A Transient Expression of Prospero Promotes Cell Cycle Exit of Drosophila Postembryonic Neurons through the Regulation of Dacapo

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Abstract
Cell proliferation, specification and terminal differentiation must be precisely coordinated during brain development to ensure the correct production of different neuronal populations. Most Drosophila neuroblasts (NBs) divide asymmetrically to generate a new NB and an intermediate progenitor called ganglion mother cell (GMC) which divides only once to generate two postmitotic cells called ganglion cells (GCs) that subsequently differentiate into neurons. During the asymmetric division of NBs, the homeodomain transcription factor PROSPERO is segregated into the GMC where it plays a key role as cell fate determinant. Previous work on embryonic neurogenesis has shown that PROSPERO is not expressed in postmitotic neuronal progeny. Thus, PROSPERO is thought to function in the GMC by repressing genes required for cell-cycle progression and activating genes involved in terminal differentiation. Here we focus on postembryobionic neurogenesis and show that the expression of PROSPERO is transiently upregulated in the newly born neuronal progeny generated by most of the larval NBs of the OL and CB. Moreover, we provide evidence that this expression of PROSPERO in GCs inhibits their cell cycle progression by activating the expression of the cyclin-dependent kinase inhibitor (CKI) DACAPO. These findings imply that PROSPERO, in addition to its known role as cell fate determinant in GMCs, provides a transient signal to ensure a precise timing for cell cycle exit of prospective neurons, and hence may link the mechanisms that regulate neurogenesis and those that control cell cycle progression in postembryonic brain development.

Introduction
In order to give rise to the diversity and specificity of cells types in the brain, cell proliferation, specification and terminal differentiation must be precisely coupled in space and time during development to ensure the correct number of cells in different populations and specify their resulting connectivity. Recent work has shown that the postembryonic central nervous system (CNS) of Drosophila is a suitable experimental model to study the genetic basis of some of these processes, including neural proliferation, cell lineage specification, and asymmetric division of neural progenitor cells, as well as tumorigenesis if these processes are perturbed [reviewed by 1–3].

The CNS of Drosophila is composed of two brain hemispheres and the ventral ganglia. The adult CB develops in the medial regions of each hemisphere, while the adult OLs develop laterally (see Fig. 1A, B for a schematic summary). Most of the cells comprising the adult brain are generated from progenitor cells called neuroblasts (NBs) that become quiescent at the end of embryonic development and that re-enter the cell cycle at different times during larval development depending on the region and cell type.

Proliferation during postembryonic development of the OL and CB has been studied extensively. Each optic lobe (OL) is generated from three neuroepithelia called the LPC (Lamina precursor cells), OPC (outer proliferation centre) and IPC (inner proliferation centre) [4,5] which give rise to the adult lamina, medulla, and lobula, respectively. OPC and IPC neuroepithelial progenitors switch from symmetric, proliferative to asymmetric, neurogenic divisions during the third instar stage [1,6,7]. Thus, most neurogenesis takes place in the OL at the end of larval development [4,5,8,9].

By contrast, most of cells of the adult CB originate from a number of scattered NBs located medially in the hemispheres, which proliferate from the first instar stage until the beginning of pupal development [4,8,10–13]. Two main different types of NBs have been found in the CB. Most of the NBs (Type I) follow patterns of proliferation similar to those of embryonic NBs, although they produce more cells in each lineage. Thus, each Type I NB divides asymmetrically several times to generate in each division a new NB and an intermediate GMC progenitor which divides once to generate two postmitotic daughters called ganglion cells (GCs) that differentiate into neurons [3,6,14] (Fig. 1C). A smaller group of Type II NBs has a different
Figure 1. Cellular Pattern of PROSPERO Protein Expression in the larval brain. A–C. Morphology, cellular organization and pattern of division in the larval CNS. A. Schematic representation of a late larval CNS which is composed of two brain hemispheres and ventral ganglia. The central brain (CB) develops in the medial regions of each hemisphere, while the optic lobes (OL) primordia are located laterally. B. Schematic drawing of a brain hemisphere showing the scattered distribution of CB NBs in the medial part and the LPC (Lamina precursor cells) and OPC (outer proliferation centre) neuroepithelia located laterally. C. The typical pattern of division of type I CB and OPC NBs. Each NB divides asymmetrically several times to generate a new NB and a ganglion mother cell (GMC) that divides once to generate two postmitotic ganglion cells (GCs). D–F. Co-expression and subcellular co-localization of PROS and MIRA in the OPC. Late third instar larval brains were immunostained with PROS and MIR antisera and analyzed by confocal microscopy. Images focusing on the same region of the OPC in three confocal sections of the same OL taken at different ventro-dorsal levels. Note the strong PROS labeling in the nuclei of the GCs located below the superficial MIR+ NBs which, apart from a few
exceptions (filled arrows), mostly lack PROS (empty arrows). Similarly, in only a few GMCs (medium size MIR+ cells located in the first internal layer, just below the NBs) did MIR and PROS co-localize (filled arrowheads) while in most of them PROS was hardly detected (empty arrowheads). G. Representation of the OPC showing the NBs located on the surface, which generate their progeny radially inside de OL. These NBs localize asymmetrically MIRA during their division. The progeny express nuclear PROS. H–J. Representative examples of CB NBs and their nearby progeny immunostained with PROS and MIRA. H. Most frequently NBs with asymmetric MIRA lacks PROS immunostaining (empty arrow). I. Nevertheless, in some cases some NBs with asymmetric MIRA showed PROS signal (filled arrow) but this is much weaker than that observed in the nuclei of the surrounding GCs. J. Notice that PROS signal is also weaker in the nuclei of single weak MIRA+ cells attached to the NBs (small arrows, putative GMCs) than in the nuclei of the surrounding GCs. K–M. Representative examples of mitotic CB NBs and their nearby progeny immunostained with PROS and Ph3. Only a few among the mitotic CB NBs showed asymmetric PROS signal (K, filled arrow) while most mitotic NBs (L, M; empty arrows. showed low if any) PROS immunosignal. Some mitotic GMCs exhibited nuclear PROS signal (M; arrowhead) although weaker than the surrounding GCs. N. Representation of a CB NB lineage which summarizes the pattern of expression of GRH, PROS and ELAV showed in panels O–P. O–P. Representative examples of CB NBs and their nearby progeny immunostained with GRH, ELAV and PROS. O. One GRH+ NB (empty arrow) and its neighbor GRH+ GMC (empty arrowhead) lack PROS labeling while all the surrounding ELAV+ GCs exhibit strong PROS signal. P. One of the less frequent cases in which a GRH+ GMC (filled arrowhead) located close to a NB, exhibited PROS signal. Q. Image showing a pair of GRH-/PROS+/ELAV- cells (small arrows) attached to a NB. Note that the rest of the progeny is GRH-/PROS+/ELAV+. doi:10.1371/journal.pone.0019342.g001

proliferative mode that involves intermediate progenitors with transit amplifying cell divisions [15–17].

During each division of embryonic NBs, the homeodomain transcription factor, PROSPERO (PROS) [18], due to its binding to the carrier protein MIRANDA (MIRA), is asymmetrically segregated from the parent NBs to its daughter GMC where it plays a key role as cell fate determinant (reviewed in [19]). In the GMC, PROS translocates to the nucleus and acts to repress the expression of cell-cycle regulators [20] and activate genes that direct terminal differentiation of neurons [21]. Recent work indicates that expression and action of PROS is similar in postembryonic Type I NBs and their GMC daughter cells [1,15,17,22–24]. However, there are several differences in the cellular pattern of PROS expression between embryonic and larval NB lineages [6] as well as in the phenotype of pros mutants in the embryonic and larval CNS [21–26]. Given these differences in expression and phenotypes, it seems likely that there might also be differences in the functional roles played by PROS during embryonic versus postembryonic CNS development.

To investigate this, we have performed a genetic, cellular and molecular analysis of the roles played by PROS in neural proliferation and neurogenesis during postembryonic development of the Drosophila brain. We focused on CB type I and OPC NB lineages. We find that a marked transient upregulation of PROS expression occurs in postmitotic GCs shortly after the division of their parent GMC. We provide evidence for the fact that this transient PROS upregulation inhibits cell cycle progression in the GCs. Furthermore, we identify the pan-neural bHLH transcription factor DEADPAN (DPN) and the cyclin kinase inhibitor DACAPO (DAP), as candidate downstream effectors of PROS in this function. In view of these findings, we discuss the implications of different roles of PROS in embryonic versus post-embryonic neurogenesis of Drosophila.

Materials and Methods

Drosophila strains and mosaic analysis

All the fly stocks used in this study were derived from Drosophila melanogaster and they were raised at 25°C on standard medium, except when particular temperature conditions were required (see below). The wt strains used were Berlin and Canton-S. Fly stocks carrying mutations, transgenes and recombinant chromosomes were: pros+/-;TM6,St [27], UAS-pros and pros+ [17] (Doc et al., 1991), UAS-cd8::GFP, UAS-nlsacGFP hs-Gal4/TM3 (Bloomington Stock Center), c820-Gal4 and c831-Gal4 [28]. To induce the overexpression of PROS, hs-Gal4;UAS-pros larvae were grown at 17°C until mid third instar stage. The level of PROS expression remained apparently normal under these conditions. Then, a short heat shock was applied at 37°C and the larvae were grown at 29°C for 15 h until wandering larval stage. Increased expression of PROS begins 6 h after the heat shock. Similarly, c831Gal4;UAS-pros larvae were grown at 17°C until mid third instar stage. Then, the temperature was shifted to 29°C for 12–15 h until wondering larval stage.

Bromodeoxyuridine (BrdU) labeling

In vivo BrdU labeling of whole-mount larval brains was carried out essentially as described previously [6] but with incubation times of 5–10 min. Larval brains of late third instar (wandering) larvae were dissected in Ringer’s solution and fixed for 3 min with modified Carney’s fixative followed by 75% EtOH for 30 min. After rehydration, the samples were denatured by treatment with 2 N HCl for 40 min and they were then neutralized by washing with phosphate buffer saline (PBS) before proceeding to the immunocytochemical analysis with an anti-BrdU antiserum (Beckton-Dickinson) and a horse radish peroxidase (HRP) coupled secondary antibody visualized with dianaminoazobenzidine (DAB).

Immunohistochemistry

Larval brains were dissected out in PBS and fixed for 30 min on ice with 4% paraformaldehyde in PBS, and then for a further 30 min with 4% paraformaldehyde, 0.1% Triton X-100 in PBS. After washing in PBS, the larval brains were sometimes dehydrated with 100% methanol and rehydrated stepwise to PBS. Brains were incubated with antisera overnight at 4–8°C in PBS containing 5% normal goat serum, 0.1% Triton X-100 and 0.02% Sodium Azide. The following primary antibodies were used: rabbit anti-Beta-Gal (Sigma immunochimicals), anti-DE-CADHERIN (a kind gift from T. Uemura); anti-activated CASPASE3 (Cell Signaling Technology), mouse and rat anti-CYCLIN E (a kind gift from H. Richardson), anti-DAP (a kind gift from I.K. Hariraran), guinea pig anti-DPN (a kind gift from J. Sheath), rat anti-ELAV (Developmental Studies Hybridoma Bank), mouse and rabbit anti-GFP (Invitrogen), rabbit anti-GRH (Almeida and Bray, 2005), rabbit anti-MIR (a kind gift from C. Gonzalez); rabbit anti-Phosphohistone-3 (PH3) (Upstate Biotechnology); mouse anti-PROS (MIR1, a kind gift from C. Doe). Fluorescent-labeled secondary antibodies (Jackson Immunchemicals) were used.
according to the manufacturer’s recommendations. For the detection of PROS, we used biotinylated secondary antibodies and Cy2 or Cy3 conjugated streptavidin or HRP-coupled secondary antibodies followed by Tyramide detection (TSA, Perkin Elmer). Immunolabeled samples were analyzed on a Leica TCS-SL spectral confocal microscope.

Fluorescent in situ hybridization (FISH)

Digoxigenin (DIG) or Fluorescein DNA labeled probes were synthesized by PCR. To prepare a pros probe, we used a 267 bp fragment corresponding to positions 4036–4303 of pros cDNA [26]. For dph we used a 253 bp fragment corresponding to positions 911–1163 of dph cDNA [31]. The hybridized probes were detected with HRP-coupled anti-DIG (Roche) and rabbit anti-Fluorescein (Molecular Probes) antibodies and visualised with secondary antibodies followed by Tyramide detection (TSA, Perkin Elmer). Immunolabeled samples were analyzed on a Leica TCS-SL spectral confocal microscope.

Analysis of cell death

Apoptotic cell death was monitored in the late third instar larval brains by immunohistochemical analysis of activated CASPASE-3, as described above.

Results

prospero expression is upregulated in new born post-embryonic neurons

Previous work on neurogenesis in the embryonic CNS has shown that PROS protein is expressed in NBs and GMCs but is lacking in postmitotic neural GCs [25,26]. In contrast, in postembryonic neurogenesis, high levels of PROS protein expression have been found in postmitotic GCs [6]. This suggests that PROS might have a novel function in postembryonic GCs. To investigate this, we first carried out a detailed immunocytochemical study of PROS expression in OPC and CB NBs in the third instar larval brain. For this we combined PROS immunolabeling with the mitotic PH3, the two NB/GMC markers, MIRANDA/MIRA and GRAINYHEAD (GRH) [32], and the pan-neuronal marker ELAV [33]. Together these markers made it possible to distinguish between PROS expression in NBs, GMCs and GCs (prospective neurons) (see Table 1). We limited our study to the OPC and CB Type I NB lineages since Type II [15,17] and mushroom bodies [34] NBs do not express PROS.

In the cells of the OPC, a marked difference in the level of PROS expression was observed in that GCs showed much stronger PROS expression than NBs and GMCs (Fig. 1D-F). Thus, high levels of PROS immunolabeling were consistently seen in the nuclei of GCs located inside the OPC. In contrast, relatively low levels of cortically localized and asymmetric PROS (together with MIRA) immunolabeling were observed in a subset of the NBs while no detectable PROS immunolabeling was observed in the remaining NBs. Similarly, the majority of the GMCs in the OPC had relatively low (or even undetectable) PROS immunostaining. Comparable findings were obtained for the CB (Fig. 1H-Q). Thus, strong and consistent PROS immunolabeling was detected in the nuclei of the postmitotic (ELAV-positive) GC progeny (Fig. 1O-Q). In contrast, relatively low (or undetectable) levels of PROS immunolabeling were seen in the majority of NBs, even in those that have asymmetrically localized MIRA (Fig. 1H-J) or expressed the mitotic marker PH3 (Fig. 1K-M). Relatively low (or undetectable) levels were also seen in most GMCs, independent of their mitotic status. Interestingly, we also frequently observed a pair of PROS+/GRH-/ELAV- cells attached to a NB (Fig. 1Q). According to this molecular marker profile, we identified these cells as recently born GCs.

Taken together, these findings indicate that in the larval brain the level of PROS expression is substantially higher in postmitotic GCs as compared to their NB and GMC progenitors. This markedly higher expression level in the progeny is unlikely to be due to the persistence of the little PROS protein detected in the GMC after being divided into the two daughter GCs. We therefore hypothesized that pros expression might be upregulated in new born neurons of the OPC and CB.

To test this notion, FISH was used to monitor the expression of pros mRNA together with the above mentioned molecular markers to distinguish cellular identities. This FISH analysis revealed a punctate distribution of pros mRNA in numerous GC-like cells in the OPC and CB (Fig. 2). Thus, in the CB PROS mRNA signal was most often observed in small MIRA+/GRH- cells located in the vicinity of NBs (Fig. 2A, B). In the OPC, we found labeled cells located in the GC layers immediately beneath the external layer of MIRA+ NBs and GMCs but rarely in more deep layers of older (differentiating) neurons, and we did not find consistent signal in most larval OL NBs (Fig. 2C). Nevertheless, we can not rule out the presence of pros transcripts in these NBs below our detection threshold. Together, these results support the idea that pros mRNA transcription is transiently upregulated in recently generated neurons (GCs). In further support of the transient nature of PROS expression in GCs, we observed that PROS protein signal in the OPC is markedly weaker after the third to fourth layer of postmitotic neurons as seen in whole mounts (e.g. Fig. 1F) and histological sections (data not shown). This strongly suggests that PROS protein is also down regulated as the GCs move to deeper layers in the OPC while differentiating into neurons.

Table 1. Differential expression of molecular markers in NBs, GMCs, and GCs.

| Marker | NB | Mitotic NB | GMC | Mitotic GMC | New born GCs | GCs |
|--------|----|-----------|-----|------------|-------------|-----|
| MIR    | ++ | Asymmetric | +++ | ++/–       | +/–         | –/+ |
| PH3    | –  | Nuclear    | +++ | Asymmetric | Cortex      | +/– |
| GRH    | –  | +/–        | +/– | –/+        | –/+         | +/– |
| PROS   | +++| Cortex     | +++ | n.d.       | ++/–        | +++ |
| ELAV   | –  | +/–        | –   | +++        | –/+         | +++ |

The level of immunostaining of the molecular markers in the different cell identities is described in an arbitrary comparative scale: absent (–), weak (+), intense (++), very intense (+++).

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Loss of prospero function increases mitotic activity and inhibits neurogenesis in the postembryonic brain

The transient upregulation of pros mRNA as well as the high levels of PROS protein in newly born neurons (GCs) suggested a possible novel role for PROS in the generation of postmitotic cells during postembryonic neurogenesis. To investigate this, we
PROS with the Gal4/UAS system [35] in immunolabeling (data not shown). Conversely, overexpression of (Fig. 3D, E G, H). Similar findings were obtained with Cyclin E compared to located underneath the layer of MIR OPC. Notice the presence of several PROS mRNA expressing cells studied the effects pros loss-of-function (LoF) and gain-of-function (GoF) on proliferation and neurogenesis on postembryonic brain development. Since pros null mutants are embryonic lethal, we first studied mitotic activity in pros−/−, a strong hypomorphic allele [27] that reduces PROS expression in the larval OLs (Fig. 3A, B). Our findings revealed a marked increase in the number of mitotically active (proliferating) cells in the larval brain of pros−/− hypomorphs compared to wt as determined by PH3 and BrdU immunolabeling (Fig. 3D, E G, H). Similar findings were obtained with Cyclin E immunolabeling (data not shown). Conversely, overexpression of PROS with the Gal4/UAS system [33] in hs-Gal4/UAS-pros larval brains resulted in a marked reduction in the number of mitotically active cells compared to wt when monitored with the same markers (Fig. 3D, F, G, I). This reduction in the number of mitotically active progenitor cells was seen both in the OPC and in the CB (Fig. 3J–Q). In the embryonic CNS, pros LoF also causes overproliferation but the supernumerary cells are eliminated by apoptosis [26]. In contrast, no increase in apoptosis was detected in the larval brain of pros−/− hypomorphs as monitored by the expression of activated CASPASE-3 (Figure S1).

To more precisely characterize the effect of pros LoF at the cellular level, we generated null-mutant MARCM NB clones in the developing postembryonic brain using prosv24. In accordance with previous reports [22–24], we found that most prosv24 mutant clones in the larval brain were significantly larger than control wt clones (Fig. 4A–C). This larger clone size was due to an increase in the number of mitotically active cells as judged by BrdU incorporation (Fig. 4F, G) and PH3 immunolabeling (Fig. 4J–L).

In the CB, two main types (A and B) of these prosv24 mutant clones were recovered in an approximate 3A:1B ratio (65 clones of 24 brain hemispheres). Clones of type A, although larger than wt clones, were similar to these in that they also contained one or two large cells located on one side of the cluster which seem to correspond to the NB and/or GMC (Fig. 4D, E-right clone). However, in addition to these large BrdU labeled cells, the mutant type A clones also contained several scattered small BrdU labeled nuclei (Fig. 4, F1, G2). Also similar to wt lineages, in type A pros clones the MIRA marker was strongly expressed in the large NB and the attached GMC but only weakly if at all in the small cells located at a distance from the NB (Fig. 4I). In contrast, type B clones were very large and mostly contained large-to-medium sized cells that were scattered throughout the clone (Fig. 4E-left clone). Moreover, all the cells of these type B clones showed strong MIRA labeling (Fig. 4H) and most of them were also BrdU-labeled (Fig. 4G1). Two comparable types of prosv17 clones were also recovered in the OPC in an approximate 4A:1B ratio (88 clones of 11 brain hemispheres). Thus, type A clones maintained the typical elongated shape of wt OPC clones, although in addition to the single mitotic NB detected in wt clones, they harbored several mitotic cells while type B clones were larger, relatively amorphous and contained multiple scattered large mitotic cells (Fig. 4J–L, O).

All type B clones and the majority of type A clones in the CB (16 clones, 5 brain hemispheres) abolished or showed a marked reduction in the expression of the pan-neuronal marker ELAV, indicating that the generation of neurons in these clones was inhibited (Fig. 4 M–O). This is in accordance with previous reports [22,24]. Nevertheless, we occasionally (1/16 clones) found small prosv17 clones that were similar in size to wt clones and in which the expression of ELAV appeared to be normal (Fig. 4P).

Taken together, these findings indicate that the LoF of pros results in an increase in proliferative mitotic activity and a decrease in the generation of neuronal cells in most postembryonic NB lineages of the CB and OPC although producing two different phenotypes possibly related to a dual role of PROS on cell cycle regulation and cell specification, as we discuss later.

prospero regulates the expression of the cyclin kinase inhibitor dacapo in nascent larval CNS neurons

To determine if PROS plays a role in inhibiting cell cycle progression in new born neurons of the postembryonic brain, we took advantage of the fact that termination of mitotic activity in the larval OL is known to be controlled by the expression of the CK1 dacapo (dap) [36]. We reasoned that if PROS could arrest proliferation in new born GCs, it might do so by regulating dap expression in these cells.

To investigate this possibility, we first determined if dap is expressed in postmitotic neurons by immunocytochemical studies
combined with cell-specific marker labeling. These experiments showed that DAP and PROS have similar expression patterns in cells of the postembryonic brain. Thus, DAP is much more strongly expressed in the nuclei of GCs as compared to NBs and GMCs where its expression is cytoplasmic and considerably weaker (Fig. 5A, A1–6). Moreover, in agreement with previous data [36], we found that *dap* mRNA was strongly expressed in scattered cells of the late third instar larval OL (data not shown).

Since this expression pattern was reminiscent of that of PROS, we analyzed the possible co-expression of *pros* and *dap* by double FISH. These experiments showed that *pros* and *dap* mRNAs are indeed co-expressed in single GC-like cells in both the CB and OPC (Fig. 5B, C). This suggests that the expression of both genes is (transiently) upregulated in newborn GCs.

Next we carried out genetic LoF and GoF experiments to determine if PROS can regulate the expression of *dap* in these...
Figure 4. Clonal analysis of the LOF of \textit{pros} unravels two differential phenotypes in the larval CNS. A,B,C. High magnification images of representative examples of \textit{wt} and \textit{prosv17} clones labeled with GFP and PROS. Note the larger size and the lack of PROS immunolabeling in the

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cells. Partial LoF in prosv24 hypomorph mutants resulted in a marked decrease in dap mRNA expressing cells in the OL and CB (Fig. 5D, E). Conversely, PROS overexpression in c831Gal4; UAS-pros postembryonic brains resulted in a substantial increase in dap mRNA expressing cells in the OL (Fig. 5D, F).

Taken together, these findings indicate that PROS regulates the expression of dap in new born prospective neurons in the postembryonic brain. This supports the notion that PROS inhibits cell cycle progression in nascent GCs through DAP.

Prospero represses the expression of deadpan in the larval NB progeny

Since dap was not identified as a direct target of PROS in a genome-wide in vivo target gene identification analysis [21], it is unlikely that PROS can regulate the transcription of dap directly. Nevertheless, since PROS is required to terminate the embryonic expression of the pan-neural bHLH transcription factor deadpan (dpn) [26] which is a suppressor of dap expression in the larval OL [36], we hypothesized that DPN might mediate the upregulation
of dap expression induced by PROS. To investigate this, we carried out LoF and GoF experiments to determine if PROS can regulate DPN expression in the postembryonic brain.

In the adult larval brain, and as reported previously [1,24,37], we observed that DPN expression in the CB was mostly restricted to NBs (Fig. 6A, G) while in the OPC we found that DPN was highly expressed in the NBs located at the surface and, depending on the ventro-dorsal/anterior-posterior position, decays more or less rapidly in the daughter cell populations that move from the periphery towards the center of the OL, as they are generated by the NBs (Fig. 6C–D). We found that the ectopic expression of PROS in NBs and their early progeny using the c831Gal4 driver (see Figure S2 for details of expression pattern) strongly reduced the expression of DPN in cells of the CB and OPC (Fig. 6A, B).

Close inspection of the OPC in these experiments revealed that DPN expression was greatly reduced or abolished in most NBs and their progeny where PROS expression was induced at high level (Fig. 6E,F). Conversely, in pros<sup>prosv24</sup> mutants we observed the ectopic expression of DPN in the progeny of some CB NBs and in most of the progeny of NBs in the OPC (Fig. 6G, H).

These results indicate that PROS represses the expression of DPN in the NBs progeny during postembryonic brain development. Since DPN is known to repress dap expression [at least in the larval OL] [36], these findings support the hypothesis that DPN mediates the upregulation of dap expression induced by PROS.

Discussion

A transient upregulation of prospero promotes the cell cycle exit of Drosophila postembryonic CNS neurons

During development, cell cycle progression must be coordinated with the regulation of cell specification and differentiation. The underlying mechanisms of coordination are likely to be particularly complex during neural development due to the enormous cell diversity in the brain. In Drosophila, these mechanisms have been well studied during embryonic CNS development. In embryonic neurogenesis, the homeodomain transcription factor PROS is expressed in the NB but it does not enter the nucleus due to its binding to the carrier protein MIRA, which localizes to the cell cortex. This interaction facilitates the segregation of PROS from the parent NB to the GMC during asymmetric NB division. In the GMC, PROS is released from its carrier and translocates to the nucleus where it plays a binary role as a cell fate determinant [38–41], and as a promoter of terminal differentiation [20,21,42].

It has been reported that PROS is similarly expressed and asymmetrically segregated during the proliferative activity of (type I) NBs in the larval CB [1,23,24,43] although it does not seem to be expressed in CB dorso-medial lineages (type II) [34] NBs. However, as we show here, during postembryonic neurogenesis, in the majority of larval CB and OPC neuronal lineages, pros expression is transiently upregulated in new born prospective neurons (GCs), in addition to its earlier expression and asymmetric segregation in some larval NBs. This is clearly different from the situation in embryonic lineages where pros is only transcribed in NBs [44,45], and PROS protein is downregulated in GCs after the division of their parent GMC [26]. A summary scheme of these different situations in embryonic versus postembryonic neurogenesis is shown in Fig. 7A.

This transient expression in most newborn postembryonic neurons shortly after the division of the GMC implies a novel role of PROS in postmitotic cells. We postulate that this role is to inhibit cell cycle progression and promote cell cycle exit. Our PROS GoF and LoF experiments support this notion. PROS GoF induces proliferation arrest and PROS LoF results in supernu-

merary cells with sustained expression of cell cycle markers, indicating an inability to withdraw from the cell cycle.

Differential roles of prospero in GMCs and GCs during postembryonic neurogenesis in Drosophila

In addition to the marked difference in PROS expression in postmitotic GCs during embryogenesis versus postembryonic neurogenesis (PROS is undetectable in embryonic GCs and high in postembryonic GCs), there are other functional differences in PROS action during embryonic versus postembryonic CNS development. For example, in pros mutant embryos, over-proliferation is followed by abundant apoptotic cell death among the supernumerary cells [20]. By contrast, we find no increased cell death in the larval OL of pros mutants. Moreover, while PROS and DAP seem to act in parallel to end the cell cycle in the embryonic CNS [20], DAP appears to act downstream of PROS in larval CNS neurons, as shown here (see discussion below). These initial findings suggest that further differences between the functions of PROS during embryonic and postembryonic CNS neurogenesis may exist and should be considered. The fact that PROS protein is present in embryonic GMCs (intermediate progenitors) but not in embryonic GCs (prospective neurons) [25,26], suggests that in the embryonic CNS, PROS initiates the end of mitotic activity in the GMC rather than in the GC. Accordingly, it has been proposed that the GMC is a transition state between the proliferating NB and the differentiating neuron that provides a window in which PROS represses stem cell-specific genes and activates differentiation genes [21]. Nevertheless, it is not well understood how the GMC can go through its terminal cell cycle in spite of the repressive action of PROS on cell cycle regulators.

Our results strongly suggest that in postembryonic neurogenesis PROS acts not only in the GMC progenitor but also in the postmitotic GCs produced by the GMC. Thus, our analysis indicates that there are two main pros expression pattern subclasses among CB type I and OPC NB lineages (Fig. 7A). For the sake of simplicity we have called them A and B. In type A, PROS is expressed in GCs after the division of GMCs while in type B, PROS is first expressed at low level in the NB and asymmetrically segregated to the GMC, and afterwards, upregulated in new born GCs. We interpret that these two subsets of expression patterns correlate well with the two main phenotypes found in pros mutant clones (Fig. 7A, B). Thus, the LOF of pros in NBs with type A PROS expression appears to preclude cell cycle exit of GCs which, consequently, continue dividing and do not differentiate, yielding a type A clone composed of a single NB, a GMC and several small mitotic cells. By contrast, in lineages with type B PROS expression, the LOF of pros seems to cause primarily a change in the fate of the putative GMC that behaves like a NB maintaining the expression of asymmetric division genes (such as MIRA) and overproliferating, to yield a type B clone composed of multiple large NB like cells (Fig. 7A, B).

Hence, we postulate that during postembryonic neurogenesis PROS functions in two sequential phases in type I NB lineages, first as cell fate determinant in some GMCs and later as cell cycle repressor in most GCs. Furthermore, we favor the idea that the different roles of PROS in postembryonic GMCs versus postembryonic GCs might be related to the higher level of expression observed in GCs compared to GMCs. Thus, high levels of PROS might be required to definitively withdraw the GCs from the cell cycle, while low levels might be sufficient to specify GMCs and modulate their cell cycle. The higher level of DAP protein in...
Figure 6. Changes in the expression of deadpan by LOF and GOF of pros. A, B. Confocal projections over 60 μm of the ventro-anterior side of the OL of wt and c831Gal4;UAS-pros late third instar larvae showing DPN and DE-CADHERIN (CAD) protein expression. Notice that many large DPN+ CB NBs (arrowheads) are missing in the c831Gal4;UAS-pros lobe which also shows an important decrease in DPN+ cells in the OPC. C, E. Single confocal images taken, as indicated in the framed areas of A and B, at a medial level of the most anterior part of the OPC. The dotted line marks approximately the border between the neuroepithelial (NE) cells and the NBs of the OPC as indicated by the sharp decrease in the expression of CAD and the increase of DPN. D, F. Schematic representations of the expression of CAD (red), DPN (blue), and PROS (green) observed in C and E, respectively. Notice that in the wt, the expression of DPN is very high in NBs (white arrowheads) and is maintained in their closest daughter cells (arrows) as they are asymmetrically generated inside the lobe. In contrast, in the c831Gal4;UAS-pros OL, although with a few exceptions (white arrow and arrowhead), DPN decays or almost disappears in most NBs (yellow arrowheads) and their closest daughter cells (second cell layer from the surface, yellow arrowheads) where PROS expression is driven at high level. These effects are in agreement with the c831-Gal4;UAS-GFP expression pattern (see Figure S2 for details). Notice that the intensity of PROS signal in the control sample OPC (C) is rather weak because the image acquisition intensity was set up at low level to avoid saturation in the PROS overexpressing sample (E). G, H. Single confocal images taken approximately at 20 μm from the ventro-anterior surface of the OL of wt and prosv24 late third instar larvae showing expression of PROS and DPN. Notice the appearance of DPN expression in the cells located inside the OPC (framed area) and LPC in prosv24, which are both deprived of DPN+ cells in the wt OL. The prosv24 CB also exhibits an increase in the presence of large DPN+ NBs located away from the lobe surface (arrowheads) and of small DPN+ daughter cells (small arrows) located nearby them.

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postembryonic GCs in relation to their parent GMCs and NBs is consistent with this hypothesis. The strong burst of PROS at the end of NB proliferation in ventral ganglia of early pupae [46] is also in agreement with the idea that high levels of PROS are required to stop proliferation. Furthermore, it has been recently shown that the misexpression of PROS at high level suppresses proliferation in type II larval brain NBs lineages without apparent change in their identity [47].

Taken together, all of these findings imply that different developmental strategies have been selected to couple cell fate decisions and cell cycle regulation during embryonic and postembryonic neurogenesis through the same effector, PROS. It is possible that this change in strategy is a consequence of the evolutionary adaptation to regulate the production of a large number of equivalent neurons in postembryonic lineages in contrast to embryonic neurogenesis where a much more limited set of specific neurons are generated in each lineage through GMC divisions.

deadpan and dacapo act in sequence downstream of prospero to regulate the cell cycle exit of Drosophila postembryonic neurons

We have here shown that PROS is coexpressed with DAP in new born prospective neurons and, moreover, we have found that...
Prospero Promotes Cell Cycle Exit of of Neurons

Prospero (pros) is sufficient and it is required for the expression of dap in these larval brain neuronal precursors. The dapp gene encodes a member of the Cip/Kip family of CKIs with homology to mammalian p27Kip1. This family of CKIs has been implicated in mediating cell cycle exit prior to terminal differentiation. They function by binding and inhibiting G1/S cyclin dependent kinase complexes (reviewed by [40]). There is compelling data supporting a role of DAP in cell cycle exit during Drosophila embryogenesis [31,49]. In Drosophila embryonic NB lineages, dapp expression becomes apparent just before the terminal neurogenic division of the GMC [31]. In contrast, we have here shown that dapp is upregulated in new born postembryonic neurons. Consistent with a role in the termination of cell proliferation, dapp expression in the larval OL has been tightly correlated with cells ending proliferation [36]. Interestingly, PROS is required to terminate cell proliferation during embryonic neurogenesis [20] and it has been shown to be involved in the regulation of dapp expression in the embryonic nervous system [42]. Thus, our results provide support to the idea that PROS promotes the cell cycle exit of post-embryonic GCs by upregulating the expression of dapp. Our data also suggest that this upregulation of dapp is mediated by inhibiting the expression of DPN (see Fig. 8 for a schematic summary). DPN is an essential panneural bHLH transcription factor, which has been previously shown to be a suppressor of dapp expression in the larval OL [36]. Indeed, the dpp gene contains consensus PROS binding sites [21] and PROS has been shown to be required to terminate the expression of dpp in the embryo [26].

Abundant data from diverse experimental systems have also implicated Prox1, the vertebrate orthologue of pros [50], in cell cycle exit regulation. Thus, studies in mice have shown that Prox1 appears to be required for the temporal expression of p27kip1 in lens fiber development [51]. Similarly, Prox1 regulates cell cycle exit in the embryonic mouse retina preceding the upregulation of p27kip1 [52]. Furthermore, Prox1 is expressed in early differentiating mouse CNS neurons [53]. Together, these data stimulate to study whether a similar sequential cascade of genes downstream of Prox1 regulates the cell cycle exit of vertebrate CNS neurons.

Supporting Information

Figure S1 The LoF of pros does not induce cell death in the larval CNS. A,B. Confocal projections taken from a ventro-anterior view of the third instar larval brain. A,C. Confocal images of a c831Gal4::UAS-GFP third instar larval brain hemisphere taken at a ventro-anterior point of view at two different levels: close to the surface (A) and approx. 30 µm inside the brain (B). D-F. Schematic representations of the neuroepithelial cells of the brain showing the expression of GFP in the MIR-+/ELAV- cells (differentiating GCs). G. Confocal images of a c831Gal4::UAS-GFP third instar larval brain taken at a medial level (equivalent to those of Fig. 6C). H. Confocal images of a c831Gal4::UAS-GFP third instar larval brain showing the expression of GFP in the MIR-+/ELAV+/MIR cells (differentiating GCs). I. Confocal images of a c831Gal4::UAS-GFP third instar larval brain showing high expression of GFP in the MIR-+/ELAV+/MIR cells (differentiating GCs). J. Confocal images of a c831Gal4::UAS-GFP third instar larval brain showing high expression of GFP in the MIR-+/ELAV+/MIR cells (differentiating GCs).

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Author Contributions

Conceived and designed the experiments: FJT. Performed the experiments: J. Colonques J. Ceron FJT. Analyzed the data: J. Colonques J. Ceron FJT. Contributed reagents/materials/analysis tools: HR FJT. Wrote the paper: FJT HR.
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