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Drosophila optic lobe neuroblasts triggered by a wave of proneural gene expression that is negatively regulated by JAK/STAT

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Neural stem cells called neuroblasts (NBs) generate a variety of neuronal and glial cells in the central nervous system of the *Drosophila* embryo. These NBs, few in number, are selected from a field of neuroepithelial (NE) cells. In the optic lobe of the third instar larva, all NE cells of the outer optic anlage (OOA) develop into either NBs that generate the medulla neurons or lamina neuron precursors of the adult visual system. The number of lamina and medulla neurons must be precisely regulated because photoreceptor neurons project their axons directly to corresponding lamina or medulla neurons. Here, we show that expression of the proneural protein Lethal of scute [L(1)sc] signals the transition of NE cells to NBs in the OOA. L(1)sc expression is transient, progressing in a synchronized and ordered 'proneural wave' that sweeps toward more lateral NEs. *l(1)sc* expression is sufficient to induce NBs and is necessary for timely onset of NB differentiation. Thus, proneural wave precedes and induces transition of NE cells to NBs. Unpaired (Upd), the ligand for the JAK/STAT signaling pathway, is expressed in the most lateral NE cells. JAK/STAT signaling negatively regulates proneural wave progression and controls the number of NBs in the optic lobe. Our findings suggest that NBs might be balanced with the number of lamina neurons by JAK/STAT regulation of proneural wave progression, thereby providing the developmental basis for the formation of a precise topographic map in the visual center.

KEY WORDS: *Drosophila*, JAK/STAT, Medulla, Neuroblast, Proneural wave

INTRODUCTION

In order to populate the central nervous system (CNS) with the appropriate number of neurons and glia, both the proliferation of neural precursor cells and timing of neural differentiation must be controlled. In the *Drosophila* CNS, neuroblasts (NBs) have been extensively studied as a model for neural stem cell development (Campos-Ortega, 1993; Goodman and Doe, 1993). Embryonic NBs delaminate as single cells from an epithelium called the ventral neuroectoderm. Neuroectodermal cells divide symmetrically in the plane of the neuroectoderm to generate identical daughter cells, but, upon differentiation into NBs, their axis of division rotates to a vertical plane (perpendicular to the neuroectoderm). NBs divide asymmetrically to generate a self-renewing NB and a ganglion mother cell (GMC), which divides again and typically generates two post-mitotic neurons (Fuerstenberg et al., 1998; Yu et al., 2006). Similar mechanisms have been described for vertebrate CNS development; progenitor cells proliferate through symmetric division in which one cell gives rise to identical daughter cells, followed by the neurogenesis in which a subset of cells becomes restricted to a neuronal or glial lineage (Anderson, 2001; Gotz and Huttner, 2005). These cells undergo asymmetric cell division in which one cell is maintained as a multipotent progenitor cell, while the other is fated to differentiate into a neuron or glia within a few rounds of cell division.

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Mechanisms that underlie the neuroectoderm to progenitor NB transition in the CNS of flies and vertebrates have been difficult to identify, in part because the transitions are not well ordered in space and time. By contrast, we find and describe here, that the development of *Drosophila* medulla neurons is a process that can be precisely described because the transition from neuroepithelial (NE) cells to NBs progresses in a synchronized and ordered manner.

The *Drosophila* visual system is composed of the retina and the optic lobe. The latter contains three optic ganglia: lamina, medulla and lobula. During embryonic development, the optic lobe invaginates from a region of head epidermis called optic lobe placode (Green et al., 1993). The optic lobe loses contact with the outer surface of the embryo and forms an epithelial vesicle attached to the brain (Green et al., 1993), and soon after larval hatching, its cells start to proliferate and separate into an outer optic anlagen (OOA) and an inner optic anlagen (IOA) (Hofbauer and Campos-Ortega, 1990). Towards the end of the first instar, the OOA adopts a crescent shape, with the opening of the crescent pointing posteriorly (Nassif et al., 2003). The OOA generates the outer medulla and the lamina neurons, while the IOA generates the inner medulla, the lobula and the lobula plate neurons. The epithelial part of the OOA is composed of a single layer of NE cells. During first and second instar stages, NE cells of the OOA proliferate by symmetric cell division. NE cells differentiate into medulla NBs and lamina precursor cells at the medial and the lateral edge, respectively (Fig. 1A-C). Medulla NBs divide asymmetrically along apico-basal axis and produce GMCs, which divide again and become medulla neurons (Fig. 1C) (Egger et al., 2007; Nassif et al., 2003; Toriya et al., 2006).

The mechanisms underlying neurogenesis have been most intensely studied in the development of external sense organs and embryonic CNS of *Drosophila*. In these systems, NBs are induced from among NE cells in a 'proneural cluster' that express 'proneural genes' such as *atonal*, *achaete* (*ac*), *scute* (*sc*) and *lethal of scute* [*l(1)sc*] (Cabrera et al., 1987; Jarman et al., 1993; Jarman et al.,

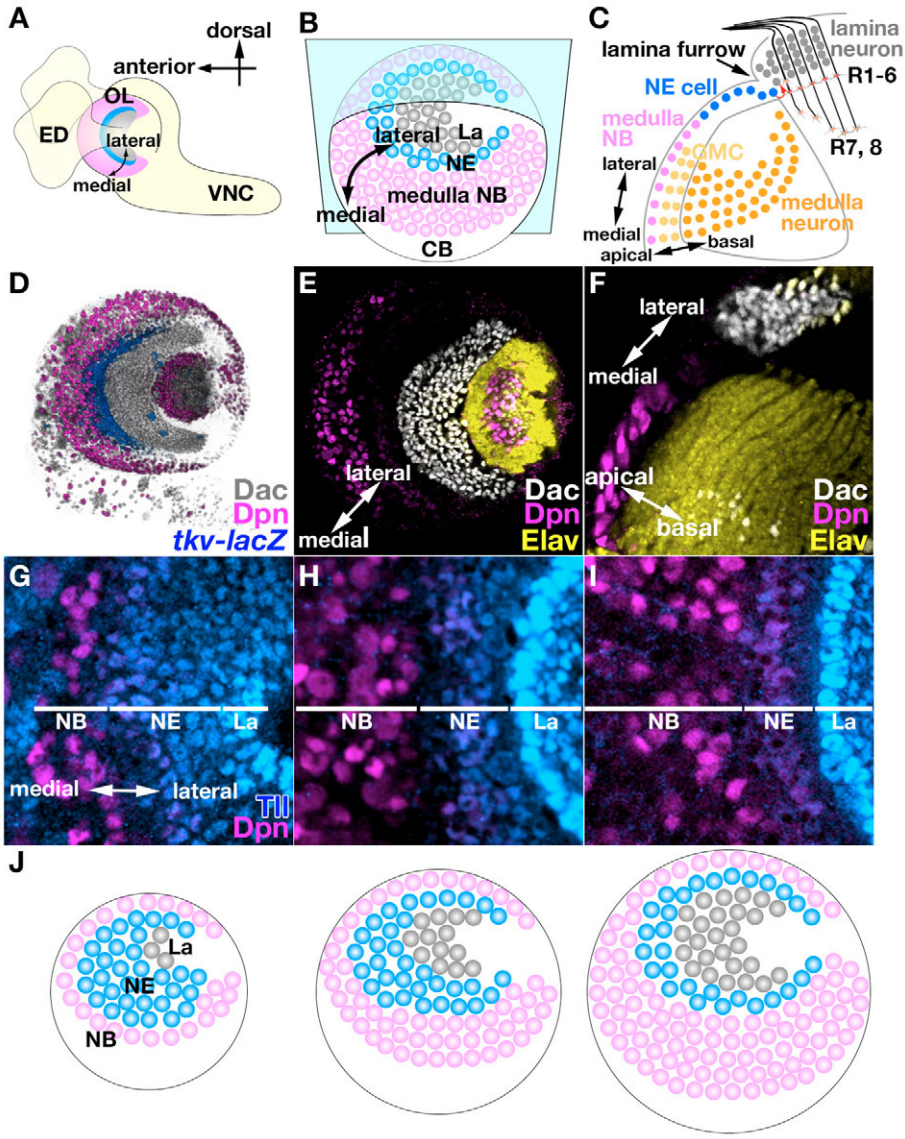

Fig. 1. Medullar neuroblast development. All images are of L3 stage unless otherwise noted. (**A**) Schematic of central nervous system. ED, eye disc; OL, optic lobe; VNC, ventral nerve cord. (**B**) Schematic of the optic lobe (ventrolateral view). Lamina neurons (La, gray), NE cells (blue), medulla NBs (light pink) and central brain (CB) are shown. (**C**) Schematic horizontal section of the optic lobe (section of blue plane in B). Lamina neurons (gray), NE cells (blue), medulla NBs (magenta), ganglion mother cells (GMCs, yellow), medulla neurons (orange), lamina furrow and R axons (R1-6 terminate in the lamina, while R7 and R8 project their axons through lamina to medulla) are shown. Cells located lateral most in the NE differentiate into lamina neurons (red arrow). (**D**) Confocal microscopic image of the optic lobe immunostained for lamina neurons (Dachshund, Dac, gray), NE cells (*tkv-lacZ*, blue) and medulla NBs (Dpn, magenta). (**E**,**F**) Lateral view (E) and horizontal section (F) of the optic lobe. Lamina neurons (Dac, white), NBs (Dpn, magenta), lobula neurons (Elav, yellow in E) and medulla neurons (Elav, yellow in F) are depicted. (**G-I**) The swath of NBs (Dpn, magenta) widens and the expanse of NE cells (Tailless, Tll, blue) decreases as the optic lobe matures. Optic lobe of early- (G), mid- (H) and late- (I) stage larvae. Tll is expressed in NE cells and lamina neurons. (**J**) Schematic of NB differentiation in G-I.

1994; Martin-Bermudo et al., 1991; Skeath and Carroll, 1992). These proneural genes encode basic helix-loop-helix (bHLH) transcription factors that dimerize with another bHLH protein Daughterless (Da) (Jarman et al., 1993; Murre et al., 1989a; Murre et al., 1989b; Villares and Cabrera, 1987). Single or several NBs are selected from each cluster by the mechanism known as lateral inhibition (Artavanis-Tsakonas and Simpson, 1991; Hassan and Vaessin, 1996). In contrast to the external sense organs and embryonic CNS, the differentiation from NE cells to medulla NBs is well ordered (Egger et al., 2007). We found a 'proneural wave' of differentiation starts from the medial edge of the NE sheet and sweeps the optic lobe from medial to lateral during third instar (L3) stage; *l(1)sc* is expressed transiently at the wave front and plays an important role in differentiation of NBs.

We also found that the Janus kinase/signal transuducer and activator of transcription (JAK/STAT) pathway negatively regulates proneural wave progression. JAK/STAT pathway is a well-conserved signaling pathway that was first identified in mammals. The *Drosophila* JAK/STAT signaling pathway is composed of several major factors: the three ligands Unpaired (Upd) (Harrison et al., 1998), Upd2 (Gilbert et al., 2005; Hombria et al., 2005) and Upd3 (Agaisse et al., 2003); the transmembrane receptor Domeless (Dome) (Brown et al., 2001; Chen et al., 2002); the JAK homolog Hopscotch (Hop) (Binari and Perrimon, 1994); and the STAT homolog Stat92E (Hou et al., 1996; Yan et al., 1996). JAK/STAT signaling is involved in many developmental processes, including segmentation in embryogenesis, eye morphogenesis, hematopoiesis and stem cell maintenance (Arbouzova and Zeidler, 2006; Luo and Dearolf, 2001; Zeidler et al., 2000). Here, we report a novel mechanism underlying NB (neural progenitor) formation and discuss its role in establishing a precise topographic map in the visual system.

MATERIALS AND METHODS

Genetics

Flies were grown at 25°C otherwise noted. *y w* and *Canton-S* flies were used as wild-type controls. The following mutant and transgenic strains were used in this study. *tkv-lacZ* is a reporter construct that has an insertion of a P element carrying *lacZ* in the promoter region of the *thickveins* gene (Tanimoto et al., 2000). *ap-lacZ* is a P element enhancer trap insertion just 5′ of the *apterous* gene and expressed in ~50 % of medulla neurons (Cohen et al., 1992). *upd-Gal4* is an enhancer trap line of *upd* (Halder et al., 1995; Tsai and Sun, 2004) and its expression was visualized by crossing with *UAS-*

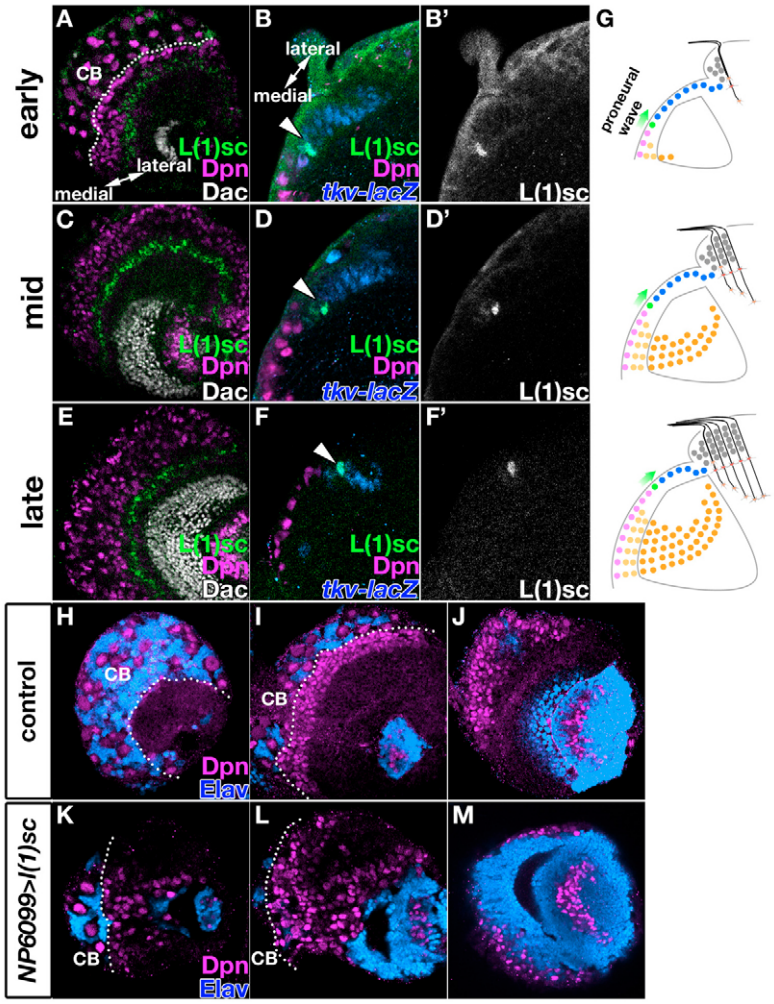

Fig. 2. L(1)sc is expressed in medial edge of the NE and can induce medulla NB differentiation.
(**A-F'**) L(1)sc is expressed in the medial edge of NE cells. Early (A,B), mid (C,D) and late (E,F) stages are shown. (A,C,E) Lateral view. Expression of L(1)sc (green), Dpn (magenta) and Dac (white) are shown. Broken white line in A indicates the border between optic lobe and central brain. (B,D,F) Horizontal section. Expression of L(1)sc (green), Dpn (magenta) and *tkv-lacZ* (blue) are shown. White arrowheads show cells expressing L(1)sc. (B′,D′,F′) L(1)sc expression in B,D,F. (**G**) Schematics of the horizontal section as in Fig. 1C. L(1)sc-expressing cells (green) and 'proneural wave' (green arrow) are shown. (**H-M**) Uniform and premature NB differentiation associated with ectopic *l(1)sc* expression driven by *NP6099* Gal4. Early (H,K), mid (I,L) and late (J,M) stages are shown. NBs are marked by Dpn (magenta) and neurons by Elav (blue). Broken white lines indicate the border between optic lobe and central brain.

GFP.nls flies. *10xSTAT-GFP* is an in vivo detector that reflects the activation of JAK/STAT signal (Bach et al., 2007). *UAS-l(1)sc* was described previously (Carmena et al., 1995). *UAS-l(1)sc* was overexpressed by crossing with *NP6099* driver (Hayashi et al., 2002; Yoshida et al., 2005). Overexpression clones of *UAS-upd* (Zeidler et al., 1999) and *UAS-hop*$^{Tum\text{-}l}$ (Harrison et al., 1995) were induced by *hs-flp; AyGal4* flies (Ito et al., 1997). *eya*^{1} is a mutant allele of *eyes absent* described by Bonini et al. (Bonini et al., 1993). Deficiency chromosome of *Df(1)260-1*, *Df(1)sc10-1*, *Df(1)sc19* or *Df(1)ase1* was recombined onto *FRT19A*. These clones were induced by *NP7340* (Hayashi et al., 2002). *UAS-flp*. *da*^{10} is an amorphic allele of the *da* mutant (Caudy et al., 1988). Clones of *da*^{10} were induced by *NP6099 UAS-flp*. *hop*^{2} is a null allele of *hop* (Perrimon and Mahowald, 1986). *Stat92E*^{85C9} is a strong hypomorphic allele of *Stat92E* (Silver and Montell, 2001) and *Stat92E*^{6346} is a putative null allele of *Stat92E* (Hou et al., 1996). *Stat92E*^{F} is a temperature-sensitive allele of *Stat92E* (Baksa et al., 2002). *Stat92E*^{85C9}/*Sta92E*^{F} and *Stat92E* ^{6346}/*Stat92E*^{F} flies were raised at 29°C. To assess the function of Stat92E, we generated clones in a *Minute* back ground with *hsflp; FRT82 ubi-GFP M(3)*^{w124} (Ferrus, 1975).

Histology

Immunohistochemistry was performed as described (Huang and Kunes, 1996; Takei et al., 2004). The following antibodies were provided by the Developmental Studies Hybridoma Bank (DSHB): mouse anti-Dac (mAbdac2-3, 1:1000), mouse anti-Arm (N2 7A1, 1:40) and rat anti-Elav (7E8A10, 1:50). Rabbit anti-Tll (1:600) was provided by East Asian Distribution Center for Segmentation Antibodies. We also used rat anti-L(1)sc (1:800, A. Carmena), guinea pig anti-Dpn (1/1000, J. Skeath), rabbit anti-Ase (1:2000, Y. N. Jan), rabbit anti-Dlg (1:1000, T. Uemura), goat anti-Horseradish Peroxidase (HRP, 1:100, Accurate Chemical and Scientific), rabbit anti-cleaved Caspase 3 (1:100, Cell Signaling Technology), mouse anti-β-gal (1:250, Promega) and rabbit anti-β-gal (1:2000, Cappel). Secondary antibodies (Jackson) were used at the following dilutions: anti-mouse Cy3, 1:200; anti-mouse Cy5, 1:200; anti-mouse FITC, 1:200; anti-guinea pig Cy3, 1:200; anti-guinea pig Cy5, 1:200; anti-rat Cy3, 1:200; anti-rat Cy5, 1:200; anti-rabbit FITC, 1:200; anti-rabbit Cy5, 1:200; anti-rabbit Alexa 546, 1:200 (Molecular Probes). Specimens were mounted with vectashield mounting media (Vector) and viewed on a Zeiss LSM510 confocal microscope.

In situ hybridization

In situ hybridization was performed as described previously (Nagaso et al., 2001). DNA template for the *upd* probe has been described previously (Tsai and Sun, 2004).

RESULTS

Development of medullar NB

During larval development, photoreceptor cells (R cells) in the eye disc project axons to the optic lobe in the brain (Fig. 1A). Axons of R1-R6 terminate in the lamina, and axons of R7 and R8 terminate in the medulla (Fig. 1C). Lamina neurons are in the lateral most aspect of the optic lobe; medulla NBs that produce large numbers of medulla neurons are on the medial side (Fig. 1B,C,E,F). Both medulla and lamina neurons derive from NE cells that constitute the surface of the OOA (Fig. 1B-F) (Nassif et al., 2003). Medulla neurons derive from NBs on the medial side of the NE sheet; lamina neurons derive directly from the lateral side of the OOA without

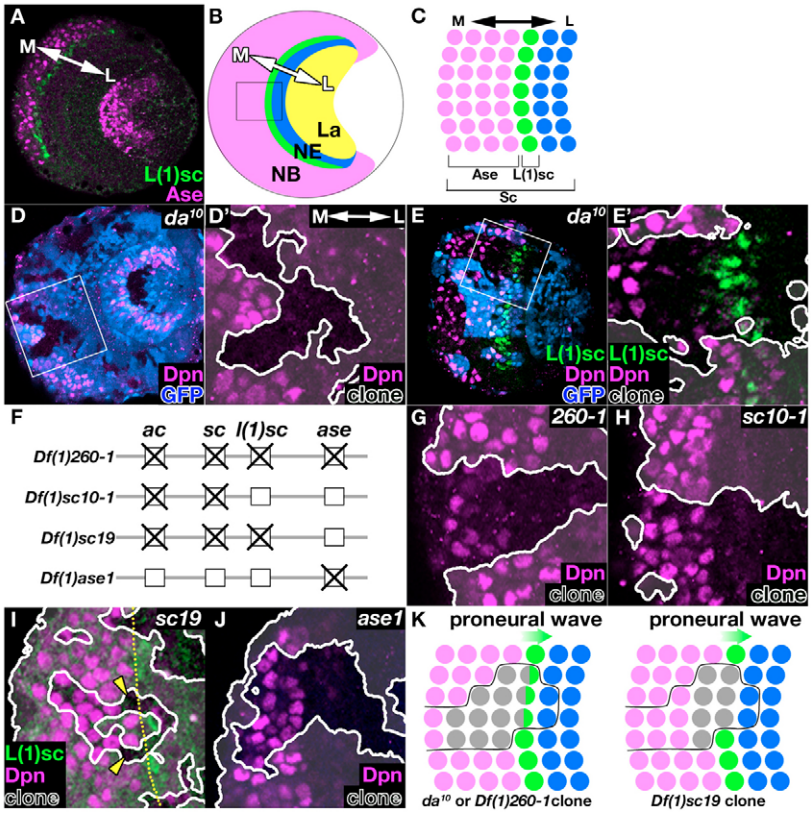

Fig. 3. Proneural genes regulate medulla differentiation. (**A**) L(1)sc (green) and Ase (magenta) are expressed in NE cells and NBs, respectively. (**B**) Schematic of lateral view of the optic lobe. Lamina neurons (yellow), L(1)sc-expressing cells (green), NE cells (blue) and NBs (magenta) are shown. (**C**) Expression pattern of proneural genes in square of B. Among the proteins of the AS-C, Sc is expressed in both NE cells and NBs, L(1)sc in the medial edge of NE cells, and Ase in NBs, respectively. (**D**,**D'**) Initiation of NB differentiation was delayed in *da*^{10} clones. Dpn expression (magenta) almost disappeared in the *da*^{10} clones, shown by the absence of GFP (blue in D) and of white in D'. (D') Higher magnification of square in D. (**E**,**E'**) Expression of L(1)sc (green) was not affected in *da*^{10} clones, shown by the absence of GFP (blue), while onset of Dpn expression was delayed (magenta). (E') Higher magnification of square in E. Clones are indicated by the absence of white signal. L(1)sc was shown in green and Dpn in magenta. (**F**) Genomic locus of *AS-C* and deficiency lines used. Deleted genes are depicted by crosses. (**G**) Onset of Dpn expression (magenta) was delayed in *Df(1)260-1* clones, indicated by the absence of white signal. (**H**) Onset of Dpn expression (magenta) was not affected in *Df(1)sc10-1* clones, indicated by the absence of white signal. (**I**) Onset of Dpn expression (magenta) was slightly delayed in *Df(1)sc19-1* clones shown by the absence of white signal. Expression of L(1)sc is shown by green. Broken yellow line shows the border between NE and NBs, and yellow arrowheads indicate cells not expressing Dpn in the clones. (**J**) Onset of Dpn expression (magenta) was not affected in *Df(1)ase1* clones shown by the absence of white signal. (**K**) Summary of the phenotypes. Onset of Dpn expression was delayed by 4-6 rows of cells in the clones of *da*^{10} or *Df(1)260-1* (left), and delayed by one or two rows in the clones of *Df(1)sc19* (right). Clones are within the black lines. NBs (magenta), L(1)sc-expressing cells (green), NE cells (blue) and cells yet to express Dpn (gray) are shown. L(1)sc expression remained in *da*^{10} clones but not in *Df(1)260-1* clones (half gray and half green circles).

formation of NBs (Fig. 1B-F) (Nassif et al., 2003). Medulla NBs are generated during L3 stages both by symmetric divisions of the OOA NE cells and progressively from the medial edge (Fig. 1G-J) (Egger et al., 2007; Nassif et al., 2003). NB generation is synchronized, forming a one-cell wide band of newly differentiated NBs to the inner slope of the U-shaped developing OOA. The NBs divide asymmetrically with their division plane oriented perpendicular to the surface and along the apico-basal axis; they produce medulla neurons basally and expand the volume of the optic lobe (Fig. 1C,F) (Ceron et al., 2001; Egger et al., 2007; Toriya et al., 2006). As the swath of NBs widens during maturation of the optic lobe, the expanse of NE cells decreases (Fig. 1G-J). On the lateral most side, NE cells receive Hedgehog (Hh) signals from innervating R axons and differentiate into lamina neurons (Fig. 1C,F) (Huang and Kunes, 1996; Huang and Kunes, 1998).

Proneural wave of *l(1)sc* expression sweeps from the medial to lateral optic lobe neuroepithelium and induces medullar NB differentiation

We determined that the proneural protein L(1)sc (Hinz et al., 1994; Jimenez and Campos-Ortega, 1990; Martin-Bermudo et al., 1991) is transiently expressed in a narrow band of 1-2 cells at the medial edge of the NE sheet (Fig. 2A-G). This band precedes NB formation and moves laterally throughout the L3 period. The expression pattern of L(1)sc led us to test the idea that L(1)sc makes NE cells competent to differentiate into medulla NBs. We overexpressed *l(1)sc* in most of the NE cells during the stages when medulla neurogenesis begins, and observed that, at early stages, the number of Deadpan (Dpn)-expressing cells on the surface of the optic lobe increased; at later stages, only medulla neurons were observed and lamina was not formed (Fig. 2K-M; 100%; *n*=20, compare with Fig.

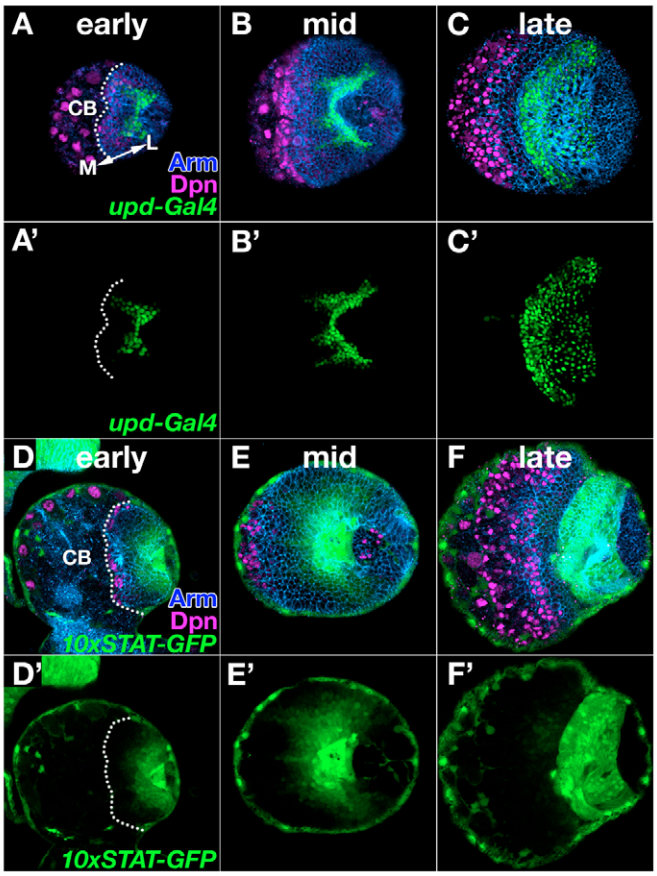

Fig. 4. JAK/STAT signal is activated in the NE cells. (**A-C′**) *upd-GAl4* visualized with *UAS-GFP.nls* is expressed in the lateral side of NE cells in early (A) to mid (B) L3 stages. In the late L3, strong *Gal4* expression is restricted to lamina neuron (C). NE cells are visualized by strong expression of Arm (blue) and NBs with Dpn (magenta). (A′-C′) Only *upd-GAl4* channel is shown. (**D-F′**) *10xSTAT-GFP* is expressed in the lateral side of NE cells during early (D) to mid (E) L3 stages. The signal is higher in the lateral side. In the late L3, GFP signal was restricted in lamina neurons (F). NE cells are visualized by strong expression of Arm (blue) and NBs with Dpn (magenta). (D′-F′) Only *10xSTAT-GFP* channel is shown. Broken white line in A and D indicates the border between optic lobe and central brain.

2H-J, see also Fig. S1 in the supplementary material). These results suggest that L(1)sc activity is sufficient to induce NBs, and the medulla and lamina neuron precursors derive from the limited number of cells in a common primordium. As L(1)sc-expressing cells lead a front that sweeps across the optic lobe and induces differentiation of NBs, we refer to the band of L(1)sc-expressing cells as the proneuroal wave (Fig. 2G). The proneural wave converts all NE cells to NBs in an ordered manner from the medial side. This program contrasts with the neurogenesis of the *Drosophila* sense organs or embryonic CNS in which only one or several NBs are selected from the NE cells.

We asked whether L(1)sc is required for NB formation. As no mutant lacking only *l(1)sc* is available, we first examined *da* mutants. *da* encodes a bHLH transcription factor that is an indispensable co-factor for all known bHLH proneural proteins (Murre et al., 1989b). In clones of cells mutant for da^{10}, an amorphic allele of *da*, expression of Dpn was strongly reduced (Fig. 3D; *n*=18). As Dpn often remained in the medial cells of the clones, we infer that Dpn expression is delayed in mutant cells. However, L(1)sc expression was not affected in mutant cells (Fig. 3E,K; *n*=13). We conclude that proneural function is required to time NB differentiation but not for progression of the proneural wave. This result also suggests that the progression of the proneural wave is independent of NB differentiation.

In order to assess the requirement for other proneural genes, we used deficiency chromosomes that delete various regions of the *achaete-scute* complex (*AS-C*; Fig. 3F). The *AS-C* contains four bHLH proneural genes: *ac*, *sc*, *l(1)sc* and *asense* (*ase*). In wild type, Ac expression was not detected; both NE cells and NBs express Sc (Egger et al., 2007) and Ase expression was detected in NBs (Fig. 3A-C). Mutant clones deficient for all the proneural genes [*Df(1)260-1* (Hinz et al., 1994)] caused the delay in Dpn expression by 4-6 rows of cells, which is indistinguishable from the *da* mutant clones, suggesting that only these four proneural genes are crucial for the proneural function in the optic lobe (Fig. 3G,K; *n*=29). As clones deficient for both *ac* and *sc* [*Df(1)sc10-1* (Campuzano et al., 1985)] did not affect Dpn expression (Fig. 3H; *n*=10), we conclude that *ac* and *sc* are not essential for NB differentiation. However, Dpn expression was delayed by 1-2 rows of cell in clones deficient for *ac*, *sc* and *l(1)sc* [*Df(1)sc19* (Carmena et al., 1995)], suggesting that *l(1)sc* is required for the timely onset of NB formation (Fig. 3I,K; *n*=36). Ase expression was not affected in these clones (data not shown). Mutant clones deficient for *ase* (Brand et al., 1993) initiated Dpn expression normally (Fig. 3J; *n*=19), suggesting that *ase* is required for timing of the normal onset of NB formation when *l(1)sc* is impaired. Sc might also have a redundant function but appropriate deficiency chromosomes are not available at present to unambiguously study the role of Sc. We also examined whether the onset of NB formation is regulated by inputs from the retinal axons because lamina development depends on arriving retinal axons (R axons) (Selleck and Steller, 1991). The 'eyeless' mutant, eya^{1} (Bonini et al., 1993), did not affect L(1)sc expression or medulla NB differentiation, suggesting that medulla neurogenesis is independent of R axon projection at least at this early phase (see Fig. S2A,B in the supplementary material). However, the medulla neuropil of this mutant was poorly organized in the later pupal stage partly because of excess cell death (see Fig. S2C,D in the supplementary material).

JAK/STAT signal is activated in NE cells

We next searched for genes that regulate proneural wave progression and determined that *upd* is expressed in NE cells (Fig. 4). Analysis of *upd-Gal4* (Halder et al., 1995; Tsai and Sun, 2004) suggests that the pattern of *upd* expression is dynamic. In the early L3 stage, *upd-Gal4* is expressed in some NE cells that express high levels of Armadillo (Arm) (Fig. 4A) (Hayden et al., 2007). *upd-Gal4* expression was restricted to the lateral side of the NE in mid L3 (Fig. 4B) and was in the lamina neuron precursors in late L3 (Fig. 4C, see Fig. S3A in the supplementary material). In the early to mid L3, expression pattern of *upd* mRNA was similar to that of *upd-Gal4* (see Fig. S3B,C in the supplementary material). But in late L3, *upd* mRNA was specifically expressed in the lamina furrow that is located at the most lateral NE cells (see Fig. S3D,E in the supplementary material). The different patterns observed in the enhancer trap expression and mRNA distribution might be caused by perdurance of the Gal4 and/or GFP reporter protein (Tsai and Sun, 2004).

To determine where the JAK/STAT signal is activated in the optic lobe, the expression of a *10xSTAT-GFP* reporter construct (Bach et al., 2007) was examined. GFP fluorescence was observed from early L3 (Fig. 4D), and was detected in the lateral side of the NE cells in

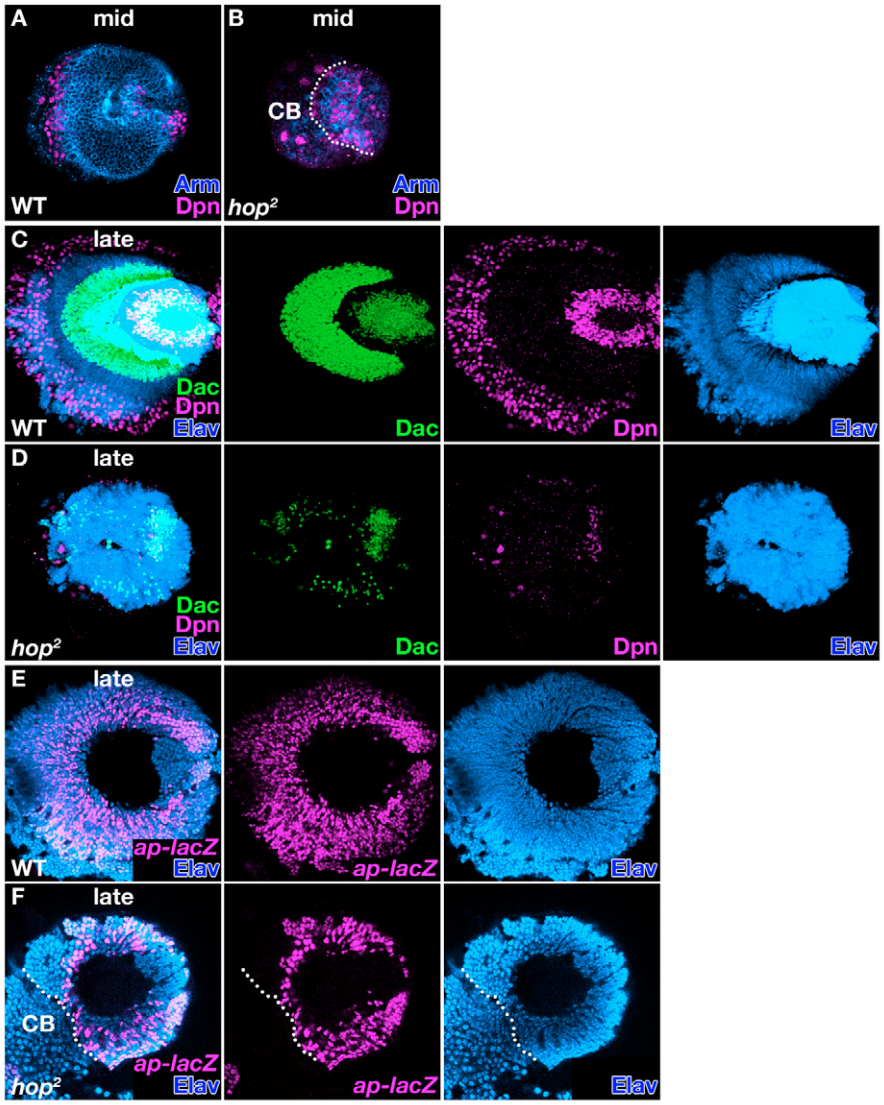

Fig. 5. JAK/STAT signal is required for the production of proper number of medulla neurons and lamina formation. (**A**,**B**) Mid L3 optic lobes of wild type (A) and *hop*^{2} (B). NE cells are marked by Arm (Blue) and NBs by Dpn (magenta). Both NE cells and NBs expressed high levels of Arm and Dpn in the *hop*^{2} optic lobe (B), while only cells at the transition from NE cells to NBs express both in the wild type (A). Broken white line in B indicates the border between optic lobe and central brain. (**C-F**) Late L3 optic lobes of wild type (C,E) and *hop*^{2} (D,F). (C,D) In *hop*^{2} mutant, NBs (Dpn, magenta) disappeared, neurons (Elav, blue) were fewer and lamina (Dac, green) was disrupted. (E,F) In *hop*^{2} mutant optic lobe, most neurons expressing Elav also express *ap-lacZ*, which is a marker for the medulla neurons, as in the wild type (F, compare with E). Broken white line in F indicates the border between optic lobe and central brain.

mid L3 (Fig. 4E). In late L3, the GFP fluorescence was weak in the NE cells and stronger in the lamina (Fig. 4F). These results suggest that JAK/STAT signaling is activated in the NE cells at least in early to mid L3, and that it is low medially and higher in the lateral NE cells (Fig. 4B,E).

JAK/STAT signal negatively regulates the progression of proneural wave

To determine whether JAK/STAT signaling has a role in NB development, we examined loss of function phenotypes for components of the JAK/STAT pathway, including Hop and Stat92E. In mid L3 optic lobes of *hop*^{2}, a null allele of *hop* (Perrimon and Mahowald, 1986), both NE cells and NBs were found to express high levels of Arm and Dpn (Fig. 5A,B; 100%; *n*=9). In the wild type, they are co-expressed only in cells at the transition from NE cells to NBs. Late L3 mutant optic lobes were smaller, had few NBs, the number of neurons was fewer and lamina neurons were absent (Fig. 5C,D; 100%; *n*=20). We infer that NBs developed prematurely in the *hop*^{2} mutant and that in the absence of the JAK/STAT signal, there was insufficient proliferation of the NE cells prior to transition to the NB fate. Similar phenotypes were observed in *Stat92E*^{85C9}/*Stat92E*^{F} or *Stat92E*^{6346}/*Stat92E*^{F} loss-of-function mutants (see Fig. S4 in the supplementary material and data not shown). To examine whether Elav-expressing neurons observed in *hop*^{2} mutant were differentiated medulla neurons, optic lobes are stained by *ap-lacZ* (Fig. 5E,F). Neurons in *hop*^{2} mutant expressed *ap-lacZ*, which indicates that though the number of NBs in *hop*^{2} was fewer than wild-type flies, NBs in the mutant optic lobe produce differentiated medulla neurons (Fig. 5F; 100%; *n*=15).

To further investigate the role of JAK/STAT signaling, *Stat92E* mutant clones were generated. Near clones of *Stat92E*^{85C9}, which is a strong hypomorphic allele of *Stat92E* (Silver and Montell, 2001), both L(1)sc and Dpn were expressed in more lateral cells than in surrounding *Stat92E*^{85C9}/+ cells (Fig. 6A,H; *n*=29), expression of Ase was present at more lateral locations, and ectopic Elav expression was observed within and near *Stat92E*^{6346} (putative null allele of *Stat92E*) (Hou et al., 1996) clones (Fig. 6B; *n*=21). These results suggest that loss of Stat92E function leads to faster progression of proneural wave and earlier initiation of medulla differentiation. Interestingly, these phenotypes were not cell-autonomous. Ectopic expression of L(1)sc was observed not only in the mutant cells but, in some cases, in adjacent *Stat92E*^{85C9}/+ cells as well (Fig. 6A′, arrowheads). When *Stat92E*^{85C9} clones were extended into the putative lamina region, Dac-expressing lamina

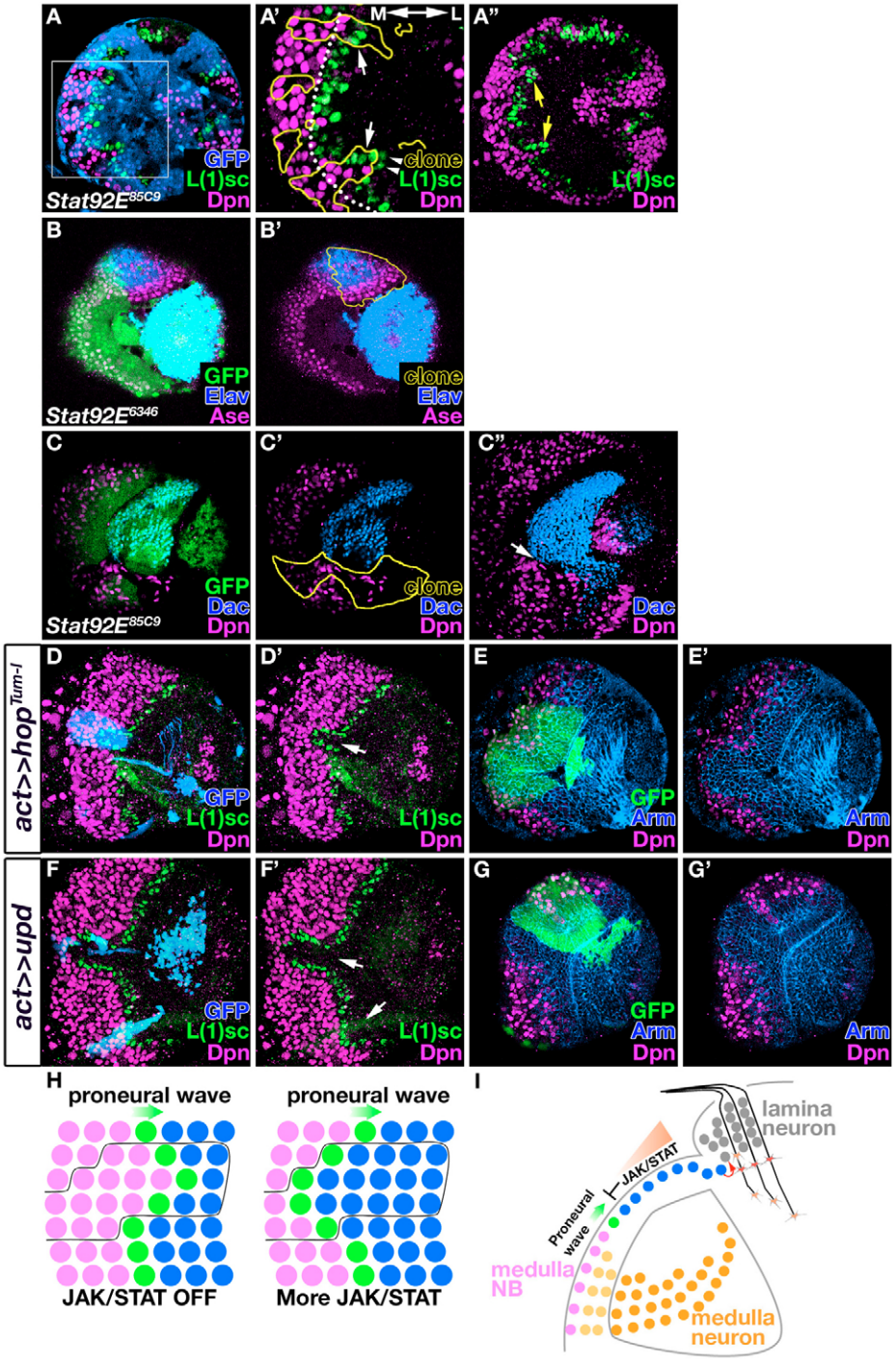

Fig. 6. JAK/STAT signal negatively regulates the progression of proneural wave. (**A-A″**) Loss of Stat92E function lead to earlier progression of proneural wave and NB development. *Stat92E*^{85C9} clones were shown by the absence of GFP (blue). (**A′**) Enlarged view of square in A. Clones were circled by yellow lines. Broken white line indicates border between NE and NB. White arrows show earlier (more lateral) expression of Dpn (magenta) and L(1)sc (green). L(1)sc was ectopically expressed in the *M(3)*^{w124}/+ cells next to the clones (white arrowheads). (**A″**) Projection of XX stacking confocal planes including A. Yellow arrows show earlier expression of Dpn (magenta) and L(1)sc (green). (**B**,**B′**) Ase (magenta) was expressed in more lateral cells and ectopic neurons (Elav, blue) were observed associated with the *Stat92E*^{6346} clones. The clones are shown by the absence of GFP (green) in B and yellow line in B′. (**C-C″**) JAK/STAT signal regulates the number of lamina neurons and medulla NBs. *Stat92E*^{85C9} clones are shown by the absence of GFP (green; circled by a yellow line in C′). (C″) Projection of XX stacking confocal planes including C. In and around the *Stat92E*^{85C9} clone, putative lamina cells [marked by Dac (blue)] were replaced by medulla NBs [marked by Dpn (magenta)], so that the lamina region was reduced in contrast to the normal optic lobe (arrow in C″, compare with Fig. 1D or Fig. 5C). (**D**,**D′**) L(1)sc (green) and Dpn (magenta) remained more medial; nearby cells expressing *hop*$^{Tum-l}$ are marked by the expression of GFP (blue) or arrowhead in D′. (**E**,**E′**) Expression of Arm (blue) remained in the *hop*$^{Tum-l}$-expressing clone. Clones were marked by the expression of GFP (green) and NBs by Dpn (magenta). (**F**,**F′**) L(1)sc (green) and Dpn (magenta) remained more medial; nearby cells expressing *upd* are marked by the expression of GFP (blue) or arrowhead in F′. (**G**,**G′**) Expression of Arm (blue) remained near the *upd*-expressing clone marked by GFP (green). NBs were marked by Dpn (magenta). (**H**) Summary of loss-of-function and gain-of-function mutation of the JAK/STAT signal. Proneural wave extends more medially with elevated JAK/STAT signaling and laterally with decreased signaling. Clones are within the black lines. NBs (magenta), L(1)sc-expressing cells (green) and NE cells (blue) are shown. (**I**) A model for the ordered formation of NBs in medulla development. JAK/STAT signaling negatively regulates the progression of the proneural wave, which induces NB formation and hence regulates the number of medulla NBs.

precursors disappeared and Dpn-expressing medulla NBs occupied the region (Fig. 6C; *n*=27). This suggests that the number of lamina neurons and medulla neurons is balanced by the JAK/STAT signal.

We next generated clones of cells expressing Hop^{Tum-l}, an active form of Hop (Harrison et al., 1995). Clones in the NE cells were associated with more medial expression of L(1)sc and delayed expression of Dpn (Fig. 6D,H; *n*=17). Cells that did not express Dpn expressed high Arm, indicating that they remained in an undifferentiated NE state (Fig. 6E; *n*=20). When *upd* was overexpressed (as above), a similar phenotype was observed (Fig. 6F,G; *n*=20 for F; *n*=10 for G). These results are consistent with the expectation that Upd acts as an extracellular activator of the JAK/STAT pathway. Note that the phenotypes were not cell autonomous and the proneural wave was continuous, even when it extended more medially (with elevated JAK/STAT signaling) or laterally (with decreased signaling). These results suggest that

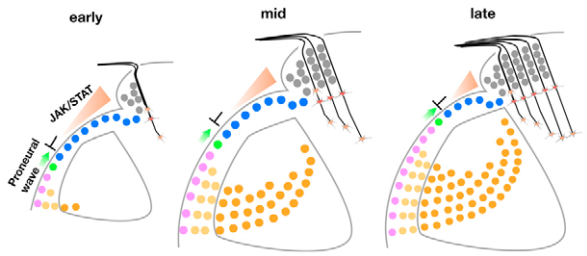

Fig. 7. A model for the progression of proneural wave. Schematic horizontal sections as in (Fig. 1C, Fig. 2G). JAK/STAT signaling is activated in the NE cells and its activation is high in the lateral and low in the medial side of NE. As the NE cells proliferate, Upd-expressing NE cells move laterally, and so does the activation of the JAK/STAT signaling. Medial NE cells freed from negative regulation of JAK/STAT signaling express L(1)sc and proneural wave moves laterally.

JAK/STAT signaling negatively regulates the progression of the proneural wave and that it does not directly regulate expression of the proneural genes (Fig. 6I). The alteration in proneural wave progression described here cannot be attributed solely to growth defects because any differences in proneural wave progression were associated with slowly growing *Minute* clones (see Fig. S5 in the supplementary material).

DISCUSSION

We report a novel mechanism of neural stem cell (NB) formation in *Drosophila* medulla development. We show that medulla development provides a novel experimental system in which stem cells are generated in a step-by-step manner. The entire development from neuroepithelium, NBs and neurons proceeds sequentially as spatially ordered arrays of progressively aged cells, and we identified a new role of JAK/STAT signaling in NB formation. We found a 'proneural wave' that sweeps unidirectionally in the NE sheet to trigger NB differentiation. This differs from the well-known but not yet fully understood mechanism called 'lateral inhibition' that singles out NBs from NE cells.

Proneural wave sweeps from the medial to lateral optic lobe and induces medullar NB differentiation

NE cells are programmed to differentiate into NBs from the medial edge of the developing optic lobe. The wave of differentiation progresses synchronously in a row of cells from medial to lateral optic lobe sweeping across the entire NE sheet; it is preceded by the transient expression of the proneural gene *l(1)sc*. As the NBs at the medial edge are oldest and the more lateral ones are youngest, developmental process of medulla neurons can be viewed as an array of progressively aged cells across optic lobe mediolaterally. This contrasts with NB formation in the embryonic CNS in which a small number of cells are selected from NE cells to become NBs, leaving the majority of NE cells to develop into non-neural cells. The optic lobe proneural wave is reminiscent of the morphogenetic furrow that moves across the developing eye imaginal disc. The morphogenetic furrow is the site where differentiation from neuroepithelium to photoreceptor neurons is initiated (Ready et al., 1976). The progression is driven by the secreted Hh expressed in the differentiated photoreceptor cells (Heberlein and Moses, 1995; Heberlein et al., 1993; Ma et al., 1993). By contrast, the proneural wave still progresses even when NB differentiation is impaired, suggesting that its progression is not driven by a factor emanating from differentiated NBs. We failed to observe progression-defective phenotypes when Hh or Decapentaplegic (Dpp) signaling was reduced (T.T., unpublished). We favor the model that the proneural wave progression is driven by an intrinsic mechanism such as a segmentation clock and is negatively regulated by JAK/STAT pathway (Fig. 7). As the JAK/STAT ligand Upd is expressed only by the most lateral NE cells, proliferation of the NE cells moves the source of ligand laterally and as a consequence releases more medial NE cells from negative regulation and allows the proneural wave to progress laterally. Alternatively, distribution of the Upd ligand and/or the response to Upd changes as the NE cells age as graded 10xSTAT-GFP activities are more prominent in the early stage. Non-autonomous action of JAK/STAT signal indicates that it does not directly regulate L(1)sc expression and there are second signal(s) that regulate the expression of L(1)sc under the control of JAK/STAT signal.

Three out of the four *AS-C* genes [*sc*, *l(1)sc* and *ase*] are expressed during medulla neurogenesis. *l(1)sc* is expressed in NE cells and *ase* in NBs, while *sc* is expressed both in NE cells and NBs (Egger et al., 2007). Deleting all *AS-C* genes causes as significant delay as *da* in NB formation but does not completely eliminate NB formation, suggesting that Da-dependent proneural gene activities are required for timely onset of NB formation. Mutation for *sc* or *ase* alone does not affect NB formation, but the simultaneous deletion of *sc* and *l(1)sc* causes the delay in NB formation and the additional deletion of *ase* further delays NB formation. *ase* expression is not altered in the absence of *l(1)sc* and *l(1)sc* is not altered in the absence of *ase*, indicating that *l(1)sc* and *ase* both contribute to the differentiation from NE cells to NBs. Although the contribution of Sc cannot be formally excluded, the highly specific expression pattern led us to infer that L(1)sc plays a major role in the proneural wave.

JAK/STAT signaling in stem cell maintenance

JAK/STAT signaling is known to regulate stem cell maintenance in the adult germline of *Drosophila* (Arbouzova and Zeidler, 2006; Fuller and Spradling, 2007). In the male testis, germline stem cells (GSCs) attach to a cluster of somatic support cells at the tip (hub) of the testis. When a GSC divides, the daughter retaining contact with the hub maintains self-renewing GSC identity, while the other daughter differentiates into gonialblast. Upd is specifically expressed in the hub cells and activates JAK/STAT signal in the GSCs to maintain stem cell state (Kiger et al., 2001; Tulina and Matunis, 2001). In the female ovary, JAK/STAT signaling is required in the somatic escort stem cells whose daughters encase developing cysts (Decotto and Spradling, 2005). Here, we show that in the optic lobe development, JAK/STAT signaling maintains NE cells in an undifferentiated state. We suggest that a common mechanism operates in both these developmental systems. Loss of Hop or Stat92E function decreases number of stem cells and ectopic expression of Upd results in over proliferation of undifferentiated cells. The cell fate may be determined by the distance of the cells from the source of ligand; the cells farther from the source commence to differentiate.

In the vertebrate CNS, NE cells first proliferate by symmetric cell divisions and differentiate into neurons and glia in later developmental stages (Anderson, 2001; Gotz and Huttner, 2005; McKay, 1997). JAK/STAT signaling has been implicated in maintenance of neural precursor cells (Yoshimatsu et al., 2006), but there is no clear evidence that those cells are in the same developmental stage as we describe here for *Drosophila*. Further

study of JAK/STAT signaling will reveal whether a common mechanism underlies stem cell development in both *Drosophila* and vertebrates, and should give new insights into vertebrate CNS neurogenesis.

Retinotopic map regulated by JAK/STAT signal

Development of a precise topographic map (retinotopic map) in *Drosophila* is known to involve regulation of lamina neuron development with respect to the incoming R axons (Selleck and Steller, 1991). The lateral NE sheet is continuous with a groove called the lamina furrow where NE cells are arrested at G1/S phase (Fig. 1C) (Selleck et al., 1992). The arriving R axons deliver Hh and liberate the arrested NE cells to proliferate and develop into lamina neuron precursors (Fig. 1C) (Huang and Kunes, 1996; Huang and Kunes, 1998). And, thus, R axons can induce the development of their synaptic partners in their vicinity to balance the number of R axonal termini and lamina neurons. However, medulla development does not depend on inputs from the R axons in the early phase. As we have shown here, both lamina and medulla neurons are derived from the continuous NE sheet. Large clones of cells mutant for the JAK/STAT signaling cause immature proliferation of medulla NBs at the expense of lamina neurons, suggesting that the number of NE cells serves as the limiting factor to generate precursors for lamina and medulla neurons. Thus, the number of medulla neurons is roughly regulated at the level of NBs whose generation might be balanced indirectly with the number of lamina neurons through regulating proneural wave progression by JAK/STAT signaling. JAK/STAT signaling therefore plays an important role in the formation of a precise retinotopic map in the visual center.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/8/1471/DC1

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