJ011578). The putative p21-binding domain is underlined with a solid line, the serine/threonine kinase domain is underlined with a broken line, and the sequence motif matching the binding site for the heterotrimeric G-protein β-subunit is shown in bold.

Supplementary materials and methods

Genetics

Fly cultures and crosses were performed according to standard procedures. Genetic markers and chromosomes have been described [S1]. All P-element insertion lines on the X chromosome (U.S. and H.J., unpublished observations) were derived from a parental strain which carried the P[lacW] element on the second chromosome. For mobilisation of the P[lacW] element, w/w; P[lacW]/P[lacW]; +/+ females were crossed to w/Y; Sp/CyO; P[ry$^+$ Δ2-3]/TM6 males. Males of the genotype w/Y; P[lacW]/ CyO; P[ry$^+$ Δ2-3]/+ were isolated and crossed to FM6/FM6 females. Single w/FM6; CyO/+; +/+ females with red pigmented eyes indicating a new P[lacW] insertion were crossed to FM7c/Y males. All X-chromosome insertions resulted in balanced stocks. Insertions on other chromosomes yielded also white-eyed flies in the progeny and were discarded. 2000 viable X-chromosome insertion lines were screened on histological sections for structural brain abnormalities compared to wild type (Berlin) as a reference strain. Four lines (*mbt*^{P1}, *mbt*^{P2} and two yet uncharacterized lines) showed changes in brain morphology. To test whether the P-element insertions were linked to the observed phenotypes, P-element excision lines were generated using the P[ry$^+$ Δ2-3] transposase. Precise excision of the *mbt*^{P1} and *mbt*^{P2} P element resulted in reversion of the mushroom body and eye phenotypes. Mutant lines generated by imprecise excision of the *mbt*^{P1} and *mbt*^{P2} P element did not show an enhancement of the mushroom body or eye phenotype in transheterozygous *mbt*^{P1} females. In addition, complementation tests between the *mbt* mutations and deficiencies created on the duplication Dp(1;4)r$^+$ f$^+$ [S2] verified the localization of *mbt* at cytological position 14E.

Transformant lines carrying the *UAS-mbt* construct were generated and mapped by standard procedures. To test whether the *UAS-mbt* transgene could rescue the defects of *mbt* flies, *mbt*$^{P1\ (P2)}$/*FM7*; *UAS-mbt*/+ animals were crossed to a collection of Gal4 enhancer trap lines that show different spatial-temporal expression patterns in the mushroom bodies ([18]; H.P. and T.R., unpublished observations). Gal4 line 238Y shows expression throughout mushroom body development [18,S3] and in the eye–antennal imaginal disc (T.R., unpublished observations) and in combination with different *UAS-mbt* transgenic lines rescued the mushroom body and eye defects of *mbt*^{P1} and *mbt*^{P2}.

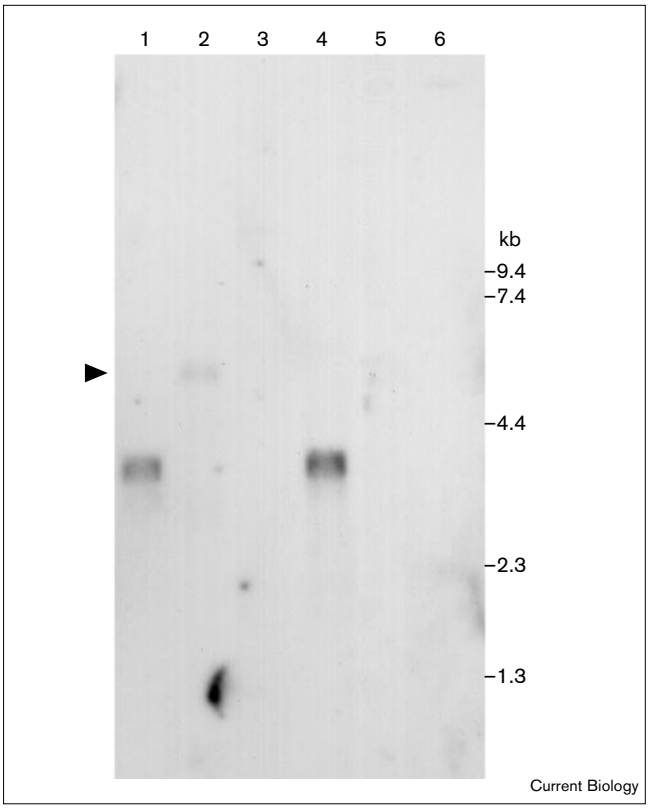
Histology

Mutant strains were isolated by mass histology [S4]. For each of the 2000 P-element lines, frontal sections (7 µm) of paraffin-embedded heads from three animals were analysed. The mushroom body neuropil and cell body volumes of *mbt* and wild-type flies were evaluated by planimetric measurements of paraffin sections stained with an anti-14-3-3 antibody [14]. The volumes of other neuropil structures were calculated from autofluorescent paraffin sections [S4]. Kenyon cell numbers were scored in randomly chosen 100 µm^2 areas of the cell body layer after staining with the anti-14-3-3 antibody. Immunostaining of frozen sections with monoclonal antibody nb181 [S5] was carried out as described previously [S6]. Semithin sections of adult *Drosophila* eyes were prepared as described previously [S7].

Molecular analysis

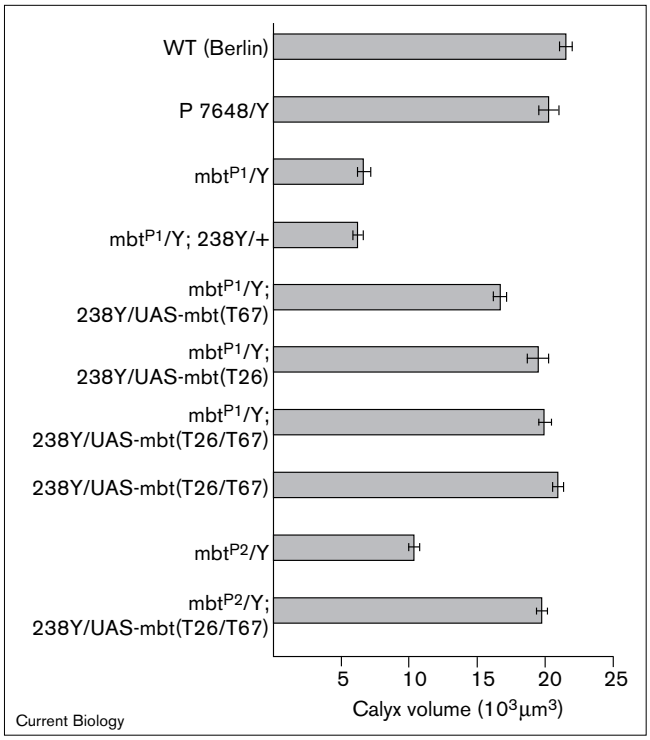
Northern blot analysis of poly(A$^+$) RNA (Pharmacia mRNA purification kit) and standard recombinant DNA procedures were performed as

Figure S3



Northern blot analysis. Poly(A+) RNA (2 μg each lane) isolated from embryos (lanes 1–3) and adults (lanes 4–6) probed with the *mbt* cDNA. In wild type, a single transcript of 3.3 kb is detected in embryos (lane 1) and adults (lane 4). In mbt^{P2}, a larger transcript (arrowhead) is seen in embryos (lane 2) but not in adults (lane 5). In mbt^{P1}, expression of the *mbt* transcript is eliminated (lanes 3,6).

Figure S4



Transgenic rescue of the *mbt* phenotype. Quantification of the calyx volumes of wild-type (WT) and mbt^{P1} males. The calyx volume in mbt^{P1} is reduced to a similar degree as the whole mushroom body neuropil (Figure S1). The calyx volume reduction in mbt^{P2} is weaker than in mbt^{P1} and could be explained by the synthesis of an aberrant transcript in mbt^{P2} (Figure S3). A randomly chosen X-chromosomal P-element insertion line (P7648) derived from the same screen was included as a further control to exclude the possibility that the observed phenotypes are due to the genetic background of the P-element strains. Expression of different *UAS:mbt* transgenes (T26 and T67 or a combination of both transgenes) with Gal4 line 238Y [18,S3] rescued the mbt^{P1} and mbt^{P2} mushroom body and eye (data not shown) defects. Expression of these transgenes with Gal4 line 238Y in a wild-type background causes no obvious phenotype in the mushroom bodies or the eye (data not shown).

described [S8]. Genomic fragments flanking the mbt^{P1} and mbt^{P2} P-element insertions sites were cloned by plasmid rescue and partially sequenced. The genomic sequences matched to several expressed sequence tag (EST) sequences (BDGP/HHMI *Drosophila* EST Project). Sequencing and restriction mapping of the corresponding cDNAs and of cDNAs isolated from an embryonic cDNA library (Stratagene) showed that all cDNAs had the same 3′ end but extended to different lengths in the 5′ direction. Sequencing of the longest cDNA was performed using the AmpliTaq DyeTerminator Sequencing kit (ABI). The genomic organisation was determined by sequencing the corresponding genomic fragments. DNA and protein databases were searched for homologous sequences using the BLAST program. The *UAS-mbt* rescue construct was produced by cloning the largest *mbt* cDNA as a *Not*I–*Asp*718 fragment into the pUAST vector [7].

References

S1. Lindsley D, Zimm G: *The Genome of Drosophila melanogaster.* San Diego: Academic Press; 1992.

S2. Falk DR, Roselli L, Curtiss S, Halladay D, Klufas C: **The characterization of chromosome breaks in *Drosophila melanogaster.* I. Mass isolation of deficiencies which have an endpoint in the 14A-15A region.** *Mutat Res* 1984, **126:**125-134.

S3. Tettamanti M, Armstrong JD, Endo K, Yang MY, Furukubo-Tokunaga K, Kaiser K, Reichert H: **Early development of the *Drosophila* mushroom bodies, brain centres for associative learning and memory.** *Dev Genes Evol* 1997, **207:**242 -252.

S4. Heisenberg M, Böhl K: **Isolation of anatomical brain mutants of *Drosophila* by histological means.** *Z Naturforsch* 1979, **34c:**143-147.

S5. Hofbauer A, Buchner E: **Does *Drosophila* have seven eyes?** *Naturwissenschaften* 1989, **76:**335-336.

S6. Buchner E, Buchner S, Crawford G, Mason WT, Salvaterra PM, Sattelle DB: **Choline acetyltransferase-like immunoreactivity in the brain of *Drosophila melanogaster.*** *Cell Tissue Res* 1986, **246:**57-62.

S7. Basler K, Christen B, Hafen E: **Ligand-independent activation of the sevenless receptor tyrosine kinase changes the fate of cells in the developing *Drosophila* eye.** *Cell* 1991, **64:**1069-1081.

S8. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laborotary Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1989.