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Tramtrack controls glial number and identity in the *Drosophila* embryonic CNS

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SUMMARY

Neurons and glia are often derived from common multipotent stem cells. In *Drosophila,* neural identity appears to be the default fate of these precursors. Stem cells that generate either neurons or glia transiently express neural stem cell-specific markers. Further development as glia requires the activation of glial-specific regulators. However, this must be accompanied by simultaneous repression of the alternate neural fate. I show that the *Drosophila* transcriptional repressor Tramtrack is a key repressor of neuronal fates. It is expressed at high levels in all mature glia of the embryonic central nervous system. Analysis of the temporal profile of Tramtrack expression in glia shows that it follows that of existing glial markers. When expressed ectopically before neural stem cell formation, Tramtrack represses the neural stem cell-specific genes *asense* and *deadpan*. Surprisingly, Tramtrack protein levels oscillate in a cell cycle-dependent manner in proliferating glia, with expression dropping before replication, but re-initiating after S phase. Overexpression of Tramtrack blocks glial development by inhibiting S-phase and repressing expression of the S-phase cyclin, *cyclin E*. Conversely, in *tramtrack* mutant embryos, glia are disrupted and undergo additional rounds of replication. I propose that Tramtrack ensures stable mature glial identity by both repressing neuroblast-specific genes and controlling glial cell proliferation.

Key words: Tramtrack, gliogenesis, *Drosophila*, CNS

INTRODUCTION

The development and correct functioning of the nervous systems of all higher eukaryotes requires the production of two cell types: neurons, and non-neuronal accessory cells or glia. Numerous studies have indicated that both cell types can be derived from the same multipotent progenitor cells. In mammals, pluripotent stem cells that can give rise both to neurons and a variety of glial types have been isolated from central nervous system (CNS) preparations (Morrison et al., 1999; Stemple and Anderson, 1992). Individual cell types are formed by successive differentiation of these multipotent progenitor cells into lineage-restricted precursor cells that can continue to proliferate, or exit the cell cycle and differentiate into one of a narrow repertoire of cell types (Raff et al., 1988; Temple and Raff, 1986).

In the *Drosophila* embryonic CNS similar bipotential precursor cells have been identified. Each abdominal segment of the lateral CNS is derived from 31 progenitor cells. Lineage studies have shown that these precursor cells either produce only neurons (neuroblasts), exclusively glia (glioblasts) or both neurons and glia (neuroglioblasts; Bossing et al., 1996; Ito et al., 1995; Schmidt et al., 1997). Two well-characterized glia-producing stem cells are the longitudinal glioblast (LGB) and the neuroglioblast 6-4. The longitudinal glioblast exclusively generates glia. It divides to produce glia that eventually ensheath the longitudinal axonal tracts of the CNS (Ito et al., 1995; Jacobs et al., 1989). However, the precise number of cell divisions that this progenitor undergoes is unclear, as are the mechanisms that regulate the final longitudinal glial number. In thoracic segments, the neuroblast 6-4 divides to generate a neural stem cell that produces four to six neurons, and a glioblast that generates three glial cells (Akiyama-Oda et al., 1999; Bernardoni et al., 1999; Schmidt et al., 1997).

Neural fate appears to be the default identity of all these progenitors. Both neuroblasts, and glioblasts and neuroglioblasts, transiently express neural stem cell markers such as the pan-neural genes *asense* and *deadpan* during their development (Bernardoni et al., 1999; Dominguez and Campuzano, 1993; Jarman et al., 1993). Further development into glia requires the activation of a glial-determining pathway. In glia of the lateral CNS, transient activation of the binary switch *glial cells missing* (*gcm*) induces glial differentiation (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Gcm expression is succeeded by expression of the Ets transcription factor *pointedP1* (*pntP1*; *pnt* – FlyBase) and the homeodomain protein *reversed polarity* (*repo*) both of which enforce the glial differentiation pathway initiated by *gcm* (Halter et al., 1995; Klaes et al., 1994; Klämbt, 1993).

The switch to glial fate requires also the simultaneous repression of the previous and alternative neuronal fate. The zinc-finger transcriptional repressor Tramtrack (Ttk) has been

suggested to perform this function (Badenhorst et al., 1996; Giesen et al., 1997). The *ttk* locus encodes two DNA-binding proteins – Ttk69 and Ttk88 – which share a common N-terminal domain, the BTB (Bric-à-Brac-Tramtrack-Broad) motif, but have divergent C-terminal zinc-finger domains and, it is assumed, DNA-binding specificities (Harrison and Travers, 1991; Read and Manley, 1992). Ttk69 is expressed in all mature glia and ectopic expression can block neuron development (Giesen at al., 1997). I analyzed the timing of Ttk69 expression in glia and found that its expression succeeds that of existing markers of glial determination. I show that Ttk69 represses the neural stem cell-specific genes *asense* and *deadpan*. Ttk69 also can control glial proliferation. Thus, in proliferating glia, protein levels oscillate in a cell cycle-dependent manner. Expression is not detectable during DNA replication, but re-initiates after S-phase. Overexpression of Ttk69 prevents cell cycle progression by inhibiting expression of the S-phase cyclin, *cyclin E* and blocks glial development. Ttk69 controls glial development by both regulating glial cell number as well as identity.

MATERIALS AND METHODS

Genetics and *Drosophila* strains

Flies were raised at 25°C. Unless specified, all mutations and genetic aberrations used are as described by Lindsley and Zimm (Lindsley and Zimm, 1992). Enhancer trap and reporter lines used were: rA87, a *gcm* enhancer trap line (Jones et al., 1995; Vincent et al., 1996); the *dpn* promoter constructs dA (+) and dA (–), which contain 5 kb of the *dpn* enhancer upstream of *lacZ* (Emery and Bier, 1995); hkb^{AI7} (Myat and Andrew, 2000); and eg^{P289} (Dittrich et al., 1997). Homozygous *ttk* mutant embryos were unambiguously identified either using antibodies against the Ttk69 zinc fingers or based on the characteristic gut and dorsal closure phenotypes.

Directed protein overexpression

Targeted expression of Ttk69 in the embryonic CNS used the Gal4-UAS system (Brand and Perrimon, 1993). Full-length Ttk69 cDNA was cloned into pUAST, placing it under the control of the Gal4 UAS. Several independent UAS-Ttk69 lines were established by P element-mediated transformation. Gal4 lines used were: *Kr*-Gal4 (expresses in T2-A4); *sca*-Gal4 (expresses in neuroblasts and their progeny) (Klaes et al., 1994); and *MZ158*0 (expresses in the longitudinal glia and its progeny, MP2 neurons and macrophages) (Hidalgo et al., 1995).

Immunocytochemistry and immunofluorescence

Embryo fixation and immunocytochemistry were performed as described in Halter et al. (Halter et al., 1995). For immunofluorescence, FITC-, Cy3- and Cy5-conjugated anti-IgG secondary antibodies (Jackson Immunoresearch) were used. Biotinylated anti-IgG secondary antibodies and FITC-, Cy5- or Cy3-conjugated streptavidin (Jackson Immunoresearch) were used where signal enhancement was required. Embryos were mounted in phosphate-buffered saline (PBS) containing 90% glycerol and 1 mg/ml phenylenediamine and viewed using an MRC 1024 confocal microscope. Digoxigenin-labeled ribroprobes were prepared and RNA in situ hybridization performed as described by Lehmann and Tautz (Lehmann and Tautz, 1994). DNAs used for probe preparation were a cDNA corresponding to *cyclin E* type II transcript.

Antibodies used were: mouse mAb 22C10 at 1:40 (Fujita et al.,

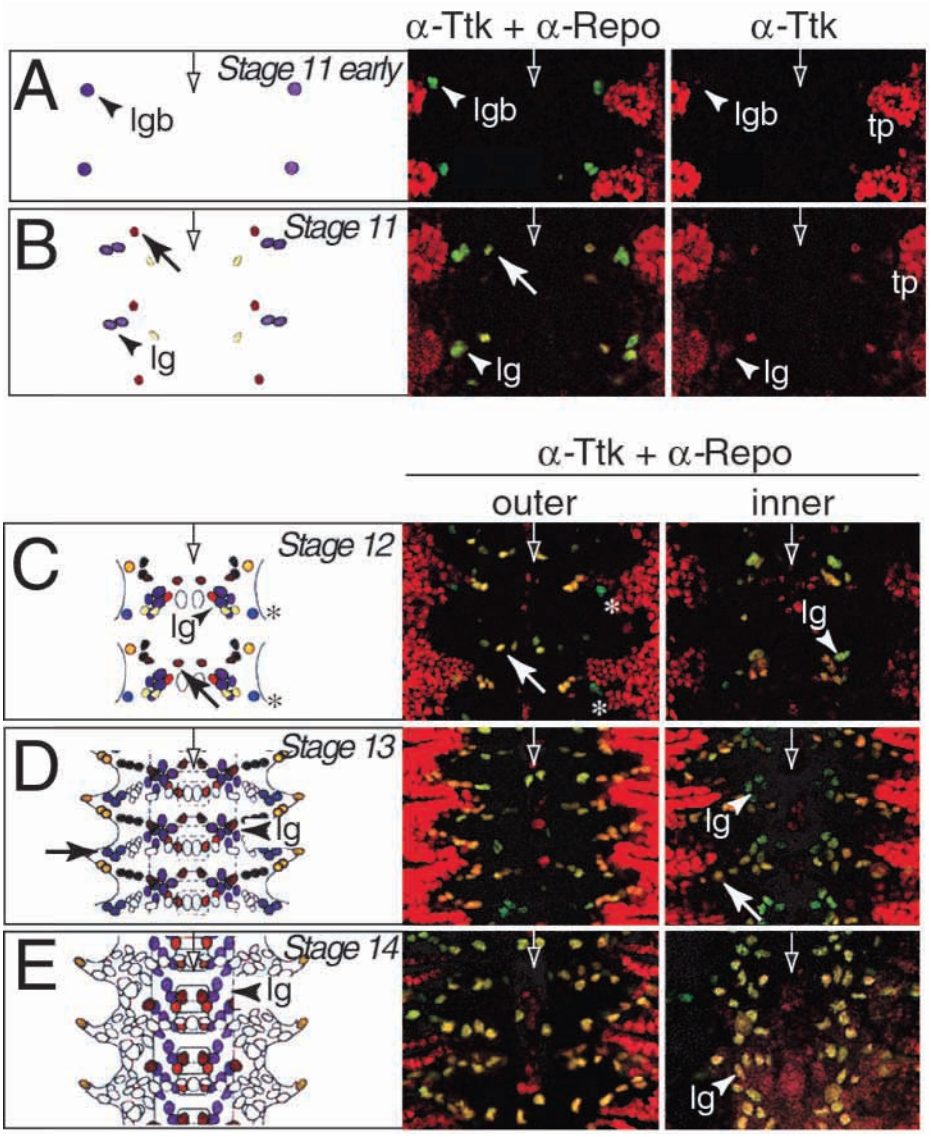

Fig. 1. Ttk69 is expressed after Repo in lateral glia. (A-E) Ttk69 (red) and Repo (green) expression in lateral glia of early Stage 11 to Stage 14 embryos. Panels show either a merge of Ttk69 and Repo (co-expression is denoted by a yellow overlap) or Ttk69 expression alone. Later stages show separate confocal sections through either outer or inner layers of the CNS, allowing overlying glial populations to be distinguished. A schematic (Halter et al., 1995) of Repo-expressing glia at every embryonic stage is shown. Anterior is upwards; open arrow, midline; lgb, longitudinal glioblast; lg, longitudinal glia (colored purple in schematic); tp, tracheal pits; asterisk, glioblast 3-1 (Schmidt et al., 1997).

1982); mouse mAb 9F8A9 and rat mAb 7E8A10 anti-Elav at 1:40 (O'Neill et al., 1994); rabbit anti-Repo at 1:100 (Halter et al., 1995); mouse mAb 990E5F1 anti-Ac at 1:40 (Skeath and Carroll, 1992); rabbit anti-Ase at 1:1000 (Jarman et al., 1993); mouse mAb anti-BrdU at 1:50 (Becton Dickinson); rabbit anti-*lacZ* at 1:1000 (Cappel); rat anti-Ttk69 at 1:50; rat anti-Ttk88 at 1:50; and rabbit anti-Ttk69 (zinc fingers) at 1:100 (Lehembre et al., 2000). Embryos were staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985).

BrdU incorporation

Bromodeoxyuridine (BrdU) incorporation was performed as described by Lehner et al. (Lehner et al., 1991) with the following modifications. After fixation, embryos were washed four times in PBT (PBS + 0.1% Triton X-100) and then incubated with two changes of 2 N HCl for 30 minutes. Thereafter, embryos were washed with multiple changes of PBT for 1 hour and immunofluorescence performed as above.

RESULTS

Temporal and spatial pattern of Ttk69 expression in the embryonic CNS glia

The 69 kDa Tramtrack isoform (Ttk69) is the only transcriptional regulator known to be expressed in all mature glia of the *Drosophila* embryonic CNS. At late stages of embryonic development Ttk69 is expressed in both midline and lateral glia (Giesen et al., 1997). As a first step to determine the function of Ttk69 in glia I analyzed the temporal profile of Ttk69 expression.

Confocal microscopy showed that Ttk69 is expressed late in glial development. In lateral glia, double labeling showed that Ttk69 is expressed after either Gcm, the ETS protein PointedP1 (not shown) or Repo (Fig. 1). For example, when Repo is first detected in the longitudinal glioblast, Ttk69 can not be detected. Later, however, low level expression of Ttk69 commences in this progenitor (Fig. 1A).

After the longitudinal glioblast divides, Ttk69 continues to be expressed at a low level while the daughters divide and migrate towards the midline (Fig. 1B-D). However, once these glia adopt their correct positions overlying the longitudinal tracts, high levels of Ttk69 are detected (Fig. 1E, arrowhead). This expression profile is recapitulated by the glioblast derived from neuroblast 3-1 (asterisk in Fig. 1C) (Schmidt et al., 1997). Initially, Ttk69 is not expressed. Ttk69 expression initiates later and finally is expressed at high-levels in the progeny glia (Fig. 1D, arrows).

In contrast, glia which express Repo and do not undergo further division express high levels of Ttk69 from the moment Repo can first be identified. An example is the cell body glia (Fig. 1B,C; arrows). In no case, was I able to detect expression of Ttk69 before that of Repo.

Neural targets repressed by Ttk

The timing of Ttk69 shows that it does not initiate glial determination. It has been proposed that Ttk69 is expressed in glia to repress neural identity genes (Badenhorst et al., 1996; Giesen et al., 1997). Lateral glioblasts transiently express neural stem cell markers during their development (Bernardoni et al., 1999) and can adopt the neuronal fate when the glial-determining pathway is not initiated in *gcm* mutants. Stable glial identity could require neuronal repression. To determine neuronal-specific genes repressed by Ttk69, I analyzed how ectopic expression of Ttk69 at various stages of nervous system development affected expression of the hierarchy of neuronal markers. This included the proneural genes of the *achaete-scute* complex, the pan-neural genes (for example *asense*) and the mature neuronal markers Elav and the antigen 22C10.

Ectopic expression of Ttk69 at any stage did not prevent neuroblast formation. Thus, expression of Ttk69 before neuroblast formation using *Kr-Gal4* does not repress the proneural genes *achaete* (Fig. 2B) or *lethal of scute* (data not shown). Strikingly, however, it does inhibit the pan-neural genes *asense* (Fig. 2C), *dpn* and *scratch*. Consequently, further neuronal development is inhibited and expression of both mature neuronal markers Elav (Fig. 2D) and 22C10 (data not shown) is ablated. Equivalent results were obtained by ectopically expressing Ttk69 in neuroblasts and their progeny using the *sca-Gal4* driver. Such expression almost completely inhibits the normal expression of *dpn* in the embryonic CNS (Fig. 2E,F; compare wild type and overexpressing lines).

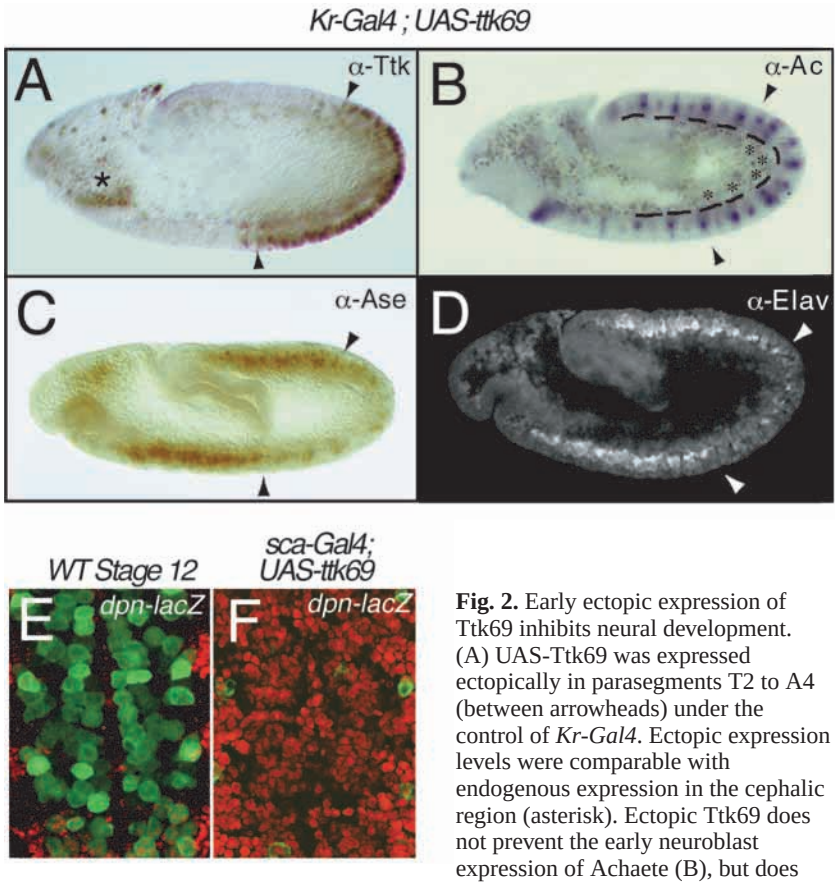

Fig. 2. Early ectopic expression of Ttk69 inhibits neural development. (A) UAS-Ttk69 was expressed ectopically in parasegments T2 to A4 (between arrowheads) under the control of *Kr-Gal4*. Ectopic expression levels were comparable with endogenous expression in the cephalic region (asterisk). Ectopic Ttk69 does not prevent the early neuroblast expression of Achaete (B), but does inhibit Asense expression (C) and the mature neural marker Elav (D). (E) Expression of a *dpn-lacZ* reporter (in green) is inhibited by ectopic expression of Ttk69 using *sca-Gal4* (F, Ttk69 revealed in red).

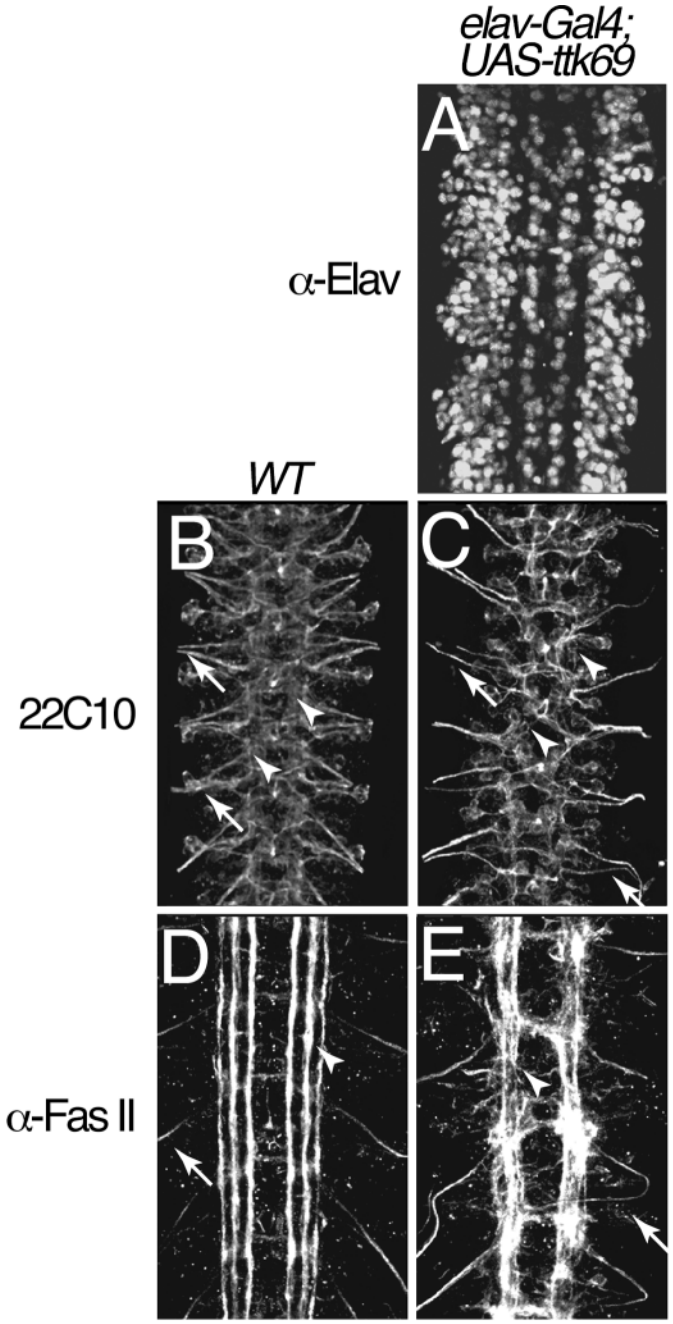

Fig. 3. Ectopic expression of Ttk69 in mature neurons disrupts axonal guidance. Late ectopic expression of Ttk69 in post-mitotic neurons, using the *elav-Gal4* driver, does not repress the neural markers Elav (A), 22C10 (C) or Fasciclin II (E). It does, however, affect the behavior of mature neurons. (B-C) MAb 22C10 staining shows that the longitudinal tracts are disrupted (arrowheads) and the segmental and intersegmental nerves exhibit fasciculation and pathfinding abnormalities (arrows). (D-E) Anti-Fasciclin II (mAb 1D4) staining confirms that the longitudinal fascicles are disorganized after Ttk69 ectopic expression and that the intersegmental nerve is misrouted and often fails to exit the CNS, instead migrating back towards the midline (arrows). The *elav-Gal4* driver also directs Ttk69 expression in neurons of the PNS. Strikingly, PNS organization was unaffected by this ectopic expression (data not shown).

If, however, Ttk69 is ectopically expressed after the normal neuroblast expression of *asense* and *deadpan*, neurons were not ablated. Thus, directed expression of Ttk69 using *elav-Gal4* (which is expressed in all post-mitotic neurons after the phase of pan-neural gene expression (Lin and Goodman, 1994)) did not repress the neural markers Elav, 22C210 or Fasciclin II (Fig. 3A,C,E). This indicates that the neural stem cell-specific genes *asense* and *deadpan* are the principal targets of Ttk69 repression in the hierarchy of neural determination. Moreover, neural identity, once conferred, cannot be reversed by Ttk69 overexpression as Ttk69 expression cannot switch neurons to the alternative glial fate.

Ectopic Ttk69 expression in mature neurons did, however, disrupt nervous system organization. Specifically, neurons showed pathfinding and fasciculation defects. As shown in Fig. 3E, the normal pattern of three longitudinal fascicles revealed using antibodies against Fasciclin II fails to form when Ttk69 is expressed in mature neurons. The Fascicles are fused and fail to separate. Pathfinding defects are also observed in the segmental nerve. These abnormalities are confirmed by mAb 22C10 staining (Fig. 3C).

Ectopic expression of Ttk69 also blocks glial formation

In the *Drosophila* embryonic and larval PNS ectopic expression of Ttk69 can convert neurons into non-neuronal cells, while, conversely, loss of *ttk* leads to the opposite transformation (Guo et al., 1995; Guo et al., 1996). In the CNS, however, neuron loss induced by ectopic Ttk69 expression does not lead to increased glial number. In fact, ectopic expression of Ttk69 in the embryonic CNS also blocks glial development. I found that normal glial development is inhibited by early overexpression of Ttk69. Thus, ectopic expression of Ttk69 before precursor formation, using *Kr-Gal4*, extinguishes the lateral glial markers Gcm and Repo in the domain of expression (Fig. 4A,B).

The block to glial development is not simply caused by the failure of stem cells to develop. Later ectopic Ttk69 expression also can inhibit glial development. Thus, longitudinal glia are ablated when Ttk69 is only expressed after the formation of the LGB using the *sca-Gal4* driver. *sca-Gal4* does not direct high levels of expression in the longitudinal glia when they are first formed (Fig. 4E, stage 11). Consequently, Repo expression in the LGB is unaffected. High level expression starts later and, by stage 12, glial number as revealed by Repo staining is reduced relative to the wild type (compare Fig. 4C,D). I confirmed these results using another driver, *MZ1580-Gal4* that induces Ttk69 expression in the LGB and its progeny (Hidalgo et al., 1995). When Ttk69 is ectopically expressed under the control of this driver, longitudinal glial number is reduced (Fig. 4F,G) although there is segmental variability in the penetrance of longitudinal glial suppression due to variability in expression levels.

Ttk69 is not detected in glia undergoing DNA replication

To understand why early ectopic Ttk69 expression inhibits glial development, I re-examined the temporal profile of Ttk69 expression in the CNS. Specifically, I analyzed Ttk69 expression in the longitudinal glia relative to replication marked by BrdU incorporation. The gross expression profile

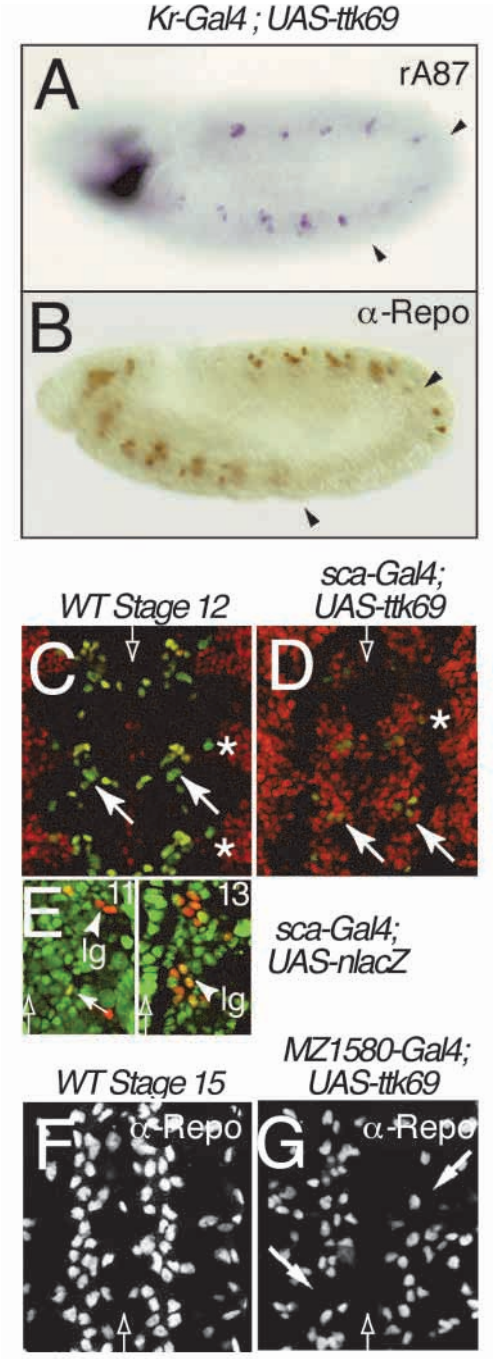

Fig. 4. Ectopic Ttk69 expression blocks glial development. Expression of the glial marker Gcm, revealed using the enhancer-trap line rA87 (A), and Repo (B) is inhibited by early Ttk69 expression using the *Kr-Gal4* driver. Later expression of Ttk69 in the progeny of glioblasts using the *sca-Gal4* driver also blocks glial development. (C) In stage 12 wild-type embryos, anti-Repo antibody staining (in green) stains between 13-15 glial cells per hemineuromere. (D) After ectopic expression of Ttk69 (revealed in red), the Repo-positive glial number is reduced to two to three cells per hemineuromere. The remaining glia are some longitudinal glia (arrows) and the glioblast 3-1 (asterisk) (E) Expression of a nuclear-targeted *lacZ* driven by *sca-Gal4* initially is low in the longitudinal glia (arrowhead, stage 11) but later increases (arrowhead, stage 13). Other glia such as cell body glia (arrows) express high levels of *lacZ* from the first. (F-G) Anti-Repo staining shows that overexpression of Ttk69 using *MZ1580-Gal4* reduces longitudinal glial number in the fully-formed stage 15 embryonic CNS (arrows in G; compare with control F). Anterior is upwards; open arrow, midline; lg, longitudinal glia.

in Fig. 1 illustrates the position of the longitudinal glia relative to the embryo co-ordinates and other glia. The longitudinal glioblast delaminates from the lateral edges of the neuroepithelium and as shall be seen, it divides symmetrically at least three times, the progeny migrating medially towards the ventral midline. The panels in Fig. 5 show high magnification of the longitudinal glia only – allowing Ttk69 expression relative to DNA replication to be clearly visualized. Ttk69 protein is undetectable in longitudinal glial cells when they undergo DNA replication as indicated by BrdU incorporation. Although the LGBs express Ttk69, Ttk69 is absent from their daughters when undergoing replication (Fig. 5, two-cell stage). However, later when these glia exit S phase, Ttk69 is again expressed at low levels. The longitudinal glia divide synchronously once more and appear to enter S phase shortly thereafter. Again, during DNA replication Ttk69 is undetectable (Fig. 5, four-cell stage), but after S phase is complete, Ttk69 expression reinitiates (four-cell stage). After this division, glia do not incorporate BrdU, and consistently express high levels of Ttk69 (Fig. 5, mature).

Ttk69 controls glial proliferation

The absence of Ttk69 from replicating glia implies that ectopic expression of Ttk69 may block normal glial development by inhibiting cell cycle progression. I examined whether ectopic expression of Ttk69 blocks entry into S-phase. BrdU incorporation is inhibited by ectopic expression of Ttk69 using the *Kr-Gal4* driver (Fig. 6A). In Ttk69-expressing segments of a Stage 10 embryo, the normal BrdU incorporation in the ventral neuroectoderm is inhibited (Fig. 6A, flanked by arrowheads). In the embryonic nervous system, entry into S phase is driven by bursts of transcription of S-phase cyclins – specifically *cyclin E*. I found that heat-shock induced overexpression of Ttk69 blocks zygotic transcription of *cyclin E* (compare wild type in Fig. 6B with *hs-ttk69* embryos in Fig. 6C). At earlier stages, maternally deposited *cyclin E* transcripts are unaffected by Ttk69 overexpression indicating that Ttk69 affects transcript synthesis rather than stability, consistent with its role as a transcription repressor. Ectopic expression of Ttk69 was induced by a 1 hour heat-shock, after which embryos were processed immediately for in situ hybridization. The rapidity of repression of *cyclin E* and the presence of multiple Ttk69 consensus recognition sites in the *cyclin E* promoters suggests that repression is direct.

As ectopic Ttk69 expression blocks replication, I examined if, conversely, loss of Ttk69 induces glial proliferation. Mutant embryos in which Ttk88 function only is affected, ttk^{1} (Xiong and Montell, 1993) are homozygous viable and have no obvious defects in their CNS development. Mutants affecting only Ttk69 or both Ttk69 and Ttk88 are embryonic lethal and the CNS is disorganized. In mutant embryos, glial development initiates correctly but, by later stages of development, glia are disorganized. In the case of the longitudinal glia, the LGBs are formed correctly and expression of Gcm and Repo is normal. However, BrdU

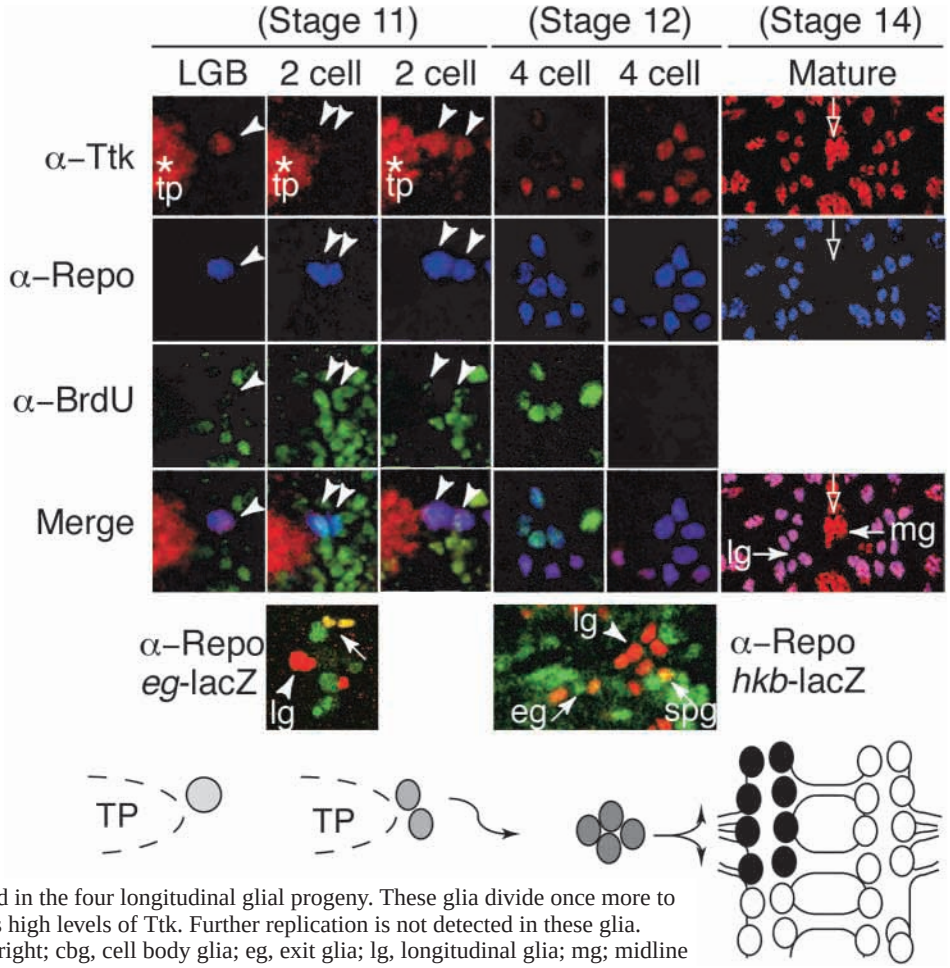

Fig. 5. Ttk69 is undetectable during DNA replication. DNA replication in glia was monitored by BrdU incorporation followed by triple labeling with anti-BrdU (green), anti-Repo (blue) and anti-Ttk69 (red) antibodies. Ttk69 is first expressed at low levels in the Repo-expressing longitudinal glioblast (LGB, arrowhead) at embryonic stage 11. The LGB divides and its progeny immediately enter S phase. During DNA replication, Ttk69 is not detected in the daughter cells (two-cell stage, arrowheads). But, once replication is complete, low level Ttk69 expression is again detected. Anti-Repo staining (red) of the *eg*^{P289} *lacZ* enhancer trap line (green; shown in the lowest panel) confirms the identity of the Repo-positive longitudinal glia relative to the Repo- and Eg-positive cell body glia (arrows). At stage 12, the longitudinal glia divide again, and immediately enter S phase and incorporate BrdU (four cell stage). Again, Ttk69 is absent during DNA replication. Two to three Repo-positive cells near the longitudinal glia do not undergo replication express high levels of Ttk69. These glia do not derive from the LGB: anti-Repo (red) and anti-*lacZ* (green) staining of *hkb*^{AI7} enhancer-trap line shows that they are probably exit glia and sub-perineural glia (lowest panel, arrows). After this stage, BrdU is not incorporated in the longitudinal glia and Ttk69 is expressed in the four longitudinal glial progeny. These glia divide once more to generate eight glial cells, all of which express high levels of Ttk. Further replication is not detected in these glia. Anterior is upwards, the midline towards the right; cbg, cell body glia; eg, exit glia; lg, longitudinal glia; mg; midline glia; spg, sub-perineural glia; tp, tracheal pit; arrows in mature panels, midline.

incorporation shows that glia undergo ectopic rounds of replication. For example, at stage 12 in wild-type embryos, the LGB has divided twice to generate four longitudinal glial precursors that can be trapped undergoing replication (Fig. 7A). In contrast, in equivalent stage *ttk* mutant embryos, between 6-7 glia can be observed to undergo DNA replication (Fig. 7B). In wild-type animals, these glia divide once more to produce the final eight glia which intermingle with at least three glia derived from other sources and migrate along the longitudinal connectives (Fig. 7C). Confocal analysis of anti-Repo stained wild-type embryos indicates that in each hemisegment there are 9.9±0.76 glia associated with the longitudinal connectives. In the equivalent stage *ttk* mutant embryos longitudinal glial number in each hemi-segment, as revealed by anti-Repo staining, is increased to 17.8±2.23 (Fig. 7D). Thus, loss of Ttk69 results in additional glial cells being generated in the longitudinal glial lineage.

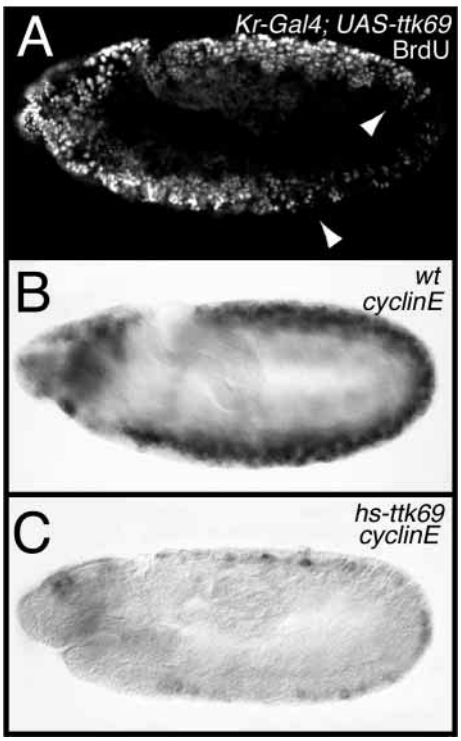

Fig. 6. Ectopic expression of Ttk69 inhibits replication and represses *cyclin E*. (A) *Kr-Gal4*-mediated expression of Ttk69 blocks BrdU incorporation in the domain of expression (flanked by arrowheads). Failure to replicate is due to repression of *cyclin E*. Expression of *cyclin E* in the wild-type CNS (B) is inhibited by ectopic expression of Ttk69 in heat-shocked *hs-ttk69* embryos (C).

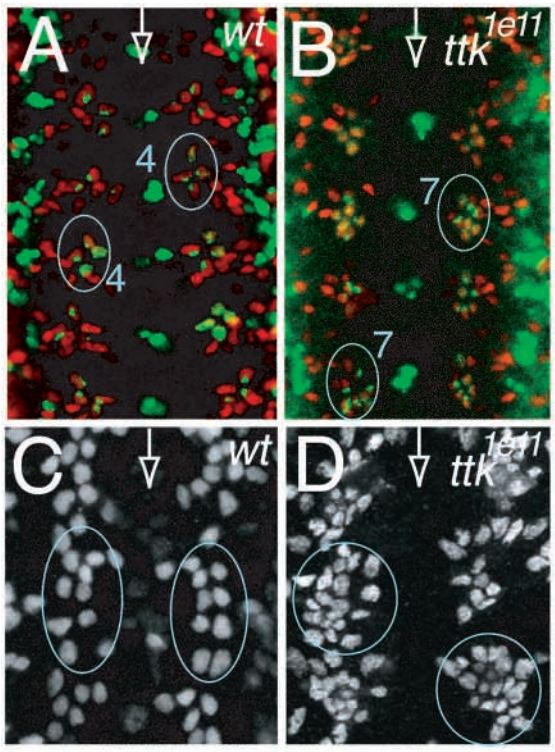

Fig. 7. Glia undergo extra rounds of DNA replication in *ttk* mutant embryos. After BrdU incorporation, wild-type (A) and *ttk*^{1e11} mutant embryos (B) were stained with anti-Repo (red) and anti-BrdU (green) antibodies. In wild-type stage 12 embryos, four Repo-positive longitudinal glia in each hemi-neuromere incorporate BrdU. By contrast, six to seven glia undergo DNA replication in mutant embryos of the equivalent stage. The number of Repo-positive glia associated with the longitudinal connectives is increased from 9.9±0.76 in wild-type embryos (C) to 17.8±2.23 in *ttk* mutant embryos (D). Data are mean±s.d. of 20 determinations. Anterior is upwards; open arrows, midline.

DISCUSSION

This investigation characterizes the expression and function of the zinc-finger transcription factor Tramtrack in the *Drosophila* CNS. Data that were obtained are consistent with Ttk69 acting to maintain glial differentiation. This is achieved in two ways. First, Ttk69 represses pan-neural genes, key elements in the regulatory hierarchy leading to neural development. Thus, the transition of multipotent progenitor cells to glial rather than neuronal fates is reinforced. Second, Ttk69 has the ability to block expression of the S-phase cyclin *cyclin E*, thus preventing entry into the cell-cycle. In this manner, the determined state is maintained.

Glial versus neuronal specification

The widespread expression of Ttk69 and its timing are consistent with Ttk69 maintaining glial identity rather than initiating glial determination. Previous investigations have shown that the two main populations of *Drosophila* CNS glia, midline glia and lateral glia, are specified by distinct mechanisms. For example, lateral glia require Gcm to initiate development, while midline glia do not (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Yet Ttk69 is expressed in both populations – the only transcription factor expressed in common. Giesen et al. (Giesen et al., 1997) have suggested that glial determination requires two independent steps: first, a proglial function, for example, Gcm (Hosoya et al., 1995; Jones et al., 1995; Vincent et al, 1996), that initiates glial development; and second, an anti-neural activity that downregulates the previous potential to form neurons. The ability of Ttk69 to repress to neuron development shows that it performs the later role and in this report I have identified the targets of neuronal repression.

In the *Drosophila* CNS, neural development requires the expression of a cascade of transcription regulators, initiating with high level expression of the proneural genes of the Achaete-Scute complex (AS-C), leading to expression of neural stem cell-specific genes *asense* and *deadpan*, and ultimately a battery of specific neural identity genes. Repressing any of these factors could inhibit neural development. Staged overexpression of Ttk69 shows that, in the CNS, Ttk69 inhibits expression of *asense* and *deadpan*. In this manner, the ability to adopt neuronal fate is blocked.

To date, in mammals, a master regulator of glial development analogous to Gcm has not been identified. Although mammalian homologs of the *Drosophila* gene *gcm* have been isolated (Kim et al., 1998), there is no evidence that they play a role in glial determination. However, recently, Notch signaling has been shown to allow some multipotent stem cells in the nervous system to differentiate preferentially into glia (Furukawa et al., 2000; Gaiano et al., 2000; Hojo et al., 2000; Morrison et al., 1999). Two downstream components of the Notch pathway have been implicated: the Hairy-E(Spl)-related bHLH repressors – Hes1 and Hes5 (Furukawa et al., 2000; Hojo et al., 2000). Both are thought to act as repressors of the neuronal specific bHLH genes *Mash1*, *NeuroD* and *Math3* (Morrow et al., 1999; Takebayashi, et al., 1997; Tomita et al., 1996). These studies, however, have not clarified whether Notch signaling acts instructively to promote glial development or whether repression of the neuronal specific bHLH genes permits multipotent stem cells to respond to other instructive signals that induce gliogenesis. If Hes1 and Hes5 act permissively, the parallels between vertebrate and *Drosophila* gliogenesis would be striking. In *Drosophila*, Ttk69 can be induced by Notch signaling (Guo et al., 1996). As seen above, Ttk69 represses the neuronal-specific bHLH gene *asense*. Ttk69 itself does not induce glial fate but blocks the alternative neuronal fate.

Control of proliferation

It is clear from the current study that, at least in some glial populations, Ttk69 has the ability to regulate proliferation. In the *Drosophila* glioblasts Ttk69 is expressed at low levels soon after glial specification, blocking neural genes. This expression is not constant, though, but oscillates during the cell cycle. Significantly, Ttk69 can not be detected when glia enter S phase and commence DNA replication. Like neuroblasts (Weigmann et al., 1997), glioblasts appear to delaminate in G2 of the cell cycle. The timing of BrdU incorporation indicates that immediately after mitosis they enter S phase and undergo DNA replication. Although Ttk69 is expressed in glia in G2, Ttk69 is not detected in glia that incorporate BrdU. As Ttk69 can repress *cyclin E* expression, the absence of Ttk69 allows replication to occur. Once replication is complete, Ttk69 is

expressed again. The BrdU incorporation experiments indicate that the LGB undergoes three synchronous cell divisions to produce eight longitudinal glia. This agrees well with a recent estimate of between 7-9 longitudinal glia obtained by DiI labeling of the longitudinal glioblast (Schmidt et al., 1997). After the third mitosis, longitudinal glia do not undergo replication but instead express higher levels of Ttk. By inhibiting *cyclin E*, high levels of Ttk69 would keep glia in G1 of the cell cycle. Similarly, differentiation of oligodendrocytes (Durand et al., 1998) and Müller glia (Ohnuma et al., 1999) is accompanied by high levels of the cyclin-dependent kinase inhibitor p27, blocking re-entry into the cell cycle.

I determined longitudinal glial number in *ttk* mutant embryos at embryonic stage 14, as the longitudinal glia first extend along the longitudinal axonal tracts. At later stages of development, glia in *ttk* mutant embryos migrate inappropriately. The regular array of glia along the longitudinal connectives is lost as glia collapse towards the midline. Antibody staining against the nuclear glial antigen Repo indicates nuclear fragmentation, which is evidence of possible cell death. It is feasible that inappropriate proliferation triggers apoptosis. When Giesen et al. (Giesen et al., 1997) examined CNSs dissected from late-stage *ttk* mutant embryos, they reported that glial number (revealed by the enhancer trap-line rI50) is reduced. Differences between glial number reported here could be due to the stage at which determinations were made.

In its ability to repress pan-neural genes and control proliferation, Ttk69 resembles the homeodomain protein Prospero. Prospero is not detected in the nuclei of neuroblasts but is detected in the nuclei of their daughter ganglion mother cells (GMCs). GMCs divide once and terminally differentiate. In GMCs, Prospero appears to be required to repress the pan-neural genes *asense* and *deadpan*, thus enforcing the transition from neuroblast to GMC (Vaessin et al., 1991). Recently, it has been shown that loss of Prospero leads to ectopic proliferation in the CNS, suggesting that it is required for exit of GMCs from the cell cycle (Li and Vaessin, 2000). Ttk69 appears to play an analagous function in glia.

Dynamic regulation of Ttk69 levels

The oscillation in Ttk69 protein levels during the cell cycle demonstrates that there is dynamic control of Ttk expression. Several mechanisms may regulate Ttk69 protein levels. Ttk69 has PEST sequences characteristic of short-lived proteins (Harrison and Travers, 1991). Moreover, both isoforms of Ttk have been shown to be targeted for ubiquitin-dependent proteolysis by a complex containing the ring-finger proteins Sina and Phyllopod (Li et al., 1997). It is possible that Ttk69 is destroyed by regulated proteolysis prior to initiation of S-phase. It is interesting in this regard that Ebi, a modifier of over-proliferation phenotypes associated with E2F/DP overexpression, has been shown to interact with Sina and Phyllopod and targets destruction of at least one Ttk isoform in vitro (Boulton et al., 2000). Another dimension is added by the recent report that Ttk69 translation can be repressed by the RNA-binding protein Musashi (Okabe et al., 2001). Translational repression by Musashi is not constitutive but appears to be regulated, in part, by signaling through the Notch pathway. The intersection between regulated translational repression and targeted proteolysis of residual protein provides exquisite control of protein levels.

In conclusion, these results demonstrate that Ttk69 maintains glial differentiation. This is achieved in two ways. First, Ttk69 represses the neural stem cell-specific genes which prevents the reprogramming of glia into neurons. Second, Ttk69 inhibits inappropriate proliferation of glia. In this way CNS organization is preserved.

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