In situ patterns of nuclear replication in brain ganglia of (12)glα mutant larvae of Drosohphila melanogaster

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Abstract. The (12)glα mutation is a recessive mutation of a recessive isogen of Drosohphila melanogaster isozyme microtubulins in the optic centres of brain of late third pariet larva. We have studied the in situ patterns of DNA synthesis in these brains by immunocytochemical detection of cells incorporating 5-bromodeoxyuridine. It was seen that (12)glα brains from younger third instar larva had lower replicating cells than in wild type larvae of comparable age but in brain ganglia of older (12)glα larvae the number of replicating cells was much higher. The spatial distribution of replicating cells in optic lobes of brain ganglia of (12)glα larvae was disturbed from early 3rd instar stage, much before the somatic growth was morphologically detectable. The stereotyped pattern of asymmetrical cell division of the neuroblasts and their progeny cells was also not seen in (12)glα brain ganglia. Therefore, it appears that the (12)glα product has an important role early in development to determine the temporal and spatial pattern of neuroblast cell division in developing brains.

Keywords. Antisense; BrU-l-antibody; neuroblasts; brain tumours.

1. Introduction

The replication programmes of different cell types of Drosohphila are regulated both temporally and spatially. Accordingly, a given cell enters a mitotic or endoreplication cycle at a defined stage of development (see Ruan and Lakhota 1990, for a recent review). DNA synthesis and cell division pattern in larval brain also occurs in a programmed manner (Poston 1950; White and Kankel 1978; Lakhota and Kumar 1989; Truman and Bate 1988). Most of the neurons of adult brain are formed and differentiated during late larval and early pupal stages (Truman and Bate 1988). The neuroblasts are formed by a stereotyped pattern of cell divisions in which the precursor neuroblasts divide symmetrically to form more neuroblasts which finally divide asymmetrically to give rise to a neuroblast and a ganglion mother cell (GMC). GMCs divide symmetrically to produce progeny and ganglion cells which differentiate into neurons. The spatial distribution of dividing neuroblasts and GMCs follows a specific pattern during larval period (Truman and Bate 1988).

Immunocytochemical detection of newly synthesized 5-bromodeoxyuridine (BrU) substituted DNA in intact tissues provides a convenient approach to examine in situ the temporal and spatial patterns of cell divisions (Schuhiger and Paffa 1967; Truman and Bate 1988). This approach has provided useful information about the spatial locations of neuroblasts and GMCs and their time course of divisions during development of brain in Drosohphila larvae (Truman and Bate 1988). The reproducibility of the patterns of locations of replicating nuclei in developing brain

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permits detection of any abnormality. In this study, we have compared the in situ pattern of location of replicating nuclei in brain ganglia of wild type 3rd instar larvae of D. melanogaster with the pattern seen in ganglia of mutant larvae that develop malignant tumours of brain during late 3rd instar stage. Among the several mutant genes that lead to tumourous growth of brain (Gatell and Mecherli 1989), one of the better known is the (1)R ph and its other mutant alleles. Larvae homozygous for (1)R ph recessive mutation appear to grow normally till day 5 (mid or early late 3rd instar stage) after which their brain ganglia begin to show tumourous growth, these larvae fail to pupate but continue as larvae for another 7-7 days during which their body becomes bloated and rather transparent. By this time their brain ganglia also assume irregular morphology. However, up to day 5 or so of larval life, the brain ganglia of these mutant larvae do not display any apparently atypical growth at morphological level. Using the technique of BrdU-labelling and in situ detection of nuclei that have incorporated BrdU, we have examined if the regulated pattern of cell divisions is disrupted during or prior to onset of tumourous growth in these mutant larvae.

2. Materials and methods
Larvae of a wild type (Oregon R) and the mutant (1)R ph or SM5 stocks of D. melanogaster were reared on standard agar-convivial-sugar-yeast media at 20°C ± 1°C. The mutant stock generates two types of larvae (for details of the mutator, see Lindsey and Gioli 1969), those that are homozygous for (1)R ph or and those which are heterozygous for (1)R ph or mutant alleles (the SM5 homozygotes do not survive). After the late 3rd instar stage, (1)R ph homozygous larvae can be distinguished from heterozygous siblings due to their transparent and bloated body and prolonged larval life. However, at earlier stages, they are very easily distinguished. The presence of orange (or orange) marker helps in identifying younger (1)R ph homozygotes since or heterozygotes have colourless Malpighian tubules.

Brain ganglia from 4- to 7-day old wild type and (1)R ph; SM5 and from 4- to 11- day old (1)R ph homozygous larvae were dissected in modified Pohl’s medium (Sinha and Lakhotia 1989) and labelled in dark with 20mM BrdU (Sigma) at 24°C for 60 min. The labelled ganglia were fixed in 90% ethanol for 30 min, hydrated through descending grades of ethanol and hydrolyzed in 1N HCl for 15 min at 24°C. Following hydrolysis, the ganglia were washed several times in PBS (125 mM NaCl, 1.5 mM Na2HPO4, 8.5 mM NaH2PO4, 1.8 mM NaHCO3, pH 7.2) and incubated with 10% goat serum for 1hr at 4°C. After removal of excess of goat serum and washing in PBS, the ganglia were incubated overnight with 1:100 dilution of anti-BrdU antibody (Boehringer, Switzerland) at 4°C in dark. Excess antibody was removed by repeated washings in PBS. Subsequently, the brain ganglia were incubated overnight at 4°C with 1:64 dilution of anti-mouse-IgG-FITC conjugate (Sigma). After the secondary antibody binding, the ganglia were washed several times in PBS and mounted on slides with a solution containing 1:4 PBS-glycerol and 4% p-phenylendiamine (Sigma). The whole mount preparations were observed under a fluorescent microscope (Leitz Ortholux) using 50 x oil and 50 x oil objective and appropriate fluorescein filter block. At least 7 ganglia of each genotype for each day of development (4 to 7 days for wild type and 4 to 11 days for (1)R ph larvae) were examined for the spatial patterns of distribution of BrdU-labelled and fluorescing nuclei.
3. Results

The brain ganglia of wild type 2nd instar larvae have a characteristic shape with two large dorsal cerebral hemispheres and a median ventral ganglion (figure 1). The dorso-lateral sides of cerebral ganglia represent the prominent optic lobes. The size of cerebral ganglia increases to some extent in wild type larvae between day 5 and day 8, when they pupate. The development of brain ganglia of 1(2)g{superscript}4/SMS larva closely followed the patterns in wild type larvae. On the other hand, brain ganglia of 4- and 5-day old 1(2)g{superscript}4 larva appeared smaller than those of same age wild type and 1(2)g{superscript}4/SMS larva but showed similar shape (see figure 1a-d). Size of brain in 1(2)g{superscript}4 larva increased rapidly from day 6 onwards but the characteristic shape continued till about day 8, after which the brain tissue was seen to be disorganized and to form large irregular mass (figure 1f-g).

Comparison of the overall number of replicating nuclei (which incorporated BrdU) and therefore showed immunofluorescence between brain ganglia of wild type and 1(2)g{superscript}4 larva, revealed that up to day 5, there were relatively fewer replicating nuclei in mutant brains (figure 1a-d). However, in 7-day old 1(2)g{superscript}4 larval brain ganglia the number of nuclei that were replicating was considerably higher than in wild type or 1(2)g{superscript}4/SMS sibs and with increasing age of 1(2)g{superscript}4 larva, the number of replicating nuclei continued to increase further (figure 1e-g).

In the 4- and 5-day old wild type larva (it is also in 1(2)g{superscript}4 or SMS heterozygotes), the replicating ganglion mother cells (GMC) formed a 2-3 cell thick concentric band in both optic lobes of brain ganglia figure 1a-c. As the larva grew older, this intensely fluorescing concentric band on each side became much wider (8-10 cell thick, see figure 1g). The proliferation centres of neuroblasts were seen at dorso-lateral positions of the brain. In addition to the proliferation centres of neuroblasts on dorso-lateral positions and the concentric bands of GMCs in optic lobes, BrdU-incorporation was also seen in some nuclei in medial parts of cerebral ganglia and in ventral ganglion. The spatial distribution of these nuclei, however, did not show any distinct pattern. The number of replicating nuclei in these other regions declined with larval growth figure 1a-c and e).

In contrast to the wild type pattern, the replicating GMCs in the 1(2)g{superscript}4 bra revealed that they were not arranged in the characteristic concentric bands in optic lobes. Thus although morphologically the brain ganglia of 4-5-day old 1(2)g{superscript}4 larva appeared similar to those in wild type larvae of the same age, the distribution of replicating nuclei was strikingly different (see figure 1a-d); the fewer replicating nuclei in the mutant brain were distributed rather uniformly over the dorso-lateral aspect of brain ganglia and the ventral ganglion (figure 1b-d). By day 7, the number of BrdU-labelled nuclei in 1(2)g{superscript}4 brain was very high but except for a faint suggestion of a concentric band of fluorescence in the optic lobe region, the large number of replicating nuclei did not show a well-defined spatial pattern (figure 1h). From day 8 onwards, the BrdU-labelled nuclei were seen all over the brain without any apparent order. As mentioned earlier, the brain also lost its characteristic shape as the tumourous growth continued (figure 1g).

The stereotyped pattern of divisions of neuroblasts and GMCs to produce neurons in insects leads to formation of clusters of replicating cells in which a large neuroblast cell is associated with smaller GMCs, pre-ganglion and ganglion cells (which ultimately give rise to neurons). These clusters can be easily identified after labelling with BrdU (see Truman and Bate 1993). Clusters of BrdU-labelled
4. Discussion

The (1;2)ab mutant is due to a deletion of about 13.1 kb DNA from the telomeric

Figure 1. Indirect immunofluorescence showing the spatial distribution of BuCl-labelled nuclei in whole mounts of brain ganglia of wild type (a, c and e) and (1;2)ab (b, d, f and g) larvae on day 4 (a, b, and d), day 5 (c), day 7 (f), and day 11 (g) (n = 300).
Figure 1. (Caption on facing page)
Figure 2. Higher magnification photomicrographs of BrdU-labeled cells from optic tectum of whole mounts of brain gilts, from wild type (a, c) and /3-2 mutants (b, d) larvae on day 4 (a, b), day 5 (c, d) and day 11 (e). Note the discrete clusters of labeled cells in wild type gilts (c, d) in each cluster, one large and a small, fluorescent nucleus (P) is associated with several smaller and brighter fluorescent nuclei. The /3-2 mutants (b, d) do not show any such discrete clusters (b, d, e) (× 2000).
region of the left arm of chromosome 2 (bands 2A–B of 2L, Mechler et al. 1985; Gattef and Mechler 1989). When homozygous, the mutant condition has profound effects on proliferation and differentiation of cells in the presumptive optic centres of brain and in imaginal discs (Gattef and Schneiderman 1967, 1974). The absence of (1)gl gene activity results in neuroblastoma and benign tumours of imaginal discs. Earlier studies (see review by Gattef and Mechler 1989) revealed that the cortex region of the brain of late (1)gl larvae was considerably enlarged due to many extra divisions of neuroblasts, GMCS and pre-pagglion cell of optic proliferation centres and that these cells failed to differentiate into optic neurons. Our present observations confirmed these results and in addition also revealed two other defects caused by the (1)gl mutation at earlier stages of development.

Thus, in the early 3rd instar larvae, the number of replicating nuclei in (1)gl larval brain ganglia was much less than in wild-type brains. The second and more significant finding of this study was that the pattern of spatial distribution of replicating nuclei was almost completely disrupted in mutant larvae from a very early developmental stage, much before any morphological evidence of the mutant genotype was apparent. This disruption of spatial pattern was evident from the absence of concentric bands of BrdU-labelled fluorescent nuclei in optical regions of the brain ganglia of 4–5 day-old mutant larvae and also from a rare occurrence of clusters of neuroblasts, GMCS and pre-pagglion cells. Such clusters of dividing sister cells were absent not only when fewer nuclei were replicating in younger mutant larvae but also when the brain ganglia were hyperactive in cell divisions in older mutant larvae. The presence of clusters of dividing sister cells in median ventral ganglion of (1)gl larvae parallels the earlier observation of Gattef and Schneiderman (1974) that this region does not participate in malignant transformation or tumourigenesis. Earlier studies of White and Kankel (1978) and of Truman and Bate (1988) suggested a very brief (~1 h) cell cycle time for neuroblasts in wild-type brain ganglia while the GMCS and other cells were found to have longer cell cycle durations. In another study (S. Roy and S. C. Lakhota, unpublished) we compared the mitotic cell cycle in wild-type and (1)gl brain ganglia and found that a sub-population of dividing cells in wild-type brain ganglia which enters mitosis within 35 to 50 min of the S-phase, is greatly absent in (1)gl brains. A vital staining of brain ganglia with toluidine blue, which preferentially stains neuroblasts (Truman and Bate 1988), revealed that (1)gl brain ganglia from day 11 larvae had a considerably reduced number of neuroblasts than on day 17 (data not presented).

In light of these and our present results, it may be suggested that typical neuroblasts with characteristic rapid and asymmetric division patterns are not present in optic lobes of cerebral ganglia of 3rd instar (1)gl larvae.

Thus the (1)gl gene appears to have a dual regulation of cell division of neuroblasts in brain ganglia: it promotes cell division in brain ganglia in younger stages but limits cell proliferation during the late 3rd instar so that the (1)gl mutant larvae have fewer replicating nuclei in brain ganglia when young but many more when tumourigenesis sets in. A direct count of cells in brain ganglia of (1)gl mutant larvae on different days also reveals that the early 3rd instar stage, the mutant brains have fewer cells (P. Sinha and A. Mishra, personal communication).

It is known (Gattef and Mechler 1989) that (1)gl gene encodes two major transcripts, one 4.5 kb and the other 6 kb. During early embryos, the 4.5 kb transcript is predominant while during mid- and late-embryogenesis,
the 6-0 kb transcript is seen. A second period of (2)gl¹ gene activity occurs in later larval life (Kliamb and Schmidt 1986; Gatoff and Mecherl 1989). It has been suggested that the embryonic expression of (2)gl¹ helps in establishing the developmental programmes being decided at that time (Gatoff and Mecherl 1989). Absence of the (2)gl¹ gene products in (2)gl² embryos leads to developmental defects in a variety of larval tissues because cell fates are not properly established. Our present results show that the programmed patterns of asymmetric divisions of neuroblasts and GMCs in specific areas of developing brain are also disrupted due to (2)gl¹ mutation. This was seen as early as day 4, much before the second period of (2)gl¹ gene activity in later larval life (Gatoff and Mecherl 1989). The altered patterns of cell divisions in (2)gl¹ brain ganglia, therefore, can be attributed to the absence of (2)gl¹ gene products during early embryogenesis. Thus among other things, (2)gl¹ gene activity during embryogenesis is also responsible for defining cell division patterns in optic centres of brain during larval life.

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References
Gatoff E and Schneiderman H A. 1967 Developmental studies of a new mutant of Drosophila melanogaster lethal malignant brain tumour (1,2)gl¹. Am. Zool 7: 360 (abstract 238)
Klatter C and Schmidt O. 1986 Developmental expression and tissue distribution of larval (2)gl¹ larval protein of Drosophila melanogaster. EMBO J. 5: 2925-2934