bHLH-O proteins are crucial for *Drosophila* neuroblast self-renewal and mediate Notch-induced overproliferation

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**SUMMARY**

*Drosophila* larval neurogenesis is an excellent system for studying the balance between self-renewal and differentiation of a somatic stem cell (neuroblast). Neuroblasts (NBs) give rise to differentiated neurons and glia via intermediate precursors called GMCs or INPs. We show that E(spl)m\(\gamma\), E(spl)m\(\beta\), E(spl)m8 and Deadpan (Dpn), members of the basic helix-loop-helix-Orange protein family, are expressed in NBs but not in differentiated cells. Double mutation for the E(spl) complex and dpn severely affects the ability of NBs to self-renew, causing premature termination of proliferation. Single mutations produce only minor defects, which points to functional redundancy between E(spl) proteins and Dpn. Expression of E(spl)m\(\gamma\) and m8, but not of dpn, depends on Notch signalling from the GMC/INP daughter to the NB. When Notch is abnormally activated in NB progeny cells, overproliferation defects are seen. We show that this depends on the abnormal induction of E(spl) genes. In fact E(spl) overexpression can partly mimic Notch-induced overproliferation. Therefore, E(spl) and Dpn act together to maintain the NB in a self-renewing state, a process in which they are assisted by Notch, which sustains expression of the E(spl) subset.

**KEY WORDS:** *Drosophila*, E(spl), Notch, bHLH-Orange, Dpn, Neuroblast

**INTRODUCTION**

Although the anatomy of the invertebrate versus vertebrate developing nervous system is very different, it is becoming appreciated that neural stem cells in these phyla are similar in terms of cell division patterns and molecular signatures (Brand and Livesey, 2011). *Drosophila* neuroblasts (NBs) are easy to identify, making them a favourable system for studying the balance between differentiation and self-renewal in neural stem cells. After delaminating from the ventral ectoderm (Hartenstein and Campos-Ortega, 1984), neuroblasts undergo multiple rounds of asymmetric divisions, each producing a renewed NB and a ganglion mother cell (GMC). GMCs divide once to produce two neurons (or glia). Upon division, embryonic NBs shrink and, by the end of embryogenesis, stop dividing and die or enter quiescence (Wu et al., 2008). In early larval life, feeding activates the InR and TOR pathways in the dorment NBs (Chell and Brand, 2011; Sousa-Nunes et al., 2011), which triggers new rounds of cell division. Two types of NBs have been described in the larval central brain and ventral nerve cord (VNC). Type I NBs, the majority, divide in the same mode as embryonic NBs. The only difference is that they regrow and sustain divisions for a longer time. Type II neuroblasts, of which there are only eight per brain lobe, are located dorsomedially and follow a more complex lineage. They divide asymmetrically to self-renew and produce immature intermediate progenitors (INPs). Upon maturation, the latter divide asymmetrically a few times to self-renew and generate a GMC. The GMC divides a final time into neurons or glia (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Izergina et al., 2009; Knoblich, 2008). The existence of two transit amplifying progenitors (INPs and GMCs) ensures that type II NBs produce a large number of differentiated progeny.

In many invertebrate and vertebrate species, bHLH-O repressors (Fischer and Gessler, 2007; Iso et al., 2003; Kageyama et al., 2007) are implicated in inhibition of neural differentiation. These proteins constitute a subclass of the basic helix-loop-helix (bHLH) transcriptional regulators containing a characteristic ‘Orange’ dimerization domain (Taelman et al., 2004). In *Drosophila*, seven genes of the E(spl) locus, encoding paralogous bHLH-O proteins, are expressed transiently in the undifferentiated neuroectoderm and inhibit NB formation; when NBs arise, they downregulate E(spl) expression (Delidakis and Artavanis-Tsakonas, 1992; Jennings et al., 1994; Knust et al., 1992; Nakao and Campos-Ortega, 1996; Wech et al., 1999). At the same time, however, nascent NBs turn on another bHLH-O gene, *deadpan* (dpn) which persists in NBs throughout embryonic and larval stages (Bier et al., 1992). Loss of *dpn* causes subtle defects in the neuronal circuitry of the animal, leading to its death in pupal stages. Loss of *E(spl)*, however, is embryonic lethal with severe NB/neural hyperplasia at the expense of epidermis, also a derivative of the neuroectoderm (Lehman et al., 1983). In vertebrates a handful of bHLH-O proteins, namely Hes1, Hes3 and Hes5, are also expressed in the neuroectoderm, accumulating within neural stem cells and downregulated in neuronal progeny (Kageyama et al., 2008). Triple *Hes1*, *Hes3* and *Hes5* knockout mice display premature neural differentiation, disruption of the neuroectoderm and a hypoplastic nervous system owing to loss of neural stem cells (Hatakeyama et al., 2004).

bHLH-O genes are transcriptional targets of Notch signalling in many contexts (Kageyama et al., 2007; Krejci et al., 2009). For example, E(spl) expression in the *Drosophila* neuroectoderm and *Hes1/Hes5* expression in mammalian neural stem cells are known to require Notch input. Despite their complementary expression patterns in the embryonic neuroectoderm, E(spl)m\(\gamma\), one of the seven E(spl) genes, and *dpn* are later co-expressed within larval NBs. Both have been reported to be targets of Notch signalling at this stage (Almeida and Bray, 2005; San-Juán and Baonza, 2011). The role of Notch signalling (and bHLH-O proteins) in post-embryonic neurogenesis in *Drosophila* is not well understood. Null clones for the pathway seem not to affect the proliferation of most...
NBs (Almeida and Bray, 2005; Monastirioti et al., 2010), whereas a more global knockdown reduces NB numbers (Wang et al., 2006) and eliminates type II lineages (Bowman et al., 2008). Conversely, overactivation of the pathway causes significant NB hyperplasia, accompanied by loss of differentiated cells (Bowman et al., 2008; Wang et al., 2006; Weng et al., 2010), although it is not clear whether this hyperplasia arises from all NBs or only from the more sensitive type II variety. The mild phenotypes resulting from Notch loss of function in Drosophila contrast with the neural hypoplasia and stem cell loss observed upon Notch knockout in mice (Imayoshi et al., 2010; Yoon and Gaiano, 2005). The paradox of the dispensability of Notch signalling in Drosophila is accentuated, considering the fact that, during mitosis, NBs have developed an elaborate mechanism to segregate asymmetrically Numb, an inhibitor of Notch signalling, to the GMC (Babaoglan et al., 2009; Guo et al., 1996; Rhyu et al., 1994; Spana and Doe, 1996; Wang et al., 2006). Mutations in numb, which are expected to result in Notch hyperactivation, lead to fully penetrant type II NB overproliferation, but only partially penetrant overproliferation in type I (Bowman et al., 2008; Lin et al., 2010; Truman et al., 2010).

In this work, we address the role of E(spl) and Dpn bHLH-O proteins in NB maintenance and proliferation, both in normal development and upon Notch-induced overproliferation. We found that both are expressed in NBs from embryogenesis onwards and have redundant functions in NB maintenance during normal development. We further showed that E(spl)mγ is a target of Notch, whereas dpm is not. Accordingly, for Notch overactivation to induce overproliferation, only E(spl) genes, not dpm, are needed.

MATERIALS AND METHODS

Fly strains

Drosophila stocks were obtained from the Bloomington Stock Center or individual laboratories.

For mosaic generation:

- w; FRT19A/FM7;
- w; FRT82B Df(1)X51/TM6B;
- w; FRT82B SeRX106/TM6B;
- w; FRT82B Df(1)X51 e SeRX106/TM6B;
- w; Su(H)G4 FRT40A/CyO;
- h s FRT82B neur1 cu e/TM6B;
- w; mh/7872 FRT2A/TM6B;
- w; FRT82B e' spdo(104)/TM3;
- FRT82B P[gro]/Df(3R)E(spl)m3 [deficiency of the whole E(spl) locus];
- FRTG13 dpm/CyO;
- FRTG13 Df(2R)dpn/CyO;
- FRTG13 mam/CyO; and
- appropriate FRT a tub-Gal80 counter-chromosomes combined with hs-FLP, a tub-Gal4, UAS-GFP.

For dpm; E(spl) double mutant clones, we constructed yw hs-FLP a tub-Gal4 UAS-nlsGFP; FRTG13 dpm/FRTG13 a tub-Gal80; FRT82B P[gro] Df(3R)E(spl)m3/2.2 FRT82B a tub-Gal80 larvae. In these, GFP is expressed upon recombination at both FRTG13 and FRT82B sites.

For ectopic expression: gh4-Gal4, UAS-E(spl)mβ, UAS-E(spl)mγ, UAS-E(spl)m6, UAS-E(spl)m3, UAS-E(spl)m5, UAS-E(spl)m7, UAS-E(spl)m8, UAS-Gal4mRNA, UAS-dpm, UAS-N-ecd, UAS-Niced, UAS-CD8-GFP and a tub-Gal80.

For mosaic analysis coupled with hyperactivation of Notch: yw hs-FLP a tub-Gal4 UAS-nlsGFP; UAS-N-ecd/ +; FRT82B P[gro]/Df(3R)E(spl)m3/2.2 FRT82B a tub-Gal80 and yw hs-FLP a tub-Gal4 UAS-nlsGFP; FRTG13 dpm/FRTG13 a tub-Gal80; UAS-Niced/ -.

Corresponding control clones were obtained by substituting the mutant chromosome with FRT82B lacZ or FRTG13, respectively.

For mosaic analysis coupled with overexpression of UAS-E(spl)mγ y w hs-FLP a tub-Gal4 UAS-nlsGFP; FRT40A Su(H)G4/FRT40A a tub-Gal80; UAS-mγ and y w hs-FLP a tub-Gal4 UAS-nlsGFP; FRT40A πMyc/ FRT40A a tub-Gal80; UAS-mγ.

Immunohistochemistry

Fixation and immunohistochemistry of embryos and larval tissues was performed according to standard protocols. Primary antibodies were rabbit-anti-β-gal (Cappel); rat-anti-Elav 7E8A10 (DSHB); rabbit-anti-GFP (Minotech); mouse-anti-GFP (Molecular Probes); guinea-pig-anti-Hey (Monastirioti et al., 2010); rat-anti-Dpn (Boone and Doe, 2008); rabbit-anti-Ase (a gift from A. Jarman, University of Edinburgh, UK); mouse-anti-Pros MR1A (DSHB); and mouse-anti-CyclE (a gift from H. Richardson, Peter MacCallum Cancer Centre, Melbourne, Australia).

To monitor E(spl) expression patterns we used the E(spl)mGFP genomic transgene (Almeida and Bray, 2005). Owing to its weak expression [probably reflecting inherently low expression of the E(spl)mγ gene], a line carrying four transgenic copies was used for embryos; a single copy was sufficient for larval experiments. In all cases, anti-GFP Ab staining was performed, as native GFP fluorescence was too weak to detect. E(spl)mβlacZ (Lecourtois and Schweisguth, 1995), E(spl)m0.5-lacZ (Cooper et al., 2000) and an E(spl)m7 genomic transgene bearing a Myc-tag (P. Piwko and C.D., unpublished) were also used.

Secondary antibodies were conjugated to Alexa488, 555, 568, 633 or 647 (Molecular Probes), or Cy3 (Jackson ImmunoResearch). Samples were imaged on Leica SP2 confocal microscope (University of Crete confocal facility).

Quantitative PCR (qPCR)

Larval brains were dissected and RNA was extracted using the RNeasy mini kit (Qiagen). cDNA was generated by RT-PCR with ImProm-II Reverse Transcription System (Promega). All qPCR reactions were performed in triplicates with QuantiTect SYBR Green PCR kit (Qiagen). Generation of specific PCR products was confirmed by melting-curve analysis. The calibration curve was constructed from serial dilutions of genomic DNA, and values for all genes were normalized to the levels of rp49. For data analysis, the second-derivative maximum method was applied and fold-induction of target cDNA was calculated. Primer sequences are available upon request.

Electrophoretic mobility shift assay (EMSA)

Full-length CDSs of dpn, mγ and mγ were cloned in pRSET-A. Proteins were produced using the T7 polymerase TnT in vitro coupled transcription-translation kit (Promega).

The E(spl)-box primers 5’-TGCTCTGTTGCACGTGTCATTAAG 3’ and 5’-TGGCATTAGACACGGCCACAG 3’ were annealed and radio-labelled using Klenow E. coli DNA polymerase. Binding was performed on ice for 20 minutes with 10 ng/μl probe, 5% glycerol, 20 mM Heps (pH 7.9), 100 mM KCl, 2 mM MgCl2, 2 mM spermidine, 1 mM DTT, 1 mM PMSF and 10 ng/μl poly-dl-dc. The reaction was electrophoresed on a non-denaturing polyacrylamide gel at 4°C. The gel was imaged using Molecular Dynamics Storm 840.

RESULTS

E(spl)mγ is expressed in both neuroectoderm and neuroblasts of the embryonic CNS

We revisited the embryonic expression of E(spl)mγ to determine whether it becomes activated in the NBs prior to larval stages. We used an E(spl)mγ-GFP transgenic line (Almeida and Bray, 2005) to detect E(spl)mγ via a GFP antibody, as the available E(spl) antibodies are not as sensitive. At stage 8, before NB segregation, E(spl)mγ is expressed in the neuroectoderm while Dpn is off. By stage 9 (Fig. 1A), the first neuroblasts delaminate from the neuroectoderm and are Dpn positive and E(spl)mγ negative, as previously described (Jennings et al., 1994). E(spl)mγ expression is first weakly detected in NBs at stage 10 (Fig. 1B, arrows) and by stage 11 all NBs express both Deadpan and E(spl)mγ (Fig. 1C).
stage 13, when NBs delamination is complete, E(spl)\(\gamma\) is lost from the neuroectoderm, but persists in the NBs (Fig. 1D). At the end of the embryonic proliferative period (stage 16) most neuroblasts decrease in size and enter quiescence. These neuroblasts remain Dpn positive but turn E(spl)\(\gamma\) off, with only a few displaying weak levels (Fig. 1E). We conclude that, after an initial downregulation at the time of NB birth, E(spl)\(\gamma\) is re-expressed in the embryonic neuroblasts in addition to the overlying neuroectoderm. NB expression is correlated with mitotic activity. It begins when the first Pros-positive cells (GMCs) are detected (Fig. 1B, arrowheads) and is downregulated at the time NBs enter quiescence (Fig. 1E). Dpn, however, is expressed before embryonic neuroblasts start dividing and remains on at the onset of quiescence; therefore, it does not correlate with mitotic activity.

bHLH-O genes are expressed in the larval CNS

We also monitored the expression of E(spl)\(\gamma\) and Dpn throughout larval life. Dpn is detected in neuroblasts of newly hatched larvae [24 hours after egg laying (AEL)] indicating that it remains active throughout quiescence (Fig. 1F). These early larval Dpn-positive NBs are small and E(spl)\(\gamma\)-negative, with the exception of five neuroblasts, one lateral and the four anterodorsal mushroom body NBs (Fig. 1F, white arrows). These four are the only NBs that never cease proliferating in the embryo-to-larva transition (Ito and Hotta, 1992; Sousa-Nunes et al., 2011). E(spl)\(\gamma\) is also found in a few Dpn-negative cells, which will give rise to the optic lobe (Fig. 1F, yellow arrow). By early L2 stage (48 hours AEL, Fig. 1G), neuroblasts increase in size, become E(spl)\(\gamma\)-positive and...
start dividing, as evidenced by the appearance of neighbouring Prospero-positive GMCs. This continues till the end of larval life (Fig. 1H-J).

We further tested whether other members of the E(spl) Complex are also activated in larval NBs. As no specific antibodies were available, we used a Myc-tagged genomic construct for E(spl)m7 (P. Pivko and C.D., unpublished) and several lacZ reporters (supplementary material Fig. S1). We found that E(spl)m8 and E(spl)m8 are expressed in larval neuroblasts, whereas E(spl)m7 is mostly expressed in the optic lobe and weakly in the neuroblasts and some surrounding cells. We did not test E(spl)m3, E(spl)m5 and E(spl)m8 owing to lack of availability of reporter lines in our lab. We conclude that, besides mγ, additional E(spl) genes are expressed in post-embryonic NBs.

The function of bHLH-O proteins in larval neuroblasts

To test the role of Dpn and E(spl) in proliferation, we performed mosaic genetic analysis in L2-L3 CNSs, where NB proliferation rate is at its highest. Type I lineages deficient for all E(spl) displayed no defects in the type of progeny they generated compared with wild type. Each mutant lineage contained a large Dpn/Ase-positive NB, 3-5 Ase/Pros-positive (Dpn-negative) GMCs and many Pros-positive (Dpn/Ase-negative) neurons (Fig. 2A-C). Type II lineages, however, displayed a slight decrease in the number of INPs compared with wild type. Immature INPs are characterized by the lack of staining for all Dpn, Ase and Pros and their proximity to the parent type II NB (Dpn positive, Ase negative). Mature INPs are slightly further from the NB and express both Ase and Dpn (Boone and Doe, 2008; Bowman et al., 2008). E(spl) type II clones contained a single Dpn-positive/Ase-negative NB, a few adjacent Dpn/Ase-negative iINPs and ~10 Dpn/Ase-positive mINPs, compared with ~25 in wild type (Fig. 2E,F). Singly mutant clones for dnp also exhibited normal type I lineages and a similar decrease in the number of INPs in type II lineages (Fig. 2D,G).

In contrast to the virtual absence of defects observed in single dnp or E(spl) clones, double dnp; E(spl) mutants displayed a dramatic phenotype. In 48/64 (75%) of type I mutant lineages, a NB could not be detected and there were fewer neurons per lineage (5.5 cells/lineage; Fig. 2H,J) compared with wild-type clones of the same age (23 cells/lineage). The remaining double mutant lineages appeared normal (22 cells/lineage; Fig. 2I,K,L). A likely scenario is that NB loss probably took place gradually over the 3 days between clone induction and fixation; up until their loss, the mutant NBs kept dividing, accounting for the (small) number of mutant neurons detected in the NB-less clones. The one-quarter of the lineages that still had not lost their NB displayed a normal size, suggesting that proliferation rate was not altered by dnp; E(spl) loss. Therefore Dpn and E(spl) possess a redundant function that is needed for NB long-term maintenance rather than for ongoing proliferation. Type II mutant clones were never recovered, suggesting that dnp; E(spl) loss of function has a more severe effect in these lineages, perhaps immediate cessation of proliferation.

We also addressed whether bHLH-O proteins are needed for NB maintenance during quiescence. For this reason, we carefully monitored NB number in larvae immediately after hatching and for the whole of larval life at 24-hour intervals. We expected that dnp loss of function might prevent NB reactivation, as it is strongly expressed during the late embryonic stages of NB quiescence. Although a reduction in larval NB numbers was found, it was rather moderate. By late third-instar, dnp+/dnp− had an average of 52 NBs (wild type=95) per brain lobe, as seen also by San-Juán and Baonza (San-Juán and Baonza, 2011), and 100 NBs (wild type=150) per VNC (supplementary material Fig. S2), suggesting that a large number of NBs can exit quiescence. Indeed almost all wild-type and dnp+/dnp− NBs had turned on Ase expression and were surrounded by Pros-positive GMCs/neurons, indicating mitotic activity, by 48 hours after hatching (supplementary material Fig. S2H). We therefore entertained the possibility that another bHLH-O factor may act redundantly with Dpn even during quiescence. Indeed, the fact that E(spl)mγ is barely detectable in late embryos does not preclude the possibility that another E(spl) family member is expressed in NBs at that time, so we asked whether loss of the E(spl) locus might enhance the dnp phenotype. As E(spl)− homozygous embryos do not hatch, owing to earlier neuroectoderm defects (Lehman et al., 1983), we could not test the complete null state for E(spl). However, upon halving the E(spl) dose in a dnp+/dnp− background, we observed a dramatic phenotypic enhancement in three respects. First, embryonic lethality was increased: dnp+/dnp−; E(spl)m8+/− larvae hatched at 31% of expected frequency, whereas the control and dnp+/dnp− genotypes hatched at 80% of expected. Second, total Dpn-positive NB numbers were strongly reduced (supplementary material Fig. S2M,N), suggesting premature NB loss around the time of quiescence. Finally, among the remaining NBs, only a few were active (Ase-positive) 24 hours after hatching, a time when most control [dnp+/+; E(spl)m8+/+] and dnp+/dnp− NBs had already entered the cycle (supplementary material Fig. S2O). Inability to reactivate NBs after hatching was accompanied by fully penetrant first-instar lethality, precluding analysis at later stages. Therefore, E(spl) cooperates with dnp to maintain the NB fate both during proliferative and during quiescent phases.

Motivated by this genetic redundancy, we hypothesized that the cohort of bHLH-O proteins expressed in NBs (at least Dpn, mγ, mβ, m8) could have the same effects on NB chromatin. As a preliminary test, we asked whether they can form DNA-binding competent heterodimers, using the electrophoretic mobility shift assay (EMSA). We tested an Eγ-box oligo [a high-affinity binding site for bHLH-O proteins (Jennings et al., 1999)] with various combinations of in vitro translated Dpn, mγ and m8 proteins. We readily detected homodimers for all proteins. Upon mixing two bHLH-O proteins, we also observed new shifted complexes that point to the existence of all possible heterodimers (supplementary material Fig. S3). These biochemical results are consistent with a redundant role between Dpn and the E(spl) bHLH-O proteins in larval NB maintenance.

Notch signalling activates E(spl)mγ and m8, but not dnp, expression in neuroblasts

E(spl) genes are common transcriptional targets of Notch signalling (Bray and Bernard, 2010). We tested Notch dependence for E(spl)m8, E(spl)mγ and dnp; there are reports of the latter two being targets of Notch (Almeida and Bray, 2005; San-Juán and Baonza, 2011). We made mosaics for null alleles of various components of the pathway and assayed lacZ-reporters of E(spl)mγ and E(spl)m8, as well as Dpn protein. A cohort of mutations were studied that affect Notch signal reception [N, Su(H), mam, spdo] or emission [Dl, Ser, neur, mib1]. With the exception of Ser and mib1, which showed no defects, disruption of the Notch pathway affected E(spl) but not dnp expression (Fig. 3; supplementary material Fig. S4; Table 1). Specifically, E(spl)mγ-lacZ was completely abolished, whereas E(spl)m8-lacZ was either abolished or strongly downregulated.
While this work was in progress, San-Juán and Baonza (San-Juán and Baonza, 2011) reported that dpn contains a NB-specific enhancer that is regulated by Notch signalling. Although seemingly contradictory to our results, they also observed that Dpn protein expression is not affected in a Su(H) background; therefore, the Notch-responsive enhancer must play only a minor role in dpn expression. We also examined Dpn expression in embryos mutant for Notch pathway components. In these genotypes, the hyperplastic NBs were strongly Dpn positive (supplementary material Fig. S5), even at late embryonic stages, consistent with the fact that dpn expression is independent of Notch signalling in embryos, as in larvae.

Despite the near extinction of E(spl)\(m^{\gamma}\) and m8 expression, no obvious effect was seen in Notch pathway mutant type I lineages in the larva: they consisted of one Dpn-positive NB, 2-5 GMCs and several neurons, as noted before (Almeida and Bray, 2005; Monastirioti et al., 2010; San-Juán and Baonza, 2011). Instead, type II clones were recovered less efficiently and showed reduced proliferation (supplementary material Fig. S6). The ‘resilience’ of type I clones to Notch disruption may be due to the persistence of Dpn (and partially m8) expression, as we showed above that Dpn and E(spl) have redundant functions. To test this hypothesis, we generated doubly mutant clones for null mam and dpn alleles, as mam mutation severely affects E(spl) expression (Fig. 3;
supplementary material Fig. S4). We observed frequent NB loss in

*dn* mam8 clones (60%), much more than observed in single

clones (0-25%; supplementary material Fig. S7), suggesting that

*dn* removal aggravates the effects of Notch pathway disruption,

as predicted.

A useful corollary from our mosaic analysis regards the cells

involved in this Notch signalling event. There were no instances of

‘exceptional’ NBs expressing E(spl)m8 in mutants known to act

non-cell-autonomously (Dl, DI, Scr and neur; collectively, 151 type

I lineages scored). We therefore conclude that the signal

responsible for m8 expression emanates from cells within the

lineage of the mutant NB, otherwise the mutant NB would be able
to respond to signal from an adjacent lineally unrelated DI cell.
The cells that signal to the NB are most likely to be the adjacent

GMCs, which stay in close contact with their parent NB. This
interpretation is also consistent with the fact that embryonic NB

m8 expression starts at the same time as GMCs are first detected
(Fig. 1).

Hyperactivation of Notch increases bHLH-O

expression levels and causes overproliferation of

larval neuroblasts

As our results suggested a role for E(spl) proteins and Dpn in

maintaining the NB and opposing premature differentiation, we

examined how these proteins behave upon overactivation of Notch

and also whether they contribute to the hyperactive Notch

phenotype of NB overproliferation. We ectopically expressed a

UAS-NΔecd transgene, a constitutively active Notch deleted for its

extracellular domain (Rebay et al., 1993; Fuerstenberg and Giniger,

1998), under the

grainyhead

gene expressed in secondary neurons

(Fig. 3). Notch pathway disruption affects E(spl)m8 but not

*dn* or cycE expression in the larval CNS.

GFP-marked clones (green) stained for β-galactosidase (E(spl)m8-lacZ) or

Hey (blue) and Dpn or CyclinE (red), as indicated. Hey is a Notch target
gene expressed in secondary neurons (Monastirioti et al, 2010). GFP is

nuclear in all panels except in Notch

hyperactive Notch

overproliferation of

larval neuroblasts

expression levels, whereas CyclinE and Dpn are unaffected. (D,E) The

same holds for Dpν10 and Dpν10

Se
[alpha]106 clones. (F,F) Se
[alpha]106 clones appear wild type. (G,G) neur1,

(H,H) mam8, (I,I) Su(H)477 and

(J,J) spdo104 clones show reduction or elimination of E(spl)m8-lacZ, but

not Dpn. Scale bars: 24 μm in A–B’,

D–J’, 20 μm in C–C’.

Table 1. Mosaic analysis of E(spl)m8 expression in the larval CNS

<table>
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<tr>
<th></th>
<th>NB my8</th>
<th>NB m8</th>
<th>% NB my8</th>
<th>NB high m8</th>
<th>NB low m8</th>
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</table>

Clones mutant for the indicated allele were scored in third instar larval CNSs. The number of clones from all regions of the CNS are shown, with the exception of the mushroom body NBs, where my8 and m8 are weakly expressed.

*Lineages with an my8-lacZ positive NB.

†Lineages with an my8-lacZ negative NB.

‡Lineages with normal levels of m8-lacZ expression in the NB.

§Lineages with reduced but detectable m8-lacZ expression in the NB.

¶Lineages with undetectable m8-lacZ expression in the NB.
Overexpression of N\(\text{\textDelta}ecd\) for 24 hours led to massive overproliferation of NBs throughout the CNS (Fig. 4B,D,F,H). Consistent with previous reports using different Gal4 drivers (Bowman et al., 2008; Wang et al., 2006; Weng et al., 2010), NB overproliferation was at the expense of neuronal differentiation (fewer Pros-positive cells; Fig. 4B,D,F,H). The supernumerary Dpn-positive NB-like cells had a smaller size than normal NBs and were not only found superficially, where NB are normally located, but also migrated into deep layers, invading neuronal territories (Fig. 4C,D). Overproliferating neuroblasts of the dorsal brain lacked Ase expression (Fig. 4N), suggesting a type II origin, and were more invasive, as they also migrated superficially around the anterior of the brain lobe, towards the ventral brain (Fig. 4P, yellow arrow). Overproliferating NBs in the ventral brain and VNC were both Dpn (Fig. 4B,D,F,H) and Ase-positive (Fig. 4P, white arrow) (type I). Besides expressing Dpn, all supernumerary type I or II NBs were also \(E(spl)\text{my}\)-positive (Fig. 4I-L). We found an increase of 2.14 and 2.04 fold, respectively, of \(E(spl)\text{my}\) and \(dpn\) RNA levels in CNSs overexpressing N\(\text{\textDelta}ecd\) for 24 hours versus control CNSs. Mean ± s.e.m. of three repeats are shown. Statistically significant differences from the controls at *\(P<0.5\) and **\(P<0.01\) are shown using a two-tailed t-test.

**E(spl) proteins play an important role in the Notch gain-of-function phenotype, but Dpn has only a minor role**

If bHLH-O proteins mediate the overproliferation effect of Notch, we expect this effect to disappear if we compromise bHLH-O protein activity. We therefore generated clones deficient for either \(E(spl)\text{my}\) or \(dpn\), where at the same time we overexpressed an activated form of Notch. We assessed both type I and type II GFP-marked lineages for hyperplastic phenotypes. We scored type I lineages as overproliferating, when they contained two or more Dpn/Ase-positive cells. Type II lineages were scored as overproliferating when they contained more than 28 Dpn-positive cells. A further category of ‘highly overproliferating’ type II NB lineages were seen containing more than 80, and as many as 400, Dpn-positive cells.

Consistent with the grh>N\(\text{\textDelta}ecd\) results, clonal overexpression of N\(\text{\textDelta}ecd\) in a wild-type background produced a high percentage of overproliferating lineages; 30-50% of type I and 100% of type II clones (Fig. 5A,C,E; Table 2). Overproliferating type I clones contained 3-63 Dpn/Ase-positive NB-like cells, usually intermediate in size between a normal NB and a GMC (supplementary material Fig. S9A). In type II lineages 38-430 Dpn-positive/Ase-negative...
cells were scored, most of which had the size of an INP, whereas up to 36 had the size of a NB. Ase-positive cells were hardly ever seen in these clones (supplementary material Fig. S9B).

Upon removal of the E(spl) genes, overproliferation by Nicd function in a wild-type background has a mild effect in type II lineages, reducing INP control clones. As noted before, loss of E(spl) positive cells were also Ase positive in sharp contrast to the E(spl)+ control clones. Three clones with mild overproliferation (35-43 Dpn-positive cells) (Fig. 5C,D). No overproliferation was detected in the VNC clones in the brain lobes displayed mild overproliferation. These clones contained a single Ase-negative NB and 25-28 mature INPs (Dpn and Ase positive). Ten to 13% of Type I and 100% of type II lineages produced overproliferating clones (Table 2): type I clones had 2-15 Dpn-positive cells) (Fig. 5F). The majority of type II clones (15/19 clones) also reverted to a wild-type phenotype upon E(spl) removal (Fig. 5B). They contained a smaller number of Dpn-positive cells per clone (2-15 cells) (Fig. 5C,D). No overproliferation was detected in the VNC clones in the brain lobes displayed mild overproliferation. These clones contained a single Ase-negative NB and 25-28 mature INPs (Dpn and Ase positive). Three clones with mild overproliferation (35-43 Dpn-positive cells) and one clone with 161 Dpn-positive cells were recovered. Even in these clones most of the supernumerary Dpn-positive cells were also Ase positive in sharp contrast to the E(spl)+ control clones. As noted before, loss of E(spl) function in a wild-type background has a mild effect in type II lineages, reducing INP number to about 10 (Fig. 2E) – and has no effect on type I lineages. So the wild-type appearance of type II UAS-N\Deltaecd; E(spl)Δ clonese is an intermediate phenotype between N\Deltaecd overexpression and E(spl) loss of function. The conclusion is that E(spl) function seems to be more important in the pathological NB overproliferation obtained upon Notch hyperactivation than it is in the background of normal Notch activity.

Analogous experiments to address the role of dpn in Notch-induced overproliferation used a different activated Notch (UAS-Nicd) (Seungnet al., 1997) for technical reasons (see Materials and methods). This transgene elicited weaker overproliferation upon clonal expression (with the same \alpha-tub-Gal4) than did UAS-\Deltaecd. Ten to 13% of Type I and 100% of type II lineages produced overproliferating clones (Table 2): type I clones had 2-15 Dpn-positive cells (Fig. 5L; supplementary material Fig. S9C), whereas Type II had 44-269 Dpn-positive cells (Fig. 5G; supplementary material Fig. S9D). With the weaker Nicd, all Type II clones, despite their hyperproliferation, contained a small number of Ase-positive cells (Fig. 5G', white arrowhead). Upon
Table 2. E(spl) proteins are necessary for Notch-mediated overproliferation of NBs in the larval brain, whereas Dpn is dispensable

<table>
<thead>
<tr>
<th>UASNΔE77; FRT82B +</th>
<th>Normal NBs</th>
<th>Overproliferating NBs</th>
<th>Highly overproliferating NBs</th>
<th>% Normal</th>
<th>% Overproliferating</th>
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<th>% Normal</th>
<th>% Overproliferating</th>
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<td>81.3</td>
<td>18.7</td>
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Table 3. E(spl)γ expression bypasses Notch to induce a hyperproliferation phenotype

<table>
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<tr>
<th>FRT40A: UAS-E(spl)γ</th>
<th>Normal NBs</th>
<th>Overproliferating NBs</th>
<th>Highly overproliferating NBs</th>
<th>% Normal</th>
<th>% Overproliferating</th>
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<td>5</td>
<td>-</td>
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<td>4.5</td>
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<th>Overproliferating NBs</th>
<th>Highly overproliferating NBs</th>
<th>% Normal</th>
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removal of dpn, the percentage of Type I overproliferating clones rose to 19-28% (Table 2), which we consider insignificant, as the vast majority contained only two Dpn-positive cells (Fig. 5J,L). Type II clones continued to overproliferate with full penetrance and only had a moderate decrease in Dpn-positive cell number (Table 2 and Fig. S1H). This analysis was performed with two null/antimorphic dpn alleles, dpnγ and dpnδ (Barbash and Cline, 1995), yielding the same results. Therefore despite the fact that abnormal Notch activity induces both dpn and E(spl), NB hyperproliferation can still occur in the absence of dpn.

E(spl) overexpression mimics the Notch hyperactivation phenotype

We next examined whether E(spl) overexpression might be sufficient to bypass Notch and induce hyperplasia in the larval CNS. We used grh-Gal4 to drive the expression of various UAS-E(spl), as well as UAS-dpn transgenes in neuroblasts. Overexpression of E(spl)β, E(spl)γ, E(spl)δ, E(spl)m3 or dpn caused overproliferation, more severe in the cases of m3, γ and δ (supplementary material Fig. S10B). E(spl)m5 or E(spl)m8 did not produce any NB hyperplasia. The effect of E(spl)γ (and others) was limited to dorsal brain lineages, including type II NBs, in contrast to the more global UAS-Nγ effects. Ectopic NBs were a mix of Ase-positive and Ase-negative ones. Overexpression of UAS-E(spl)γKNEQ (supplementary material Fig. S10D), a mutant that lacks the ability to bind DNA (Giaigtzoglou et al., 2003), caused no overproliferation.

For further analysis, we selected one of our most potent transgenes, UAS-E(spl)γ, and asked whether it is sufficient to cause overproliferation even in the absence of Notch signalling. To that end, we compared E(spl)γ overexpressing clones in a wild-type versus Su(H) mutant background. In a wild-type background, γ overexpression produced a moderate level of overproliferation in type I NBs (16% of clones in the brain and 4.5% in the VNC, see Table 3) and fully penetrant (96%) overproliferation in Type II clones (Fig. 6A). The latter contained 28-487 Dpn-positive cells (supplementary material Fig. S11). The effect of E(spl)γ overexpression was similar to the activity of the weaker UAS-Nicd transgene described above. In E(spl)γ-overexpressing Su(H) mutant clones, overproliferation could still be observed in both type II and
type I clones (Fig. 6D-F) at the same frequencies (Table 3). Although type I clones were essentially indistinguishable from those generated in a wild-type background, type II clones contained significantly fewer Dpn-positive cells (28-158; supplementary material Fig. S11). We conclude that E(spl)mγ is sufficient to cause NB overproliferation in the absence of Notch. In type II lineages, Notch signalling can boost this activity, indicating some degree of interdependence/synergy between E(spl)mγ and other Notch downstream effectors.

DISCUSSION

We have presented an analysis of the expression and function of two types of bHLH-O proteins expressed in Drosophila neuroblasts, Dpn and the E(spl) family. Our main conclusions are that (1) these two types of factors have distinct expression modalities – E(spl)mγ and m8 are targets of Notch signalling, whereas Dpn is not; (2) these factors have redundant functions to maintain NBs in a self-renewing state in normal development, yet (3) in a pathological NB hyperproliferation context, Dpn and E(spl) have distinct functions.

Different modes of bHLH-O expression in neuroblasts

It was heretofore thought that embryonic NBs are cells that escape from Notch signalling, which is only perceived in the surrounding neuroectoderm. Only in the later post-embryonic period, were the NBs thought to respond to Notch (Almeida and Bray, 2005; San-Juán and Baonza, 2011). We have shown that this happens much earlier, already in the embryonic NBs. Soon after the NB delaminates, a time when it sends, but does not receive, a Notch signal, it starts asymmetrically dividing. Our results are consistent with the daughter GMCs sending a Delta signal back to their sister NBs, thereby initiating E(spl) expression. E(spl)mγ expression ceases when the NB enters quiescence, only to restart when proliferation resumes.

Dpn, another bHLH-O protein, is also expressed in NBs, but much less dynamically. Its expression initiates upon NB delamination from the neuroepithelium and persists throughout its life. Dpn does display some degree of dynamic expression, as it is rapidly turned off in the iINPs, only to be reactivated upon maturation. Our loss-of-function data (Fig. 3; supplementary material Fig. S4) clearly indicate that it is not a target of Notch in the NB, in contrast to E(spl). Paradoxically, dpn is induced upon Notch hyperactivation (Fig. 4). This could be an indirect effect mediated through E(spl). Indeed, E(spl)mγ overexpression can induce ectopic dpn expression (Fig. 6). Still, dpn does harbour a Notch-responsive enhancer that drives expression in larval NBs (San-Juán and Baonza, 2011). This same region scored positive for Su(H) binding in a ChIP-chip approach in a cell line of mesodermal origin (Krejci et al., 2009). How this enhancer contributes to the overall expression pattern of dpn will be a matter of future analysis.

The function of bHLH-O proteins: proliferation or anti-differentiation?

Despite their different expression modalities, Dpn and E(spl) have redundant functions in the larval NBs, as only double mutant clones show proliferation defects. These mutant NBs do not stop...
proliferating immediately, rather gradually terminate their cycling within a few days following homozygosing of the mutant alleles (Fig. 2). We propose that Dpn/E(spl) keep the NB in an undifferentiated state and proliferation is a consequence of the ability of these cells to respond to mitogens. Upon Dpn/E(spl) loss, this state becomes unstable and prone to switch to a terminally differentiated state. This transition takes a few days, probably reflecting the time needed to accumulate pro-differentiation factors. A redundant role of Dpn/E(spl) in maintaining the undifferentiated state also during quiescence transpired from our genetic analysis of NB re-activation after embryogenesis. Whereas dpn+/-/E(spl)+/+ NBs quite successfully re-entered the cell cycle, dpn-/-:E(spl)+/+ NBs were unable to do so, despite trophic growth factor stimulation.

E(spl) expression has been associated with the less differentiated of two alternative outcomes in other instances. For example, during NB formation, E(spl) genes are expressed in the undifferentiated embryonic neuroectoderm and not in the NBs. The same happens in the optic lobe neuroepithelium (Egger et al., 2010; Wang et al., 2011). In this work, we have presented evidence for a similar role for Dpn/E(spl) in the NB. Excessive Dpn/E(spl) activity in GMCs/iINPs can revert these partially differentiated cells back to a NB-like fate. For this reason, NB asymmetric divisions must ensure that Dpn and E(spl) are never expressed in the GMC or iINP. Regarding E(spl), we propose that this is ensured by the directionality of Notch signalling (GMC to NB). Dpn is also never seen to accumulate in the GMCs/iINPs, suggesting a repression mechanism at work in these cells, e.g. via Pros (Choksi et al., 2006; Southall and Brand, 2009). These modes of transcriptional control are probably combined with active protein clearance by degradation.

An anti-differentiation role has also been proposed for vertebrate homologues of Dpn/E(spl), the Hes proteins. Hes1, Hes5 and Hes3 are all expressed in proliferating neural stem cells of the embryonic CNS. Upon Hes knockout, neural stem cells prematurely differentiate resulting in a hypoplastic nervous system, with increasing severity as more Hes genes are lost (Hatakeyama et al., 2004). In an interesting analogy, only Hes1 and Hes5 are direct targets of Notch signalling (Nishimura et al., 1998). Another example where anti-differentiation during quiescence is mediated by high Hes1 expression are cultured fibroblasts and rhabdomyosarcoma cells (Sang et al., 2008). Similar to what we observed with Dpn/E(spl)-mutant embryos, a quiescence trigger, like serum depletion, can result in irreversible cell-cycle withdrawal, if Hes1 activity is compromised.

The Notch/bHLH-O axis in normal and pathological neurogenesis

Our results have shed light on the paradox of why Notch loss of function has only minor effects in larval neurogenesis, whereas its hyperactivation causes significant overproliferation (Bowman et al., 2008; Lin et al., 2010; San-Juán and Baonza, 2011; Truman et al., 2010; Wang et al., 2006; Weng et al., 2010). Notch loss of function decreases E(spl) expression, leaving Dpn levels unaffected. Furthermore, Notch pathway disruption does not seem to directly affect NB proliferation, as Cyclin E expression is not eliminated (Fig. 3). Therefore, Notch signalling from the GMC/iINP to the NB acts to ensure robustness in NB maintenance, in collaboration with Dpn.

When Notch signalling is aberrantly activated in the GMCs/iINPs, both type I and type II lineages overproliferate, although the former do so with lower penetrance (fewer lineages) and expressivity (smaller clones) (Table 2). Yet, for both types of lineages, E(spl) genes are necessary (Fig. 5) and sufficient (Fig. 6) to implement overproliferation. This is consistent with our hypothesis that ectopic E(spl)/Dpn activity in the GMCs/iINPs inhibits their differentiation and makes them competent to respond to mitogenic stimuli.

Why are Type II lineages more sensitive than type I lineages to Notch gain of function? A crucial difference between these NBs is the lack of expression of Ase in type II, as its artificial reinstatement can revert the latter to type I-like behaviour (Bowman et al., 2008). It was recently demonstrated that Ase downregulates E(spl) expression (Southall and Brand, 2009). It is even possible that Ase antagonizes E(spl) proteins post-transcriptionally, as the two can interact (Alifragis et al., 1997) and we have documented extensive antagonistic interactions between E(spl) proteins and Sc, a protein related to Ase, in different contexts (Giagtzoglou et al., 2003; Giagtzoglou et al., 2005). Thus, N hyperactivation will probably cause a smaller increase in E(spl) levels/activity in type I cells, compared with type II. If resistance to differentiation stimuli depends on the level of E(spl)/Dpn activity, this would account for the relative resilience of type I lineages to Notch-induced overproliferation.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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