**Drosophila** Hey is a target of Notch in asymmetric divisions during embryonic and larval neurogenesis

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**SUMMARY**

bHLH-O proteins are a subfamily of the basic-helix-loop-helix transcription factors characterized by an ‘Orange’ protein-protein interaction domain. Typical members are the Hairy/E(spl), or Hes, proteins, well studied in their ability, among others, to suppress neuronal differentiation in both invertebrates and vertebrates. Hes proteins are often effectors of Notch signalling. In vertebrates, another bHLH-O protein group, the Hey proteins, have also been shown to be Notch targets and to interact with Hes. We have studied the single *Drosophila* Hey orthologue. We show that it is primarily expressed in a subset of newly born neurons, which receive Notch signalling during their birth. Unlike in vertebrates, however, Hey is not expressed in precursor cells and does not block neuronal differentiation. It rather promotes one of two alternative fates that sibling neurons adopt at birth. Although in the majority of cases Hey is a Notch target, it is also expressed independently of Notch in some lineages, most notably the larval mushroom body. The availability of Hey as a Notch readout has allowed us to study Notch signalling during the genesis of secondary neurons in the larval central nervous system.

**KEY WORDS:** *Drosophila*, Hey, Notch, Asymmetric cell division, bHLH-O, Cell fate

**INTRODUCTION**

Among the superfamly of basic-helix-loop-helix (bHLH) transcription factors, several structurally distinct classes are discerned. One of these, the bHLH-Orange (bHLH-O) class (Fischer and Gessler, 2007; Iso et al., 2003), is characterized by the ‘Orange’ domain, a protein interaction domain perhaps serving as an extended dimerization surface (Taelman et al., 2004). bHLH-O proteins are important developmental and physiological regulators in processes ranging from neurogenesis to circadian rhythm control.

In a number of invertebrate and vertebrate species, bHLH-O repressors are known to inhibit neural differentiation. In *Drosophila*, the seven E(spl) bHLH-O proteins are expressed in the neuroectoderm, where they inhibit cells from differentiating as neuroblasts (NBs) (Jennings et al., 1994; Nakao and Campos-Ortega, 1996). In vertebrates, a number of Hes bHLH-O proteins, notably Hes1, Hes3 and Hes5 in the mouse, are also expressed in the neuroectoderm; in this case it is the neural stem cells that express the Hes genes, which are subsequently downregulated in the differentiating neuronal progeny (Kageyama et al., 2008). Triple *Hes*1, *Hes*3, *Hes*5 knock-out causes premature neural differentiation, disruption of the neuroepithelium and a hyperplastic nervous system owing to stem cell depletion (Hatakeyama et al., 2004). In *Drosophila*, loss of the entire *E(spl)* locus results in supernumerary NB specification from the neuroectoderm and a hyperplastic nervous system (Lehman et al., 1983). Despite these differences, owing to the different mode of neural precursor specification between vertebrates and insects, the generalization can be made that E(spl)/Hes proteins antagonize neuronal differentiation. At most developmental settings across metazoan phylogeny, neural expression of E(spl)/Hes genes is a direct response to Notch signalling (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Ohtsuka et al., 1999).

Expression of another subfamily of bHLH-O genes has been detected in the progenitor cell zones of the developing vertebrate central nervous system (CNS). These genes encode the three Hey proteins, so named after a characteristic tyrosine residue in their C-terminal domain (Hairy/enhancer-of-split like with a Y); they are also known as Hrt, Herp, Hesr, Chf or Gridlock (Kokubo et al., 1999; Leimeister et al., 1999). Although neural defects are minor in Hey knock-out mice, overexpression studies have suggested that Hey and Hes proteins might synergize with each other in suppressing neural differentiation and maintaining the neural stem cell fate (Sakamoto et al., 2003). *Hey1* has even been linked to the pathogenesis and aggressiveness of gliomas (Hulleman et al., 2009). Hey knock-out mice have highlighted their roles in developmental processes outside the nervous system, in particular, heart and vasculature development (Fischer et al., 2004; Kokubo et al., 2005). In these contexts, all three mammalian Hey genes appear to respond to Notch signalling, similar to E(spl)/Hes genes in neurogenesis. Biochemical data support Hes-Hey heterodimer formation (Iso et al., 2001; Taelman et al., 2004), raising the possibility that these two subclasses of bHLH-O proteins might synergize in some developmental contexts as Notch effectors.

The *Drosophila* genome contains a single *Hey* orthologue (Kokubo et al., 1999), which had not been studied to date. We decided to characterize it in the hope of better understanding the process of neural precursor specification, based on the assumption that, by analogy to vertebrates, Hey might display protein-protein interactions with E(spl). To our surprise, Hey was not co-expressed with the E(spl) proteins in the neuroectoderm, rather was restricted...
to differentiating neurons, suggesting a radically different role in neurogenesis than we had assumed. Once NBs are specified in Drosophila, they undergo cycles of asymmetric cell divisions that give rise to a secondary precursor, called a ganglion mother cell (GMC), in addition to self-renewing. GMCs divide once to give rise to two neurons or, less often, glia. The majority of GMC divisions are asymmetric, with the fates of the two daughters dictated by unequal levels of Notch signalling. The ‘A’ sibling neuron requires high Notch signalling, whereas the ‘B’ sibling neuron downregulates Notch reception, which is usually achieved by asymmetric segregation of a Notch inhibitor, Numb, into the nascent ‘B’ neuron (Skeath and Thor, 2003). We describe a complex pattern of Hey expression in relation to these divisions during both neurogenic phases of the animal, early embryogenesis and larval life, where thousands of new neurons are added to generate the adult CNS (Maurange and Gould, 2005). In all sibling pairs that we could identify, Hey was expressed in the ‘A’ neuron. Genetic analysis confirmed that Hey is a Notch target gene in most instances. Our results extend the Hey-Notch relationship to Drosophila in support of an ancient connection between bHLH-O genes and Notch activity and, for the first time, implicate a bHLH-O protein in the process of GMC asymmetric division.

**MATERIALS AND METHODS**

**DNA constructions**

Cloning of Drosophila Hey was done by PCR amplification from an embryonic cDNA library using gene-specific primers: Forward (EcoRI): gccggaatcatggatcacaacatg; Reverse (Xhol): taactcgacgtaggcaaatcttc. Amplified sequences were cloned into Bluescript and pGEM-T easy vectors. To ectopically express Hey in vivo, we subcloned the cDNA (EcoRI/Xhol) into the pUAST vector (Brand and Perrimon, 1993) and used it to transform flies.

**Fly strains**

All fly stocks were obtained from the Bloomington Stock Centre, the Exelixis Collection at Harvard or individual laboratories (see acknowledgements). The enhancer trap line AJ96-lacZ was used to identify the dMP2/vMP2 neurons (Menne and Klambt, 1994). K33-lacZ, an enhancer trap in E(spl)Hm7 – FlyBase (Cooper et al., 2000) was used to mark larval NBs. Mutant backgrounds were: Hey^{6665/CyO}; w; Df(2R)ED1735/CyO; w; Df(2R)Exel6055/CyO; mb, FRT2A/TM6B/CyO; hs-FLP; FRT40A; UAS-Hm74; UAS-GFP, 1/30,000 (Minotech); mouse anti-Repo 8D12, 1:25 (DSHB); guinea pig or mouse anti-Hey 1:1000 (this study); rat anti-Dpn, 1:1 (gift from C. Doe); rat anti-E-cadherin 1:20 (DSHB); rabbit anti-GFP, 1:30,000 (Minotech); mouse anti-Repo 8D12, 1:25 (DSHB); guinea pig or mouse anti-Hey 1:1000 (this study); rat anti-Dpn, 1:1 (gift from C. Doe); rat anti-E-cadherin 1:20 (DSHB); rabbit anti-Ase 1:1000 (gift from M. Arjan); and rabbit anti-Numb 1:1000/1:100 (gifts from Y.-N. Jan and J. Knoblich). Detection was done using secondary antibodies conjugated to Alexa 488, 555, 568, 633 or 647 (Molecular Probes), or Cy3 (Jackson ImmunoResearch). Embryos and tissues were imaged on a BioRad Radiance 2100 or Leica SP2 confocal microscope.

**RESULTS**

**Hey is transiently expressed in a subset of embryonic neurons and glia**

We amplified a full-length Hey cDNA from a Drosophila cDNA library, which we used as a probe for in situ hybridization (Fig. 1A), and for cloning in prokaryotic expression vectors. We then used bacterially expressed full-length Hey protein to raise anti-Hey antibodies. There were no obvious differences between the RNA and protein patterns. Hey protein showed nuclear accumulation, as expected for a transcription factor, and was primarily detected in a segmentally repeated pattern within the CNS (Fig. 1A,B) starting at stage 10. Later, more Hey-positive cells gradually appear in the CNS. The neuroectodermal epithelium, where the related E(spl) bHLH-O proteins are expressed already starting at stage 8 (Jennings et al., 1994), is devoid of Hey expression, which instead is detected at deeper levels overlapping with the GMC-immature neuron marker Pros (Fig. 1C,D) (Vaessin et al., 1991). From double-staining with the neuronal antigen Elav (Robinow and White, 1991) it was clear that the vast majority of Hey-positive cells represent neurons (Fig. 1E,F) rather than GMCs, confirmed as lack of colocalization with the NB/GMC marker Asense (Brand et al., 1993) (Fig. 1G,G′). Besides neurons, Hey expression was detected in a subset of Repo-positive glia of the CNS (Halter et al., 1995) (Fig. 1H,H′) and peripheral nervous system (PNS; not shown). Of note, Eve staining, which was used to visualize particular neurons (see below), also marks the dorsally located pericardial cells (Frasch et al., 1987; Su et al., 1999). No Hey immunoreactivity was detected within or near these heart precursors (Fig. 1C), contrary to the strong expression of mammalian Hey genes during cardiogenesis. Finally, a few Hey-positive cells per segment were detected in the embryonic PNS (Fig. 1A,B). Most of these were also neurons, by virtue of being Elav-positive (data not shown), but were not characterized further.

We used lineage-specific markers to characterize Hey expression in more detail. One was Even skipped, which marks a subset of neurons (Skeath and Doe, 1998): the aCC/pCC sibling pair, the RP2 motoneuron, the cluster of U motoneurons and the cluster of EL interneurons. Another was the AJ96-lacZ enhancer trap (Menne and Klambt, 1994), which marks the MP2 precursor and its progeny, the dMP2/vMP2 neurons. With AJ96-lacZ, we detected strong Hey accumulation in vMP2 but not in dMP2 (Fig. 2B). We could even detect weak Hey expression shortly before mitosis of the MP2 progenitor during late stage 10 (Fig. 2A). Among the Eve-positive neurons (Fig. 2G,H), pCC and the U neurons expressed Hey. aCC, RP2 and the EL neurons were Hey-negative. At stage 11, the sibling of RP2, RP2si, a smaller cell, which only transiently expresses Eve, was Hey-positive (Fig. 2G). Hey expression in all these neurons appeared transient. For example, whereas immunoreactivity in vMP2 was strong at stage...
12, it was downregulated and barely detectable by stage 14 (Fig. 2C). Similarly, by stage 14 no Hey could be detected in pCC cells, although it was still expressed strongly in some of the later-born U motorneurons (Fig. 2H). Transient Hey expression was also observed in the two identical progeny of MP1, a midline precursor, which are marked by Odd (see Fig. S1 in the supplementary material).

Most of the neurons described above belong to well-characterized lineages, in which sibling fates arise through differential Notch signalling. In each of the RP2/RP2sib, aCC/pCC and dMP2/vMP2 pairs, the second cell requires Notch signalling in order to acquire the ‘A’ fate, distinct from that of its sibling cell (‘B’ fate) (Skeath and Doe, 1998; Spana and Doe, 1996). Also in the U lineages, which arise from sequential GMCs from neuroblast NB7-1 (Cleary and Doe, 2006), the U neurons require Notch, whereas their Eve-negative Usib neurons do not. All Notch-requiring cells, namely RP2sib, pCC, vMP2 and the U cells, robustly express Hey, whereas none of their ‘B’-fate siblings do so. This raises the possibility that Hey is expressed in response to Notch.

Hey is a target of Notch signalling in the embryonic CNS

We used mutations that perturb Notch signalling to address whether Hey expression is regulated by Notch. As Notch is involved in a number of developmental decisions before neuron birth, most notably NB lateral inhibition (Lehman et al., 1983), it is expected that Notch-null embryos will exhibit a complex phenotype, which might obscure a later effect on Hey. We therefore turned our attention to mutations that have no defects in lateral inhibition, but disrupt Notch signalling specifically at later asymmetric cell divisions. Although Mastermind is an essential nuclear cofactor in Notch signalling (Bray, 2006), the hypomorphic mam^04615^ allele has sufficient activity to carry out lateral inhibition normally, but fails during asymmetric cell divisions (Skeath and Doe, 1998). spdo is dispensable for lateral inhibition, but its disruption abolishes Notch signalling, specifically in asymmetric cell divisions (O’Connor-Giles and Skeath, 2003; Skeath and Doe, 1998). In homozygous embryos for either mam^04615^ or spdo^55^ we detected a dramatic loss in Hey immunostaining (Fig. 2E,I,L,P) compared with wild-type embryos of the same stage. In an AJ96-lacZ background, we could not detect Hey in either of the MP2 progeny neurons, in agreement with fact that this division is now symmetric, producing two dMP2 (Hey-negative) cells (Fig. 2E). Staining spdo^55^ embryos with Eve reveals that Hey is absent from the symmetric aCC/aCC pair, that arises because of pCC-to-aCC cell fate switching. Finally, transformations of RP2sib into RP2 (two Eve-positive cells instead of one) and U into Usib (Eve-negative) result in the disappearance of Hey expression in RP2/RP2sib and U/Usib lineages as well (Fig. 2I). Similar Notch dependence of Hey expression is likely to occur in most other neuronal lineages, explaining the global reduction in Hey-positive cells.

In a converse experiment, we elicited ectopic Notch activity in ‘B’ neurons by using loss-of-function alleles of numb, which normally inhibits Notch signalling within ‘B’ neurons (Spana and Doe, 1996). In the severe numb^2^ mutant (Skeath and Doe, 1998), asymmetric GMC divisions become symmetric, giving rise to two ‘A’-type
neurons, a phenotype opposite to that of mam and spdo. In agreement with Hey being a target of Notch, numb2;AJ96-lacZ embryos contain two Hey-positive vMP2 cells at the expense of the dMP2 siblings (Fig. 2D). Examining Eve-positive lineages, a ‘B’- to ‘A’-type switch is evident with loss of Eve staining at the RP2 (‘B’-type) position and an increase in Eve staining at the U (‘A’-type) location (Fig. 2I). All of the supernumerary U cells are Hey-positive throughout the CNS of numb2 embryos compared with wild-type ones of the same stage. Taken together with the results from mutants with reduced Notch pathway activity, the numb phenotype confirms the responsiveness of Hey expression to Notch signalling in most CNS lineages.

We also studied a genotype with more severe disruption of Notch signalling. A double-null mutant for Di and Ser, the genes encoding the only two Notch ligands in Drosophila, displays a strong neurogenic phenotype (Lehman et al., 1983). This refers to a severe hyperplasia of CNS neurons, resulting from the inability of nascent NBs to laterally inhibit their neighbours. Despite displaying a large increase in total neuronal numbers, Di Ser embryos show a decrease in Hey-positive neurons, consistent with their Notch signalling defect (Fig. 2N). As a comparison, we used Df(3R)E(spl)522.2, a deficiency for the entire E(spl) complex. This harbours seven bHLH-O genes that are crucial targets of Notch in lateral inhibition but are not needed for Notch signal transduction per se. Indeed, Df(3R)E(spl)522.2-homozygous embryos display a neurogenic phenotype as severe as that of Di Ser embryos; however, in this case the number of Hey-positive cells is increased, paralleling the global increase in neurons (Fig. 2O). This supports the notion that Notch signalling goes on in GMC divisions in the absence of E(spl), resulting in Hey expression. The fact that a few Hey-positive cells persist in Di Ser, mam or

Fig. 2. Hey expression in wild-type and mutant backgrounds. (A-E) AJ96-lacZ line stained for Hey (green) and β-gal (red) to mark the vMP2/dMP2 cells in wild-type (A-C) and mutant backgrounds (D,E). (A) Hey expression in the MP2 lineage starts at stage 10 within the undivided MP2 neuroblast. (B) In stage 12 embryos Hey is expressed in vMP2s, the anteriorly located AJ96-positive cells, but not in dMP2s. (C) In stage 15 embryos Hey expression is turned off. (D) In the numb genetic background, which induces transformation of dMP2 into vMP2, Hey is expressed in both AJ96-positive cells (stage 12). (E) The opposite (Hey absence) is observed in spdo embryos, in which vMP2 is transformed into dMP2 (stage 12).

Note the scarcity of Hey-positive cells (also in I) compared with equivalently staged wild-type (B) or numb (D) embryos. (F-I) Hey expression in Eve-positive lineages in wild-type (F,G) and mutant (H,I) backgrounds. (F) A stage 11 embryo montage showing deep focal planes with aCC/pCC and RP2/ RP2sib pairs marked with Eve (red). Hey is expressed in pCC (arrow) but not aCC. RP2 does not express Hey in contrast to the smaller RP2sib (arrowhead) which is still Eve-positive at this stage. In some segments a Hey/Eve-positive U cell (asterisk) is evident. (G) A stage 15 embryo montage of superficial focal planes to visualize U and EL lineages. Eve marks the U and EL cells. Hey is expressed in the U cells (arrows) but not in the EL cells (arrowheads). (H) In a stage 15 numb embryo more cells within the U-cluster are labelled with Eve, as Usub is transformed into U. (I) Loss of Notch signalling in a spdo embryo (stage 12) results in two RP2s (arrowheads) and two aCCs (arrows) per hemisegment. Eve-positive U cells are transformed to Usub cells (Eve-negative or weakly positive near the aCC pairs). Persistent Eve-positive cells at the U position (asterisk) are either undivided GMCs or Usub cells that have not yet extinguished Eve expression. None of these cells express Hey. Hey expression is limited to a few midline cells and a cluster of lateral cells. (J-P) Ventral views of stage 15 embryos (J-L) and sagittal views of stage 12 embryos (M-P) of different genetic backgrounds stained for Hey. (J) Wild-type; (K) numb2; (L) spdo55; (M) wild-type; (N) Df(3R)E(spl)522.2; (P) mam04615. Note the increased number of Hey-positive neurons in the E(spl) and numb embryos compared with that of wild type embryos. Conversely, there are fewer Hey-positive neurons in Di Ser, mastermind and spdo embryos. Anterior is up (A-L) or to the left (M-P). Scale bars: 55 μm in A-L; 130 μm in M-P.
spdo mutant embryos could be either due to residual Notch signalling in these mutant backgrounds, or to a Notch-independent mode of Hey expression in specific cells.

**Hey is expressed in the larval CNS in response to Notch**

During larval stages, many NBs resume asymmetric divisions to produce large numbers of additional neurons in both the central brain and ventral nerve cord (VNC). This second burst of proliferation gradually ceases by pupariation, after which the post-embryonic (or secondary) neurons fasciculate with the pre-existing embryonic ones and remodel their projections in the process of building the adult CNS (Maurange and Gould, 2005; Truman et al., 2004). In third-instar larval CNS, we detected strong Hey accumulation in groups of cells positive for the neuronal marker Elav, but negative for the NB/GMC marker Ase (Fig. 3A). The Hey-positive cells are among the secondary neurons based on the following additional criteria, besides their lack of Ase and expression of Elav: (1) They are near the surface of the CNS; a group of Hey-positive cells was found adjacent to each NB/GMC cluster (Fig. 3A,B); (2) They have short axonal projections visualized by anti E-Cadherin/Shg (Fig. S2); and (3) They are GFP-positive when a NB lineage is positively marked as little as one day before fixation, indicating recent descent from the marked NB (Fig. 3B; see Fig. S2 in the supplementary material). Hey-positive immature neurons were also seen in large numbers within the optic lobe proliferation centres in two broad swaths below Ase-positive (Hey-negative) progenitors (Fig. 3C,D). As in the embryo, larval Hey expression is transient. Very few Hey-positive neurons were detected in the abdominal ganglion at late third instar (Fig. 3C), where imaginal neurogenesis has already ceased, although younger feeding larvae do contain Hey-positive cells in that region (data not shown). Furthermore, as neurogenesis ceases in other regions, such as the central brain after pupariation, the number of Hey-positive neurons decreases dramatically. By two days into pupation, only four mushroom body (MB) NBs per brain hemisphere continue to produce Hey-positive cells (Ito and Hotta, 1992) (Fig. 3E,F).

Although the majority of Hey-positive cells in the larval CNS are neurons, we also detected two instances of Hey-positive non-neuronal cells. One was in the dorsolateral brain, where a few cells were found positive for both Hey and Ase (see Fig. S3 in the supplementary material). These Hey/Ase double-positive cells were occasionally seen to label with phospho-histone H3-Ser10 (PH3), a mitotic marker. By virtue of their small size and characteristic anatomical location, we propose that these are the mushroom body (MB) GMCs. Indeed, in two day old pupal brains, where, as noted before, only the four MB NBs are actively proliferating, each of the four MB Hey-positive clusters includes 2-4 Ase-positive GMCs, in addition to many Ase-negative neurons (Fig. 3F). The second exception is a number of glial cells, which were revealed by Repo staining. Whereas surface glia are Hey-negative, many optic lobe glia located in the inner and outer proliferation centres are Hey-positive (Chotard and Salecker, 2007) (Fig. 3D).

To address the dependence of Hey on Notch signalling in the larval CNS, we generated MARCM mosaic clones (Fig. 4A) mutant for various Notch pathway components. In lineages null for the Notch receptor, no Hey immunoreactivity was observed (Fig. 4C). The same was true for clones for a null Su(H) allele (Fig. 4E). Su(H) is the transcription factor via which intracellular cleaved Notch is targeted to its downstream genes (Bray, 2006); therefore, Hey expression in post-embryonic neurons of the central brain, VNC and optic lobe is activated via the canonical Su(H)-dependent Notch pathway. Despite the fact that Notch signalling was abolished by these null alleles of Notch or Su(H), NB proliferation was not markedly perturbed and mutant GMCs...
(marked by Pros or Ase) and neurons (marked by Pros or Elav) were formed in apparently normal numbers (Almeida and Bray, 2005) (Fig. 4E-G). Strikingly, in MB lineages, Hey in both GMCs and neurons was unaffected in Notch or Su(H) loss-of-function genotypes (Fig. 4D; see Fig. S3 in the supplementary material), making the MB a region that expresses Hey in a Notch-independent manner. The newly characterized PAN lineages (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008), however, behaved as most NBs do, displaying loss of Hey upon disruption of the Notch pathway (see Fig. S4 in the supplementary material).

To gain a more complete picture of Notch signalling in secondary neurons we analyzed null mutant clones of the signal-sending machinery, namely Dl, Ser, neur and mib1, as well as the accessory

Table 1. Mosaic analysis of Hey expression in the larval CNS

<table>
<thead>
<tr>
<th></th>
<th>NB Hey+</th>
<th>NB Hey–</th>
<th>% NB Hey–</th>
<th>OL Hey+</th>
<th>OL Hey–</th>
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<tr>
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<td>4</td>
<td>18</td>
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</tr>
<tr>
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<td>100</td>
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<tr>
<td>Su(H)Δ47</td>
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<td>71</td>
<td>100</td>
<td>0</td>
<td>16</td>
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<tr>
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<td>96</td>
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<td>72</td>
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<td>1</td>
<td>3</td>
<td>5</td>
<td>0</td>
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<td>58</td>
<td>85</td>
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</table>

The number of neuroblast (NB) lineages that contain at least one Hey-positive cell are shown in the column NB Hey+. Column NB Hey– refers to NB lineages without Hey-positive cells. Under these columns, we have included clones from the central brain and ventral nerve cord (VNC), excluding the mushroom body (MB) region. Optic lobe (OL) outer proliferation centres were scored separately. As individual clones were hard to discern, we scored the number of brain hemispheres where marked clones in the optic lobe contained Hey-positive cells (OL Hey+) or where clones were devoid of Hey-positive cells (OL Hey–).
factor **spdo** (Table 1). In the central brain and ventral nerve cord, clones doubly mutant for **Dl Ser** displayed a marked absence of Hey-positive cells (Fig. 4K), with only 4% of the clones containing a small number of Hey-positive cells. The incidence of Hey-positive mutant cells is probably due to the non-autonomy of the **Dl Ser** mutations, namely the fact that mutant cells can still receive a signal sent from adjacent wild-type cells. The fact that this only happens rarely suggests that secondary neurons, in most cases, receive the signal from adjacent neurons of the same lineage, possibly even their immediate siblings. Contrary to the central brain/VNC, the medulla precursor neurons in the optic lobe displayed more widespread non-autonomous behaviour in **Dl Ser** clones. In all of these clones there were a few Hey-positive mutant cells, in contrast to **Notch or Ser(II)** clones, where none of the mutant cells expressed Hey (Fig. 4H-J). Mosaics null for **Dl or neur**, were qualitatively similar to **Dl Ser**, lacking Hey-positive neurons (Fig. 4F,J,L,N). One difference was in the central brain/VNC, where clones with Hey-positive cells were encountered more frequently, 28% and 21% for **Dl and neur**, respectively. This might be owing to low level residual signalling in these genetic backgrounds. In the case of the **Dl mutation**, this is probably due to **Ser**, considering the result from the **Dl Ser** double mutation. In the case of **neur**, the most likely candidate for supplying residual activity is Mib1, as these two ubiquitin ligases have been shown to have overlapping functions (Pitsouni and Delidakis, 2005; Wang and Struhl, 2005). **Ser** or mib1-null mutations did not affect Hey expression (Fig. 4M,P), suggesting that their role is only a minor supportive one in the present context.

Finally, we tested clones mutant for the tetraspamin Spdo. An accessory protein required only in instances of Notch signalling associated with asymmetric cell divisions (O’Connor-Giles and Skeath, 2003; Skeath and Doe, 1998). These clones also lacked Hey immunoreactivity, implicating Spdo in the genesis of secondary neurons (Fig. 4G). Fifteen percent of the brain/VNC clones did contain a few Hey-positive cells (Fig. 4O), as did most optic lobe clones. As the **spdo**null allele is a probable null, the non-autonomy observed might reflect a requirement for **spdo** in signal emission, similar to **Dl and neur**, although evidence to date places Spdo function in the signal receiving cell (Hutterer and Knoblich, 2005; Langevin et al., 2005; O’Connor-Giles and Skeath, 2003; Roegiers et al., 2005), making such an interpretation unlikely. Alternatively, escapers could be explained by perdurance of the wild-type **spdo** RNA and protein that were present in the progenitor cell that was still **spdo**null before clone induction.

In conclusion, our mosaic analysis lends support to a mode of Notch-dependent asymmetric cell division in the birth of secondary neurons for most CNS lineages. This cannot be unequivocally demonstrated because, unlike in the embryo, specific ‘A versus B’ neuron markers are lacking for the larval stage. Yet, the Notch-dependence, and especially the Spdo-dependence, of Hey expression is consistent with asymmetric GMC division, where one cell escapes Notch signalling by inheriting Numb, whereas the other receives a Spdo-aided Notch signal and, as a result, adopts a different fate. Indeed, most Hey-positive cells in the larval CNS display low or undetectable levels of Numb accumulation (see Fig. S5 in the supplementary material).

**Hey participates in asymmetric neuron fate establishment in the embryo**

*A piggyBac recessive lethal insertion, WH-f06656, was isolated in the first intron of Hey during the Exelixis screen (Thibault et al., 2004). Using our antisera, we were unable to detect any Hey protein in homozygous embryos (Fig. 5A) and we therefore refer to this insertional data as *Hey**f06656*. To determine whether the lethality is due to disruption of Hey or some distantly linked secondary mutation, we tested the *Hey**f06656* chromosome for complementation against two deficiencies in the *44A* region, **Df(2R)exel0655** and **Df(2R)ED1735**. In both cases, no heterozygotes were obtained confirming that lethality maps at or near the Hey locus.

The lethal phase of *Hey**f06656*/D individuals was determined to be late embryonic/early larval. Between 40% and 85% (depending on the experiment) of these individuals hatched, but most died within the first instar. The hatched larvae were less active than their wild-type (*Hey*+/-) siblings, and a small number even went through the first larval moult before dying. No obvious cuticular defects were detected in the dead embryos and larvae. We stained homozygous mutant embryos for various neuronal markers to assess CNS integrity. No consistent defects in the anatomy of longitudinal, commissural and peripheral nerve tracts were seen (data not shown).

We further determined whether particular neuronal fates might be affected. If Hey is needed to transduce the Notch signal which distinguishes ‘A’- from ‘B’-type neurons, we would expect the Hey mutant embryos to have a phenotype similar to **spdo** mutant ones, namely vMP2>dMP2, RP2sib>RP2 and U>Usib cell fate switches. Yet, Eve staining of Hey mutants revealed the presence of a single RP2 and a normal looking U-cluster per hemisegment (Fig. 5B). Similarly, 22C10 staining, which reveals the pioneer axonal tracts of dMP2 (points anteriorly) and vMP2 (points posteriorly), did not detect any defect in Hey mutants (data not shown). This was confirmed by staining for Odd, a protein expressed specifically in the dMP2 neurons and the two MP1 midline neurons (Spana et al., 1995). Besides the MP1s, only one cell per hemisegment (dMP2) was Odd-positive in *Hey**f06656* embryos and not two as we would expect had there been a vMP2>dMP2 fate switch (Fig. 5C). We conclude that **Hey** is not strictly required to realize the ‘A’ cell fate, despite its ‘A’-specific expression pattern. Alternatively, **Hey** could be required for ‘A’ versus ‘B’ fate determination but it might act redundantly with another Notch target so that the single **Hey** knockout produces only a slight defect, consistent with the variable embryo-larval lethality observed.

If the latter hypothesis is correct, we would expect that ectopic overexpression of Hey might cause an opposite ‘B’>‘A’ fate switch, similar to those seen in *numb* loss-of-function embryos, where endogenous **Hey** is overexpressed (Fig. 2). To test our hypothesis, we generated UAS-*Hey* transgenic flies and induced overexpression in various GMCs and their progeny by using the *fiz.ng-Gal4* line (Lin et al., 1995). This driver is expressed, among other neurons, in aCC/pCC, RP2/RP2sib and dMP2/vMP2 cells, but not in the U or EL lineages (Fig. 5D,E). This would place Hey ectopically in aCC, RP2 and dMP2, in addition to bolstering its levels in pCC, RP2sib and vMP2. Eve staining of *fiz.ng-Gal4*:**UAS-Hey** embryos revealed a loss of RP2 in 52% of the hemisegments (73/140 hemisegments from 9 embryos) suggesting a fate switch RP2 (Eve-positive)>>RP2sib (Eve-negative) (Fig. 5G). Odd staining revealed loss of dMP2 in 70% of hemisegments (35/50 hemisegments from 2 embryos), consistent with a dMP2 (Odd-positive)>>vMP2 (Odd-negative) transformation (Fig. 5I). Although Hey expression is also driven ectopically in the aCC cell, its putative transformation into a pCC cell fate could not be detected as Eve labels both siblings. From the above results, we conclude that Hey has the ability to switch neuronal fates from the ‘B’ to the ‘A’ fate in the absence of Notch signalling. Therefore, at least in these asymmetric GMC divisions (MP2 and GMC4-2), Hey expression appears sufficient to implement the Notch fate-determination effect.
In the process of the above gain-of-function experiments, we explored different Gal4 drivers to ectopically express Hey. We never observed any suppression of neurogenesis in either the embryo or the larva/adult using near-Gal4, dpp-Gal4, 253-Gal4 or Eg-Gal4 (see Fig. S6 in the supplementary material; data not shown). This is contrary to the amply documented loss of neural elements obtained when bHLH-O proteins of the E(spl)/Hes family are overexpressed. In our controls, we ectopically expressed E(spl)m7 and hairy and, in both cases, we observed significant suppression of neurogenesis. We conclude that Drosophila Hey has significantly diverged from the Hairy/E(spl) family in both structure and function and is involved in neuronal fate decisions rather than in regulating the number of neuronal precursors.

DISCUSSION
Hey is a transducer of the Notch signal in GMC asymmetric cell division

We have analyzed the expression pattern and function of the single Hey gene in Drosophila. We detected Hey almost exclusively in the CNS in young postmitotic neurons and glia, specifically those that receive a Notch signal at birth. It has long been appreciated (Buescher et al., 1998; Skeath and Doe, 1998; Spana and Doe, 1996; Udoloph et al., 2001) that Notch signalling plays an important role in the acquisition of neuronal/glial cell fate after GMC division, with most GMCs producing two different progeny, an ‘A’ cell with high Notch activity and a ‘B’ cell with no Notch activity. Still, no Notch target genes had been identified in this process. We now show that Hey is such a target gene in many, and perhaps all, GMC asymmetric divisions. Our conclusions are based on the expression pattern of Hey, its response to Notch pathway perturbation and on the ability of ectopic Hey to block development of RP2 and dMP2, two ‘B’-type neurons.

Although we have good evidence that Hey expression can recapitulate the effect of Notch signalling, Hey loss-of-function has only a mild phenotype. The trivial possibility that the transposon insertion allele used has residual activity is unlikely as (1) no Hey protein is detectable in homozygous mutants and (2) the Hey<sup>F06656</sup> allele results in recessive lethality. Nevertheless, the issue will be permanently decided with the generation and analysis of more Hey alleles. The alternative hypothesis, which seems more probable, is that one or more additional factors besides Hey can also act as nuclear effectors downstream of Notch in the ‘A’ GMC progeny. No Hey paralogues exist in the D. melanogaster genome, but
structurally divergent proteins, even outside the bHLH-O family, could share similar functional characteristics. At the moment, we have no good candidate for such a factor; however, we have excluded a number of bHLH-O factors that do not seem to be co-expressed with Hey in neurons, namely E(spl)IR7 and m8, Hairy and Dpn (Fig. 1D; data not shown).

Besides GMCs, a number of other neural progenitors, namely NBs, sensory organ precursors (SOPs) and SOP progeny cells, all undergo asymmetric cell divisions with Notch involvement (Knoblich, 2008; Lai and Orgogozo, 2004). We could not detect Hey expression in either the NB/GMC pair or in the SOP progeny cells of external sensory organs (Fig. 1; Fig. 3; Fig. 4; data not shown), suggesting that Hey expression is turned on exclusively in GMC asymmetric divisions. Hey-positive glia could also be the progeny of asymmetrically dividing GMCs (Udolph et al., 2001). It is yet unclear which cells might be the immediate progenitors of the few Hey-positive PNS neurons.

**Notch signalling is intimately related with bHLH-O genes**

Until the present work and the recent paper by Krejci et al. (Krejci et al., 2009), the only *Drosophila* bHLH-O genes known to be targets of Notch were the seven of the *E(spl)* complex. Hey and two other bHLH-O genes, *dpn* and *Her*, had been predicted as candidate Notch targets based on nearby clustering of putative Su(H) binding sites, the DNA elements via which activated Notch is tethered to its target genes (Rebeiz et al., 2002). Although *Her* does not seem to be a true Notch target (Rebeiz et al., 2002), Krejci et al. (Krejci et al., 2009) have shown that *dpn* is a Notch target in the muscle-progenitor-like *Drosophila* DmD8 cell line; an in vivo context for such a response has yet to be determined. Together with *Hey*, this makes a total of 9 out of 13 bHLH-O genes in the *Drosophila* genome which are regulated by Notch. It should be stressed that Notch has a number of additional (non-bHLH-O) targets, depending on the species and cellular context, but few, if any, show such widespread association as the bHLH-O genes. The latter are activated by Notch in a multitude of unrelated contexts, such as neuroectoderm, mesoderm, wing epithelium, leg segmentation (Bray, 2006; Lai, 2004) and now GMC asymmetric cell divisions in *Drosophila*, and in neural progenitors, presomitic mesoderm, cardiogenesis and vasculogenesis in vertebrates (Aulehla and Pourquie, 2008; High and Epstein, 2008; Louvi and Artavanis-Tsakonas, 2006).

In addition to its widespread Notch-dependent expression, we have detected a clear instance of Notch-independent expression of *Hey* within the GMCs and neurons of the MB precursors (Fig. 4D; see Fig. S3 in the supplementary material). Other examples where *Hey* expression does not correlate with known events of Notch signalling are the MP2 NB and the two MP1 midline neurons (Fig. 2A; see Fig. S1 in the supplementary material). It is also clear that in embryos with severe Notch signalling defects we still observe a small number of Hey-positive cells in the CNS (Fig. 2N), suggesting that there are additional neural lineages, where *Hey* is likely to be expressed independently of Notch. Analysis of the cis regulatory regions of *Hey* should shed light on Notch-dependent and Notch-independent enhancer elements.

**Hey function has diverged despite structural conservation**

The bHLH-O family has undergone considerable diversification during evolution (Simionato et al., 2007). Although sequence analysis can unambiguously assign genes to this family, it cannot identify orthologues in distantly related species. A classic example is the *Drosophila* to mammals comparison, where no clear orthologue relationships exist between Hairy, Dpn and the seven *E(spl)* in *Drosophila* and Hes1, 2, 3, 5, 6 and 7 in mammals, suggesting that the diversification of these proteins occurred separately after divergence of protostomes and deuterostomes. Hey proteins are the singular exception, being particularly well conserved. The bHLH domain of *Drosophila* Hey shows 97-98% similarity to that of its mammalian counterparts. This might lead one to expect substantial conservation of Hey function, which, strangely enough, was not observed.

First, mammalian Hey genes have a very broad expression pattern, including presomitic mesoderm, embryonic heart, vascular precursors, developing brain and spinal cord, neural crest etc (Kokubo et al., 1999; Leimeister et al., 1999). Fly Hey, by contrast, seems confined within the CNS and PNS. Although there is complexity in its expression, as documented here with its contextual Notch dependence/independence, the great majority of its expression pattern seems to be in the newly born Notch-dependent ‘A’-type neurons. The absence of Hey expression from the developing *Drosophila* heart is most striking, given the foremost importance of *Hey* genes in vertebrate cardiogenesis. A second indicator of functional non-conservation comes from comparing the role of *Hey* within the nervous systems of mammals versus *Drosophila*. In the former, *Hey* has been proposed to act in the maintenance of progenitor fate and to antagonize neuronal differentiation, similar to Hes proteins (Sakamoto et al., 2003). In fact, it has been proposed that Hey-Hes heterodimers mediate these effects. In the fly, we could not detect Hey expression within progenitor cells, with the few rare GMC exceptions, noted above. We could not even detect Hey- *E(spl)* or Hey-Dpn co-expression, although we did not test all seven *E(spl)* genes for lack of specific reporter lines. To overcome any doubt, we made functional tests by ectopically expressing *Hey*. Instead of suppressing sensory organ formation, it mildly increased the number of bristles, showing an opposite phenotype from that of *E(spl)* or *hairy* ectopic expression (see Fig. S6 in the supplementary material). We are therefore confident that Hey does not antagonize neural differentiation in the fly.

This leaves us with the puzzle of why Hey is so strongly conserved. Perhaps some yet uncharacterized molecular aspect of its role in chromatin recognition/transcriptional regulation is conserved, despite considerable diversification in cellular and developmental contexts. These contexts have diverged greatly between insects and vertebrates, the only unifying theme being their developmental contexts. These contexts have diverged greatly between insects and vertebrates, the only unifying theme being their conserved, despite considerable diversification in cellular and developmental contexts. These contexts have diverged greatly between insects and vertebrates, the only unifying theme being their conserved. Perhaps some yet uncharacterized molecular aspect of its role in chromatin recognition/transcriptional regulation is conserved, despite considerable diversification in cellular and developmental contexts. These contexts have diverged greatly between insects and vertebrates, the only unifying theme being their conserved. Perhaps some yet uncharacterized molecular aspect of its role in chromatin recognition/transcriptional regulation is conserved, despite considerable diversification in cellular and developmental contexts. These contexts have diverged greatly between insects and vertebrates, the only unifying theme being their conserved. Perhaps some yet uncharacterized molecular aspect of its role in chromatin recognition/transcriptional regulation is conserved, despite considerable diversification in cellular and developmental contexts. These contexts have diverged greatly between insects and vertebrates, the only unifying theme being their conserved. Perhaps some yet uncharacterized molecular aspect of its role in chromatin recognition/transcriptional regulation is conserved, despite considerable diversification in cellular and developmental contexts. These contexts have diverged greatly between insects and vertebrates, the only unifying theme being their conserved. Perhaps some yet uncharacterized molecular aspect of its role in chromatin recognition/transcriptional regulation is conserved, despite considerable diversification in cellular and developmental contexts. These contexts have diverged greatly between insects and vertebrates, the only unifying theme being their...
Supplementary material

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References


