Normal Function of the mushroom body defect Gene of Drosophila Is Required for the Regulation of the Number and Proliferation of Neuroblasts

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In the developing central nervous system of Drosophila, proliferation follows a reproducible and well-described spatial and temporal pattern. This pattern involves a defined number and distribution of neural stem cells (neuroblasts), as well as a precisely regulated time course of division of these neuroblasts. We show that mutations in the mushroom body defect (mud) gene interfere with the regulation of this pattern in a rather specific manner. In the abdominal neuromeres a subset of neuroblasts prolongs the period of proliferation. Additional daughter cells persist into the imaginal. Similar defects are expressed in the anterior ventral nerve cord and in the lateral central brain region. In the mushroom body cortex, however, mutations in mud affect the proliferation pattern by increasing the number of neuroblasts. These additional neuroblasts behave like normal mushroom body neuroblasts according to their time course of proliferation and the specification of their progeny.

INTRODUCTION

As shown for many different species, organogenesis requires a precisely regulated spatial and temporal pattern of cell proliferation (e.g., Sulston et al., 1983; Hartenstein and Campos-Ortega, 1985; Foe, 1989; for review see Gurdon, 1992). This is especially true for the most complex organ, the central nervous system (CNS); see Fig. 1 for a schematic presentation of the following description). During CNS development in Drosophila, proliferation starts at embryonic stage 8, when the first embryonic neuroblasts (eNB) delaminate from the ventral neurogenic region of the ectoderm (Poulson, 1950; Hartenstein and Campos-Ortega, 1984; Hartenstein et al., 1987; Doe et al., 1988, Doe, 1992). After their delamination eNB divide and give rise to a chain of smaller ganglion mother cells, which in turn divide into two postmitotic ganglion cells (Hartenstein et al., 1987). Following stage 12 the number of proliferating neuroblasts (NB) decreases (Prokop and Technau, 1991). In the late embryo (stage 17) and first larval instar, DNA replication can no longer be detected in the ventral nerve cord (vNC) (Truman and Bate, 1988; Prokop and Technau, 1991; Smith and Orr-Weaver, 1991). In the thorax most NB interrupt their proliferation for at least 35 hr (Truman, 1990; Prokop and Technau, 1991). About 30 hr after larval hatching (ALH) the first thoracic NB start replicating again and eventually a segmental pattern of 47 postembryonic NB (pNB) can be detected. Thoracic pNB divide until 20 to 30 hr after puparium formation (APF) (White and Kankel, 1978; Truman and Bate, 1988). In contrast to the thorax, the number of abdominal pNB is strongly reduced. Also the proliferation period is shorter than in other regions of the CNS, and the G2 following the first S-phase is extremely prolonged (Truman and Bate, 1988; Taylor and Truman, 1992).

The segregation of pNB from the procephalic lobe, which gives rise to the brain, is more complex. The number of these NB seems to increase by equal divisions of NB (Hartenstein and Campos-Ortega, 1984). Most of them stop dividing until the late stage 17 embryo. However, five NB (one lateral NB, L-NB; four mushroom body NB, MB-NB) have been described that maintain proliferation into postembryogenesis (Truman and Bate, 1988; Prokop and Technau, 1991). The other pNB in the larval brain start proliferation between 8 and 50 hr ALH and vanish about 20 to 30 hr APF (White and Kan-
Fig. 1. Schematic presentation of the BrdU experiments referred to in the text. The time scale indicates hours AEL and hours ALH, respectively. (A–K) Different experiments with varying times of BrdU application (hatched bars), of subsequent chase periods (dotted lines), and of fixation and staining for BrdU (arrows). Black bars below the time scale indicate the periods of DNA replication in different NB populations according to Hartenstein and Campos-Ortega (1984), Truman and Bate (1988), Prokop and Technau (1991), Ito and Hotta (1992), and Taylor and Truman (1992). The scheme at the bottom demonstrates the cellular events taking place, at least in the thoracic neuromeres: The embryonic NB segregate from the peripheral ectoderm, start to proliferate, and bud of ganglion mother cells, which themselves divide symmetrically into two postmitotic ganglion cells. The NB reduce their size during each embryonic division. During late embryogenesis and early postembryogenesis they keep mitotically silent for at least 35 hr, enlarge again as postembryonic NB, and resume proliferation in the second instar until the pupal stage. Abdominal NB (A3–7): scattered central brain NB (Brain); lateral NB (L-NB); mushroom body NB (MB-NB); thoracic NB (T1–3); terminal NB (Term).

kel, 1978; Truman and Bate, 1988; Ito and Hotta, 1992). Only the four MB-NB continue proliferation throughout the pupal stage (Ito and Hotta, 1992).

We were interested in mutations affecting the proliferation pattern of NB subsets. Since we suspected that specific proliferation defects may result in altered morphology of the CNS, we analyzed viable Drosophila mutants, in which CNS morphology is affected.

One of these mutants is mushroom body defective (mud). Several alleles of mud have been detected in screens for structural brain mutants (Heisenberg, 1980; Fischbach et al., 1987). In mud flies the antennal lobe is enlarged and the central body appears misshaped. Most severe is the mushroom body (MB) phenotype: the number of Kenyon cell bodies, occupying the cortex region above the calyx, is increased in mud. The number of fibers, forming the larval peduncle, can be nearly doubled. In the imago, the MB-neuropil consists of an enlarged calyx at the expense of the peduncle and lobe system, partly due to false reorganization during metamorpho-
sis (Heisenberg, 1980; Technau and Heisenberg, 1982; Fischbach et al., 1987).

In this paper we demonstrate that mutations in the Drosophila gene mud affect the proliferation pattern in the CNS. In a subset of NB, located laterally in the neurons, the mud mutation suppresses mitotic arrest. In the dorso-caudal brain region, however, the mud mutation leads to an increased number of NB, which become specified as MB-NB.

MATERIALS AND METHODS

Stocks

The mud locus maps to 12E9–11 on the first chromosome (Lindsay and Zimm, 1992). So far four alleles of mud have been found: mud1 (former designation: mud1ss, Heisenberg, 1980), mud2, and mud4 were induced by EMS in the laboratory of M. Heisenberg; mud5 (former designation: mud5usse) was isolated by hybrid dysgenesis (Fischbach et al., 1987). All mud alleles are semilethal. Homozygous female flies are rarely found in FM7-balanced stocks, and hemizygous males occur at a much lower frequency than FM7 males. Most experiments have been carried out with mud5 stocks balanced over attX or FM7 and with an FM7-balanced mud5 stock. For allelic comparison we used FM7-balanced mud1, mud2, and mud3 stocks that have been repeatedly outcrossed to Canton S (kindly provided by J. S. deBelle, Würzburg). Attempts to outcross mud5 have not been successful (J. S. deBelle, personal communication).

The transformant lines 2–25 (fourth chromosome) and 3–107 (third chromosome) have been generated by the enhancer trap technique by C. Klämbt (Klämbt et al., 1991).

Application of Bromodeoxyuridine (BrdU)

BrdU was applied by injection into embryos, by feeding larvae or imagines with a diet containing BrdU, or by in vitro incubation of brain tissue.

Staged embryos were dechorionated with a needle, stuck to a coverslip, dried, and covered with fluorocarbonoil (see also Prokop and Technau, 1993). Five to ten nanoliters of a 15 mM solution of BrdU (Sigma) in 0.2 M KCl was injected into embryos at defined stages (embryonic stages according to Campos-Ortega and Hartenstein, 1985). Afterward the embryos were reared into the late embryonic stage 17, into larval, or into imaginal stages.

In larval and imaginal stages BrdU was applied by feeding animals with standard medium containing BrdU. Depending on the length of pulses the concentration ranged between 1 and 10% of a BrdU solution (33 mM BrdU in 40% ethanol, according to Truman and Bate, 1988). Newly hatched larvae were collected every 30 min and transferred to the medium for several hours (at 25°C) or for the whole larval period. Afterward they were either directly stained (pulse experiments) or transferred to standard medium without BrdU, to allow further development (pulse and chase experiments). Newly hatched flies were collected two times a day and fed a diet, containing BrdU, for about 8 days (at 25°C).

Pupal brains were removed from the pupal cases and transferred for 3 hr to Schneider's medium (Gibco) containing 5 mM BrdU.

Staining for BrdU Incorporation

At postembryonic stages the CNS were removed, fixed in Carnoy's solution, and rehydrated. Late embryos (tracheae already filled with air) were mechanically removed from the vitelline membrane and washed in PBT. Either the CNS were isolated prior to fixation and treated as mentioned above or the whole embryos were fixed in Carnoy's solution and rehydrated. In the latter case the embryos were rehydrated and their tips were cut off to allow penetration of the antibodies later. In most cases specimens were stained for BrdU incorporation according to Truman and Bate (1988) using monoclonal antibodies against BrdU (Becton–Dickinson; Gratzner, 1982) and HRP-coupled secondary antibodies (Dianova). Alternatively we used a biotinylated secondary antibody (Dianova) diluted 1:500 in PBT containing 1% milk powder (low fat) followed by an incubation step with ABC elite kit (Vector Laboratories). This staining procedure detected a more complex pattern of specific BrdU incorporation than the former technique (see Discussion). Specimens were embedded in Araldite (Serva).

Preparation of Sections

CNS were prepared and fixed in Carnoy's solution for 30 min, rehydrated, and, in some experiments, stained for BrdU as described above. Subsequently, the CNS were fixed in 6% glutaraldehyde in phosphate buffer for several hours, transferred to 2% osmiumtetroxide in phosphate buffer for several hours, carefully dehydrated with ethanol, shortly transferred to xylene, embedded in Araldite (Serva), and cut into 20-μm serial sections.

Toluidine Blue Staining

The pattern of NB can be differentially stained using toluidine blue solution. CNS of either stage 17 embryos or larvae were prepared. According to Truman and Bate (1988) they were incubated in the staining solution (Altmann and Bell, 1973) for 1.5 hr at 50°C. Fixation and differentiation in Bodian's fixative (change Bodian's...
5–10 times) were monitored under the dissecting microscope and stopped after about 30 min. Specimens were embedded in Araldite.

**Ablation of pNB Using Hydroxyurea (HU)**

Larvae were fed for 0–5 hr ALH (25°C) with standard medium containing 30–50 mg HU/ml diet. In contrast to treatment of late larvae (Broadie and Bate, 1991), the viability is not affected when early L1 larvae are treated even with high concentrations of HU. It also does not retard or interfere with further development. Afterward larvae were allowed to develop on standard medium without HU.

**Staining for β-Galactosidase Activity**

CNS were dissected out in Ringer’s solution, fixed for 10 min with 4% paraformaldehyde in PEM buffer (0.1 M Pipes, 2 mM MgSO₄, 1 mM EGTA, pH 6.9), and washed in oxidation buffer (150 mM NaCl, 1 mM MgCl₂, 3.3 mM K₃[Fe(CN)₆] in 200 mM phosphate buffer, pH 7.2). To avoid precipitation of X-Gal, the staining solution was prepared as follows: oxidation buffer was warmed to about 60°C, 1% X-Gal stock solution (20% X-Gal in dimethyl sulfoxide) was added, and the staining solution was shaken rigorously and slowly cooled to room temperature. Fixed tissues were incubated in staining solution at 37°C for several hours, dehydrated, cleared for a minute in xylene, and embedded in Araldite.

**Cell Countings and Planimetric Measurements**

BrdU-labeled cells were counted on a video monitor onto which CNS of either larvae or imagines were projected by a video camera mounted on an Axioiopan (Zeiss).

Planimetric measurements of β-gal-labeled mushroom bodies were carried out by scanning camera lucida drawings, counting the pixels inside the drawn area with the help of the Photoshop program (Adobe Systems, Inc.), and converting these data into micrometers squared.

Planimetric measurements of the brain volume of imaginal mud¹ males were carried out on 7-μm serial sections (kindly provided by J. S. deBelle, Würzburg; sections were prepared according to Heisenberg and Böhl, 1979). The sections were projected onto a video monitor and measured with the help of a program developed by R. Wolf (facilities kindly provided by R. Wolf and M. Heisenberg, Würzburg).

**RESULTS**

**A Subset of NB in the vNC of mud Mutants Prolongs Their Proliferation Period**

NB in the vNC of *Drosophila melanogaster* follow a very precise temporal and spatial proliferation pattern. All of them stop dividing until embryonic stage 17, and some of them resume proliferation during the second larval instar (Truman and Bate, 1988, Prokop and Teichnau, 1991; Fig. 1). Thus, during late embryonic and early larval stages, no BrdU incorporation can be detected in the vNC of the wild type.

Mutations in the mud gene affect this regulation (Figs. 2 and 3). In contrast to wild type, larvae of all four mud alleles incorporate BrdU into cells of the vNC upon BrdU application for several hours ALH (Exp. C, 1; see Fig. 1 for schematic presentation of the experiments mentioned). These effects are less severe for the non-
outcrossed mud stock but very strong for the mud' (Fig. 3b), mud' and mud stocks that have been purged of modifiers by repeated outcrossing (see Materials and Methods). A temporal profile of mutant proliferation behavior carried out in an altX-balanced mud stock suggests a decline of BrdU incorporation with increasing age: animals that have been injected with BrdU at the late embryonic stage 17 (about 16-17 hr after egg laying; Exp. A, 1) carried up to 210 labeled cells; animals fed with BrdU 0-5 hr ALH (Exp. C, 1) up to 70 cells; animals fed 5-12 hr ALH (Exp. D) up to 40 cells; and animals fed 12-17 hr ALH (Exp. E) up to 25 cells (Fig. 2; see also Fig. 5).

In all cases labeled cells were arranged as small clusters in a lateral and ventrolateral row along each side of the vNC. As the pattern was the same following BrdU injections into late stage 17 embryos and additional feeding in the early larva (Exp. B, 1-3), the labeled clusters of both stages seem to be identical. If larvae in these experiments were chased into the second larval instar (Exp. B, 2), BrdU-labeled thoracic clusters contained a single, large, less densely labeled nucleus, which most likely belongs to an enlarged NB (Fig. 4a). As no such cell can be detected during earlier stages, these putative NB seem to follow a normal time course of shrinkage and reenlargement (Hartenstein and Campos-Ortega, 1984; Truman and Bate, 1988; see also Prokop and Technau, 1991). When chased into the third larval instar (Exp. C, 3), the BrdU-labeled cells occupied positions near the border between the embryonically and postembryonically derived cortex regions, which is a typical position of ganglion cells born in the late embryo or early larva (Fig. 4b; see also Truman and Bate, 1988). Furthermore, the majority of BrdU-labeled cells in the mutant vNC persisted through metamorphosis (Fig. 4c): In early mud' larvae that have been pulse-labeled with BrdU from 0 to 4 hr ALH (Exp. C, 1), a total of 35 to 70 (n = 7) cells was labeled in the thoracic and abdominal vNC. Animals of the same batch that have been chased into the adult stage (Exp. C, 4) carried 25 to 75 (n = 6) labeled cells in corresponding regions.

To find out which kind of NB or cells might be affected in their replication behavior, we fed BrdU throughout larval life (Exp. F) and analyzed the labeling pattern in the abdominal neuromeres A3-7, which is less complex than that in the more anterior vNC. For the abdominal region Truman and Bate (1988) described only three pNB in typical positions of each hemineuromere, termed vm-, vl-, and dl-pNB (Figs. 3c, 6c, and 3g). When larvae, hemizygous for mud' were fed BrdU throughout larval life (Exp. F), the number and location of abdominal BrdU-labeled clusters were comparable to wild type (Figs. 3d, 3f, and 3h). However, in the vm and dl clusters the number of labeled cells was significantly increased: while in the wild type the vm-, vl-, and dl-pNB produce not more than 5, 5, and 15 cells, respectively (Truman and Bate, 1988), the number of cells was variably increased (up to more than doubled) in mud' for the vm and dl clusters (but seemed to be normal for the vl cluster; Fig. 3f). No cluster contained more than one NB (recognizable by its large nucleus: 4 to 4.5 μm in wild type and mud), suggesting that each cluster represents a single clone.

Thus, a defined fraction of NB in the vNC of mud seems to exhibit proliferation defects, leading to an increased cell number. To test how far proliferation might be affected also at later larval stages, we gave defined BrdU pulses during the third instar. In one experiment larvae were fed with BrdU 65-67 hr ALH, when all pNB in the wild type are dividing. Immediately after this short pulse they were fixed and stained (Exp. G): in mud' as well as in wild type, vm-NB were labeled together with up to two, and dl-NB with up to three, progeny cells (data not shown). Thus, as the number of labeled cells was not increased, proliferation in mud appears normal during this time period. In a second experiment BrdU was fed from 88 hr ALH until the wandering larval stage (Exp. H). During this period wild-type pNB in the vNC cease proliferation (Truman and Bate, 1988). In vNC derived from these experiments the dl clusters generally comprised 5 to 7 cells in the wild type, but up to 18 cells in mud'. The vm clusters were no longer labeled in wild type. In most preparations of mud' however, we found up to 9 cells in these clusters. Hence, mud' larvae show prolonged BrdU incorporation in the vm- and dl-NB also at the end of the postembryonic proliferation period.

Taken together, our data suggest that abdominal vm- and dl-NB in mud ignore the mitotic arrest during late embryogenesis and early postembryogenesis and prolong their postembryonic proliferation period by this generating of enlarged clusters of progeny cells. In the anterior vNC it is not possible to define the affected NB in similar detail due to the dense NB pattern in that region. However, late embryonic and early larval proliferation defects suggest that they correspond to the abdominal vm- and dl-NB.

NB in the Ventrolateral Region of the Central Brain of mud Prolong Their Proliferation Period

In the brains of wild-type larvae that have been fed with BrdU 0-4 hr ALH (Exp. C, 1 and 2), there are five NB dividing (Truman and Bate, 1988; Fig. 1). These NB do not shrink during late embryogenesis and stain continuously with toluidine blue. Four of them, named MB-NB, lie dorsocaudally (see below for detailed description). The fifth, named L-NB, lies ventrolaterally (Fig.
Fig. 3. Incorporation of BrdU into the larval vNC at different developmental stages. Wild-type larvae on the left and mud individuals with strong phenotypes (mud<sup>+</sup> in (b), mud<sup>-</sup> in (d and f) and mud<sup>-</sup> in (h)) on the right derive from the same experiments. In all photos anterior is to the left. (a and b) Horizontal views of vNC of early first instar larvae that were fed with BrdU for several hours ALH (Exp. C, 1). Cells of the vNC incorporated BrdU only in mud. They mainly lie in two rows on each side of the vNC (indicated by arrows). (c and d) Ventral views of vNC of third instar larvae that were fed with BrdU throughout larval life (Exp. F). The pattern of labeled cell clusters (corresponding to the pattern of NB) is not altered in mud. Arrowheads point toward one dl cluster, respectively. (c and d) A ventrolateral view of the abdominal neuromeres of the same preparations as in (c and d). The position and number of abdominal clusters (vm, vl, dl) is normal in mud but the number of
5c; Ito and Hotta, 1992). However, in the ventrolateral hemispheres of all four mud alleles, treated in the same way (Exp. C, 1 and 2), more separated cell clusters (in most cases 4–5) are labeled with BrdU (Fig. 5d). One of these clusters is the L-NB cluster. The additional 3–4 clusters lie anterior and posterior to the L-NB. If these mud larvae are chased into imaginal stages (Exp. C, 4), the supernumary groups of labeled cells come to lie in latrofrontal positions (Fig. 5d). The number of labeled progeny of the L-NB is not increased, at least in mud² (Fig. 6a).

Injection of BrdU into late stage 17 (16.5 hr after egg laying) wild-type embryos (Exp. A, 1, 2, and 4) also leads to BrdU incorporation into the L-NB cluster. In addition, in the embryonic brain 3–4 further clusters are labeled in the lateral hemisphere (Exp. A, 1 and 2). When chased into the adult stage (Exp. A, 4), they come to lie in latrofrontal positions of the brain (Fig. 5a). This pattern of proliferation in the brain of late wild-type embryos resembles that of early mud larvae (Exp. C, 1, 2, and 4; see above). Injection of BrdU into late stage 17 mud² embryos (16.5 hr after egg laying; Exp. A, 1, 2, and 4) results in about 11 BrdU-labeled cell clusters (L-NB and MB-NB not included) spread over the whole hemisphere, about 7–8 more than in wild type (Fig. 5b). A comparable pattern can be seen in wild-type brains of animals that have been injected in embryonic stage 16 (14.5 hr after egg laying; data not shown).

Taken together, these data suggest that at least a fraction of NB, in the brain of mud embryos, prolongs the time period of proliferation, like some NB in the vNC do. Those 3–4 NB in the ventrolateral hemisphere, which are the latest to vanish before hatching in wild type, prolong their proliferation into postembryogenesis in mud.

In mud, Surplus NB Divide in the Dorsocaual Brain Region

In mud the proliferation behavior in the dorsocaual brain region is affected. This was shown by feeding wild-type and mud² animals with BrdU 0–4 hr ALH and counting the labeled cells in the dorsocaual brain region of the imagos (Exp. C, 4). While counts in the wild type and females heterozygous for mud² vary between 45 and 69 labeled cells, the numbers range between 49 and 120 in mud² males and females. Thus, the number of BrdU-labeled cells, in the dorsocaual brain region of mud² animals, varies between numbers typical of wild type and twice that number (Fig. 6a). Variability even occurs between both hemispheres of the same individual. When BrdU was injected into late stage 17 mud² embryos, counts in the dorsocaual brain region of the imagos (Exp. A, 4) also revealed a comparable increase in labeled cells (Fig. 6a).

In the late embryonic and early larval dorsocaual brain region of wild type, BrdU incorporation is due to proliferation of 4 NB (Figs. 7a and 7b; Truman and Bate, 1988), which contribute cells to the MB and are therefore called MB-NB (Prokop et al., 1991; Ito and Hotta, 1992). To find out about the origin of the additional cells in the dorsocaual brain region of the mutants, the number of NB in that brain region was counted at different stages of development: In late stage 17 embryos or first instar larvae, mutant for mud² or mud⁴ (both not outcrosssed), BrdU as well as toluidine blue staining revealed 4–15 NB (Figs. 7d and 7e). This variation might explain the variable total number of labeled cells in the dorsocaual brain region (see Fig. 6a). In many preparations of late embryonic or early larval mud CNS, the NB in the dorsocaual brain region were reduced in size: whereas the diameters of nuclei of BrdU-labeled MB-NB were about 4.5 μm in the wild type, they varied between 2.8 and 4.5 μm in mud.

For the wild type, Ito and Hotta (1992) found that the 4 MB-NB are the only NB that continue proliferation into late pupal stages (Fig. 1). Accordingly, when wild-type brains were exposed to BrdU for 66–69 hr APF (Exp. I), 4 labeled NB were found above the dorsocaual part of the MB neuropil (calyx; Fig. 7e). However, in mud² and mud⁴ pupae many more NB incorporated BrdU or labeled with toluidine blue. They were lying above the calyx, which in most cases was significantly enlarged or formed a huge extra mass of neuropil (Heisenberg, 1980; Teichnau and Teichnau, 1982; Fischbach et al., 1987). In one extreme case of a mud² female pupa, derived from an FMR-balanced stock, the number of NB was 8 in the left and 35 in the right hemisphere (Fig. 7f). However, the number of BrdU-labeled cells, associated with (and likely to be derived from) each NB, in the same animal was comparable to wild type (up to 5 cells). In a few of these preparations, labeled NB were also found in other brain regions, most often in the antenial lobe region. Feeding BrdU to wild-type and mutant imagines (Exp. K) no longer led to incorporation into

BrdU-labeled cells in the vm and dl clusters is significantly increased. Specimens in (a–f) have been labeled using an HRP-conjugated secondary antibody. (g and h) Dorsal views of vNC of late third instar larvae treated like the individuals in (c and d), but stained with ARC elite kit (see Materials and Methods). (g) In these preparations an additional set of about 40 BrdU-labeled glial cells per abdominal neuromere is detected in the wild type (Prokop, 1993; Urban et al., 1993). (h) In mud the glial pattern appears normal, and the pNS-clusters (arrowheads) are enlarged in a manner similar to that in (d). Scale bar, 30 μm in (a and b); 50 μm in (c and d); 20 μm in (e and f); 40 μm in (g and h).
Fig. 4. BrdU-labeled cells at different developmental stages in mud. (a) A close-up of the thoracic region of a mutant second instar larva that has been treated with BrdU in the late embryo and for several hours ALH (Exp. B, 2). BrdU-labeled cells are arranged in clusters. Each cell
neural cells except for a few (up to 4) glial cells in the brain region (data not shown; see also Ito and Hotta, 1992).

Taken together, these data suggest that there are surplus NB in the dorsocaudal brain region of mud that lie in the cortex above the calyx and seem to mimic the proliferation behavior of wild-type MB-NB.

**Surplus Neuroblasts in mud Are Additional MB-NB**

Several lines of evidence demonstrate that the additional NB in the dorsocaudal brain of mud are additional MB-NB:

1. The enhancer-trap strains 2-25 and 3-107 (kindly provided by K. Fischbach; Klambt et al., 1991) express β-gal specifically in the MB cortex (S. Tix, personal communication; Schneider and Fischbach, 1992): in brains of strain 2-25, β-gal expression is found in the entire MB cortex from embryonic stages onward (not shown); in 3-107 it is found not before the third larval instar in 4 small cell clusters closely associated with the MB-NB (Fig. 8, a). When crossed into mud⁶ mutant background, 2-25 as well as 3-107 showed a significantly increased number of β-gal-labeled cells in the late third instar (Fig. 8c). At least in mud⁶, 2-25 individuals a slight increase of the β-gal-labeled dorsocaudal brain area was already measured in freshly hatched larvae, again suggesting that the mutant phenotype is already expressed in the embryo (Fig. 6b). Feeding BrdU to these larvae 0-4 hr ALH (Exp. C, 1) and subsequent double staining for BrdU and β-gal demonstrate that both markers are colocalized in the same area (data not shown).

For an allelic comparison, 2-25 was crossed into all four mud alleles and the sizes of the labeled dorsocaudal brain regions were analyzed in the late third instar (Fig. 6b): the outcrossed mud⁴, mud⁵, and mud⁶ stocks especially showed drastically enlarged labeled areas ranging from wild-type values to the fourfold size. The phenotypes of non-outcrossed FM7-balanced strains of mud⁸ and mud⁶ were less severe, suggesting the presence of modifiers in these stocks. Heterozygous females always were comparable to wild-type controls.

2. To test whether the surplus β-gal staining in mud is attributed to cells derived from the additional NB in the dorsocaudal brain region, we ablated them with HU. HU affects only cells that are actively dividing (Timson, 1975; Truman and Booker, 1986; Broadie and Bate, 1991). Larvae were treated with HU 0-5 hr ALH to specifically ablate the few brain NB that are dividing at that stage. Afterward they were reared into the late larval stage, stained, and analyzed: In 3-107 no MB-specific staining was detected in wild type or in mud⁶ background (Fig. 8e). In 2-25 the number of labeled cells was significantly reduced following HU ablation (Fig. 6b); however, a patch of β-gal-labeled cells remained. This remaining patch resembled the labeled area in L1 larvae and accordingly was larger in mud⁶ background than in wild type. Hence, these labeled cells are likely to be of embryonic origin (Fig. 6b; Hinke, 1961; Technau and Heisenberg, 1982).

We conclude that the surplus NB lineages, observed in the dorsocaudal brain of early mud larvae, express MB-specific markers and are responsible for the increased number of β-gal-labeled cells in the MB cortex.

3. We also analyzed the imaginal brain morphology of HU-treated animals. Following early larval HU ablation, the MB neuropil was no longer detectable in 20-μm sections of wild-type brains (Fig. 8f). This result confirms that the four MB-NB in each wild-type hemisphere give rise to intrinsic cells (Kenyon cells) of the MB (for further discussion see Ito and Hotta, 1992). In the same preparations, the antennal lobe was also significantly reduced, demonstrating that the L-NB (which also divides in the early larva) contributes part of the cells of the antennal lobe cortex (data not shown). In untreated mud flies, the MB cortex as well as the calyx is significantly enlarged (Figs. 8d and 9; Heisenberg, 1980; Fischbach et al., 1987). However, as in the wild type, 20-μm sections of imaginal mud⁶ brains did not reveal any MB structures when specimens had been treated with HU in the early larva.

Taken together, these data show that the increased number of fibers in the larval peduncle of mud (Technau and Heisenberg, 1982), as well as the enlarged MB neuropil and MB cortex in mud flies (Heisenberg, 1980; Fischbach et al., 1987), derives from a surplus number of MB-NB in the dorsocaudal brain region.

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4 In these preparations also, surplus proliferation in the vNC seems to be colocalized or at least closely related with a segmental pattern of β-gal-expressing cells.
**Fig. 5.** BrdU incorporation in the brain of wild type and *mud*. CNS of wild type are shown on the left and CNS of *mud* on the right. (a and b) Animals that have been injected with BrdU in the late embryo (Exp. A, 2 and 4); (c and d) animals that have been fed with BrdU 0–4 hr ALH (Exp. C, 2 and 4). Labeled animals were either raised into the second instar larva (photos, lateral view, anterior to the left) or into the imaginal stage (camera lucida drawings, frontal view). In the larval and adult hemispheres of wild type and of *mud*, labeled cells are found in the dorsocaudal brain region (in photos: open arrows, in drawings: stippled area). (c) In wild-type animals, one further NB in the ventrolateral brain region incorporates BrdU in the early larva (large black arrow). This is the L-NB, which contributes part of the cells of the lateral cortex of the antennal lobes (al). The L-NB can be detected in all the other preparations, too (large black arrows in a–d). (d) In early *mud* larvae, additional cell groups in the ventrolateral hemisphere incorporate BrdU (small arrows in d) that come to lie in laterofrontal positions in the imaginal...
The Brain Volume in mud Is Significantly Increased

We described proliferation defects in mud leading to additional neural cells which obviously persist into imaginal stages. To find out how far these additional cells might be simply added to the normal brain architecture, we planimetrically determined the volume of the central brain neuropil in adult Canton S males as well as in outcrossed mud$^+$ males (serial sectioned preparations kindly provided by J. S. deBelle, Würzburg).

The volume of the MB neuropil in the wild type (including the calices, the peduncles, and $\alpha$- and $\beta$/γ-lobes of both hemispheres) was $(0.16 \pm 0.02) \times 10^6 \mu m^3$ (Fig. 9). For unknown reasons these values are nearly five times higher than previously reported $(0.034 \times 10^6 \mu m^3)$; Hinke, 1961. Although the lobes and peduncles were not detectable in most mud preparations, the total volume of the MB neuropil in eight measured mud$^+$ males was always larger than in wild type and varied from values close to the wild type up to 10-fold.

The mean volume of the brain neuropil (without the optic lobe and MB neuropil) in eight Canton S males was $(3.89 \pm 0.28) \times 10^6 \mu m^3$ (Fig. 9). These data are in good accord with former descriptions $(3.66 \times 10^6 \mu m^3)$; Hinke, 1961. The mean brain volume taken from eight mud$^+$ males was $(5.58 \pm 0.36) \times 10^6 \mu m^3$. Thus, the central brain volume was increased by 40% in mud, but shows similarly low variability to the wild type.

Taken together, the volumes of both the MB neuropil and the remainder of the central brain are significantly increased in mud compared to wild type. This effect is much stronger and more variably expressed in the MB than in the surrounding central brain neuropil. Surplus MB neuropil in mud derives from a variable number of additional MB-NB (see previous chapters). These supernumerary MB-NB do not seem to arise at the expense of other NB, considering the enlargement of the other central brain neuropil. We do not know whether the enlargement of these other regions is also due to an increased number of NB or just to the prolongation of NB proliferation described before.

DISCUSSION

mud Affects Proliferation Control of a Subset of NB

In the vNC and in the ventrolateral brain regions of all four known mud mutants, cells incorporate BrdU at developmental stages when these regions in the wild type do not become labeled. Especially the simple proliferation pattern in the abdominal segments A3–7 allowed for a detailed analysis of the mud proliferation defect: in A3–7 two NB per hemineuromere, the vm- and dl-NB (Truman and Bate, 1988), do not respect the periods of mitotic quiescence. Instead, they extend their proliferation from the embryo into postembryogenesis and prolong their larval proliferation period into the wandering larval stage.

In the anterior vNC and in the ventrolateral hemispheres, there are also BrdU-labeled cell clusters in mud that do not exist in early wild-type larvae. The complex postembryonic NB pattern makes the analysis difficult in these regions. However, the similar size and position of BrdU-labeled clusters in the anterior vNC and in A3–7 suggest a similar proliferation defect in these regions. This would mean that the clusters in the anterior vNC also originate from prolonged proliferation of a normal subset of NB (perhaps representing vm- and vl-NB) and not from surplus NB. In the ventrolateral hemispheres the similarity between the pattern of labeled cell clusters of early mud larvae and of late wild-type embryos also suggests prolonged proliferation of a normal subset of NB. Supernumerary cells from these divisions persist into the imaginal and contribute at least partly to the enlargement of the central brain neuropil (without the MB). We do not know whether further defects may also be responsible for this enlargement.

These data suggest that mud interferes with proliferation control of a subset of NB located in the lateral CNS. This is evident for A3–7 and is likely to be also the case in other neuromeres. The regulation of the cell cycle is well studied (for reviews see, e.g., Lewin, 1990; Nurse, 1990; Pines and Hunter, 1990; Glover, 1991), but little is known about the mechanisms coordinating the mitotic pattern in complex tissues like the CNS (e.g., Booker and Truman, 1989; Selleck and Steller, 1991; Selleck et al., 1992; Taylor and Truman, 1992; Ebens et al., 1993). The gene *string* (stg) was reported to mediate the pattern of the first three postblastodermal cell cycles during *Drosophila* embryogenesis by regulating the $G_2/M$ transition (Edgar and O'Farrell, 1989, 1990; O'Farrell et al., 1989; Lehner, 1991). However, most embryonic cells, when they cease dividing, arrest in a $G_1$-phase, and there must be a coordinating mechanism that prevents entry into S-phase (Edgar and O'Farrell, 1990; for reviews see Lewin, 1990 and Lehner, 1991). Also NB seem
to arrest in a G1 phase when they stop proliferation in the late embryo. This is suggested by the observation that pNB undergo an S phase before they start their first postembryonic mitosis (Truman and Bate, 1988) and points toward the possibility that mutations in mud might affect G1 arrest of specific NB.

Ebens et al. (1993) demonstrated that NB proliferation in the vNC can be nonautonomously regulated via glial cells. However, we did not find any abnormalities in the glia pattern of mud larvae which could account for the observed proliferation defects (Figs. 3g and 3h; unpublished results).

NB affected in proliferation control in mud do not stain with toluidine blue (data not shown), as most NB normally do (Truman and Bate, 1988). However, in the wild type, the v/-, vl-, and dl-NB are also weakly or not labeled (Truman and Bate, 1988; our own observations). Furthermore, in the hemispheres of late wild-type embryos toluidine blue staining is found only in the MB-NB and L-NB, but hardly in those 3–4 late NB, which in mud seem to prolong their proliferation into early postembryogenesis (data not shown). Thus, low affinity for toluidine blue might be a common feature of those wild-type NB, the proliferation control of which is most severely affected in mud.

mud Affects the Number of MB-NB

Another type of NB affected in mud are the MB-NB. Here, however, mud does not affect the proliferation control, but the number of specific NB. We presented several pieces of evidence that surplus NB in the dorsalcaudal brain region represent additional MB-NB: they behave like wild-type MB-NB in their period and frequency of proliferation, and their daughter cells express MB-specific markers, which are no longer detectable when these NB are ablated with HU. Furthermore, the daughter cells of additional NB are likely to be Kenyon cells, as the enlarged MB neuropil in mud (Heisenberg, 1980; Fischbach et al., 1987) can no longer be found following HU ablation.

Planimetric measurements of the brain neuropil suggest that the increased number of MB-NB is due to additional cells becoming determined as NB and is not due to males from outcrossed mud alleles show a significant increase in size, whereas males from non-outcrossed mud and mud7 stocks show a less severe phenotype. Measurements for mud7 (open dots) are less reliable due to a dominant modifier suppressing β-gal expression in 2–35 and further enhancer-trap lines. Males were separated from females by the size of their gonads. Due to the lack of FM7-blue balancers with postembryonic β-gal expression, mutant males could not be separated from FM7 males.
transformation of neighboring NB: Compared to wild type, we found a reproducible increase rather than a reduction of neuropil volume in the central brain of mud⁴ males when the MB neuropil was subtracted. This would mean that mud expresses a specific irregular hyperplasia (Goss, 1966). However, we do not know whether additional NB also account for the hyperplasia of central brain neuropil other than the MB neuropil.

There are two periods in which NB patterns become established: in the early embryo, when NB segregate from the neurogenic region of the ectoderm (Hartenstein and Campos-Ortega, 1984) and in the larva, when a
subset of these NB becomes reactivated as pNB (Truman and Bate, 1988; Truman, 1990; Prokop and Technau, 1991). The *mud* mutation already interferes with the embryonic NB pattern: At least in *mud* the number of NB and of BrdU-labeled cells is increased in the dorso-caudal brain of the embryo, and similarly the β-gal-la-
beled dorso-caudal brain area in freshly hatched *mud*; 2-25 larvae appears slightly enlarged. Once established,
the additional NB persist into the pupa and behave like wild-type MB-NB. As it was not possible to reliably count the MB-NB at earlier embryonic stages (unpublished results), it remains unclear at what time the MB-NB number increases. One possibility might be additional equal divisions of early NB, as they have been reported for the procephalic lobe of wild type (Hartenstein and Campos-Ortega, 1984). Additional symmetric divisions during embryogenesis could also explain the fact that MB-NB in late mutant embryos or early mutant larvae are smaller than those in wild type.

We described at least two different phenotypes of NB regulation: on the one hand lateral NB in the CNS prolong their proliferation period; on the other hand the number of MB-NB is increased. As mentioned above the first phenotype might be due to a failure in establishing a $G_1$ block, the latter to a failure in switching from a mode of equal divisions into stem cell behavior. It remains open as to how far these proposed mechanisms might have a common molecular basis. In addition to

[Fig. 9. The volume of the brain neuropil in adult wild type and mud males. Bars on the left side represent the volumes of the central brain neuropil of eight wild type and eight mud males. Black portions indicate the subfraction of the MB neuropil (calyx, peduncles, and α-, β-, and γ-lobes of both hemispheres). Mutant MB lacking the peduncles and lobes are darkly shaded. Brightly shaded portions represent the brain volume (without the MB and the optic lobes). Single bars on the right side represent the mean values of the central brain without the MB and optic lobe neuropil.]

these defects, the organization of the adult brain neuropil is severely affected in mud (Heisenberg, 1980; Fischbach et al., 1987). However, this might be a secondary defect due to the altered proliferation. Similar suggestions have been made for neuropil disorder in anachronism mutants of Drosophila (Ebens et al., 1993). Most severely affected is the MB neuropil in mud: following their reorganization during metamorphosis Kenyon cells tend to coil up in the calyces and fail to establish peduncles and lobes (Heisenberg, 1980; Technau and Heisenberg, 1982; Fischbach et al., 1987). This phenotype might be the consequence of the overproduction of Kenyon cells by additional MB-NB (see also Fischbach et al., 1987). Interestingly, the presence of peduncle- and lobe-like structures was only observed in mud males which express a less severe hyperplasia of the MB (Fig. 9).

From embryonic stages onward surplus MB-NB in mud seem to be specified to produce surplus Kenyon cells. The number of these cells seems not to be reduced by regulating mechanisms (e.g., cell death mediated by cell interactions). HU ablation of the MB-NB and the L-NB resulted in loss of corresponding neuropil structures, which were obviously not replaced by progeny of neighboring NB (see also Witten and Truman, 1991). Furthermore, these HU ablations affect specific behavioral aspects, like learning (correlated with the loss of the MB-NB) and odor recognition (correlated with the reduction of the antennal lobes) (deBelle and Heisenberg, 1994). These observations strongly support the idea that specific (postembryonic) NB give rise to specific types of progeny (see also Doe and Goodman, 1985; Witten and Truman, 1991; Huff et al., 1989; Doe, 1992; Udolph et al., 1993). For the other affected NB in the ventrolateral brain region and in the vNC of mud it is unknown what kind of daughter cells they produce in the wild type and what type of cell is produced by their prolonged proliferation in mud. We only know that these cells are able to persist into the adult. Addressing the identity of the cells derived from prolonged proliferation might shed some light on the question how far proliferation control is linked to differentiation processes.

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REFERENCES


neuroblasts in *Drosophila* to a male or female fate is dependent on
Truman, J. W. (1990). Metamorphosis of the central nervous system of
Truman, J. W., and Bate, C. M. (1988). Spatial and temporal patterns
of neurogenesis in the CNS of *Drosophila melanogaster*. Dev. Biol.
Truman, J. W., and Booker, R. (1986). Adult-specific neurons in the
mon precursor for glia and neurons in the embryonic CNS of *Dro-
sophila* gives rise to segmentspecific lineage variants. Development
118, 765–775.
and postembryonic development of glial cells in the ventral nerve
cord of *Drosophila*. In “Gene, Brain, Behaviour. Proceedings of the
21st Göttinger Neurobiologie Conference” (N. Elsner and M. Heisen-
movement in the formation of the imaginal nervous system in *Dro-
expression in postembryonic lineages in the moth *Manduca sexta*.