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Conservation of the Notch signalling pathway in mammalian neurogenesis

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SUMMARY

The *Notch* pathway functions in multiple cell fate determination processes in invertebrate embryos, including the decision between the neuroblast and epidermoblast lineages in *Drosophila*. In the mouse, targeted mutation of the Notch pathway genes *Notch1* and *RBP-Jk* has demonstrated a role for these genes in somite segmentation, but a function in neurogenesis and in cell fate decisions has not been shown. Here we show that these mutations lead to altered expression of the Notch signalling pathway homologues *Hes-5*, *Mash-1* and *Dll1*, resulting in enhanced neurogenesis. Precocious neuronal differentiation is indicated by the expanded expression domains of *Math4A*, *neuroD* and *NSCL-1*. The *RBP-Jk* mutation has stronger effects on expression of these genes than does the *Notch1* mutation, consistent with functional redundancy of *Notch* genes in neurogenesis. Our results demonstrate conservation of the Notch pathway and its regulatory mechanisms from fly to mouse, and support a role for the murine Notch signalling pathway in the regulation of neural stem cell differentiation.

Key words: mouse, neurogenesis, Notch pathway

INTRODUCTION

Neurogenesis in vertebrates occurs by the regulated withdrawal from the cell cycle of a homogeneous population of progenitor cells in the neural tube (McConnell, 1981). For example, in the mammalian cerebral cortex, prospective neurons individually cease division, migrate centrifugally and differentiate. This process is reiterated throughout development, generating radially arranged layers of neurons, with the last-born neurons in the outermost layer (McConnell, 1995). Clearly, this process has to be controlled both spatially and temporally, in order to generate the correct number of neurons in different regions of the developing central nervous system (CNS). It is thus essential to understand the mechanisms that regulate the birth of neurons in the mammalian CNS.

In *Drosophila*, neurogenesis is initiated by the separation of neural progenitors (neuroblasts) from progenitors of the epidermis (epidermoblasts). Prior to this separation, neuroblasts and epidermoblasts are intermingled in the neurogenic ectoderm (Campos-Ortega, 1993). Each cell in this region has the potential to become either a neuroblast or an epidermoblast and has to choose between these developmental fates (Technau and Campos-Ortega, 1986). Cell-to-cell interactions involving direct contacts between neighboring cells are essential for the proper separation of these two lineages. Thus, a prospective neuron inhibits its neighbors from also developing into neurons, a mechanism termed lateral inhibition (Heitzler and Simpson, 1991; Greenwald and Rubin, 1992). Lateral inhibition also regulates the number of cells that become neurons, as well as their spatial arrangement. The products of the so-called neurogenic genes participate in this cell communication process. A typical neurogenic phenotype is defined by lack-of-function mutations that cause the expansion of the nervous system at the expense of the epidermis (Lehmann et al., 1981). The genes *Delta* (*Dl*), *Notch* (*N*), *Suppressor of Hairless* (*Su(H)*) and the *Enhancer of split* (*E(spl)*) complex, belong to the neurogenic group.

Notch encodes for a large membrane-spanning protein (Wharton et al., 1985) that acts as receptor for the membrane-bound ligands Delta (Vässin et al., 1987) and Serrate (Fleming et al., 1990). Genes related to *Notch* and *Delta* have been identified in several different species. All known ligands for Notch-related receptors are membrane-bound (Artavanis-Tsakonas et al., 1995; Greenwald, 1994), and biological assays indicate that Notch signalling occurs only between cells that are in direct contact with each other (Heitzler and Simpson, 1991). At present, there are two models of Notch signal transduction to the nucleus. In the first model, ligand binding is thought to cause the translocation of the transcription factor Su(H) from the cytoplasm to the nucleus (Fortini and Artavanis-Tsakonas,

1994). In the second model, ligand binding is thought to induce proteolytic processing of Notch and translocation of a fragment of Notch to the nucleus, where it binds to and activates Su(H) (Jarriault et al.,1995). A recent study in *Drosophila* indicates that differential subcellular localization of Su(H) is not essential for its function (Gho et al., 1996). Which model is correct remains unresolved.

Genetic and molecular data have shown that the genes of the *E(spl)* complex are the first target for Su(H) after Notch signalling (Lecourtois and Schweisguth, 1995; Bailey and Posakony, 1995). In vertebrates, homologues to all these Notch pathway genes exist, including *Delta* (Chitnis et al., 1995; Henrique et al., 1995; Bettenhausen et al., 1995), *Serrate* (Lindsell et al., 1995; Myat et al., 1996), *Notch1-4* (Weinmaster et al., 1991, 1992; Lardelli et al., 1994; Uyttendaele et al., 1996), *RBP-Jk* (Recombination signal sequence Binding Protein for *Jk* genes) homologue of *Su(H)* (Furukawa et al., 1992; Schweisguth and Posakony, 1992) and *Hes-1-5* (*Hairy* and *Enhancer of split* homologues; Sasai et al., 1992; Takebayashi et al., 1995). Moreover, these genes are expressed in the CNS as well as other regions of the body (Chitnis et al., 1995; Henrique et al., 1995; Bettenhausen et al., 1995; Franco del Amo, 1992; Reaume et al., 1992; Sasai et al., 1992; Akazawa et al., 1992). Experimental studies in the embryonic chick retina (Austin et al., 1995), the *Xenopus* embryonic CNS (Chitnis et al., 1995) and mammalian cells in culture (Nye et al., 1994) have suggested that the Notch signalling pathway functions in vertebrate neurogenesis. However, the phenotypic analysis of mouse mutations in either *Notch1* (Swiatek et al., 1994; Conlon et al., 1995) or its putative downstream effector *RBP-Jk* (Oka et al., 1995), did not reveal a role for the Notch pathway in neurogenesis, largely because both mutations cause embryonic death around day 9 of development, just as neuronal differentiation is beginning.

In this paper, we present the results of an investigation of the role of Notch signalling in mouse neurogenesis. First, we show that the RBPJK protein of the mouse embryo is predominantly localized to the nucleus and shows no obvious variations in cellular localization in *Notch1* mutants. Secondly, we show that *Hes-5*, *Mash-1* and *Dll1* are targets of the Notch signalling pathway. Thirdly, we show that more cells express early neuronal differentiation markers in *RBP-Jk* and *Notch1* mutants, suggesting that activation of Notch signalling negatively regulates the formation of neurons in the neural tube of the mouse. Lastly, we demonstrate that the *RBP-Jk* mutation causes a more severe neurogenic phenotype than the *Notch1* mutation, indicating that there may be functional redundancy of the different Notch proteins of the mouse. This provides further evidence that the Notch signalling pathway, its regulatory mechanisms and its role in neurogenesis are conserved from fly to vertebrates.

MATERIALS AND METHODS

Genotyping

RBP-Jk and *Notch1* mutant embryos were obtained by mating females and males heterozygous for *RBP-Jk* (Oka et al., 1995) or *Notch1* (Conlon et al., 1995), targeted mutations, respectively. Embryos were genotyped by PCR analysis of the yolk sacs. Primers and conditions were as described previously.

Generation of anti-RBPJK-specific antiserum

Polyclonal antibodies were raised against a RBPJK polypeptide containing the first 276 aa of the protein. The RBP (1-276) polypeptide was produced in BL-21 (protease-deficient) bacteria using the pET-15b expression vector (Novagen). Clones containing the RBP expression construct were induced with IPTG to express the recombinant protein at high levels. Recombinant protein was purified to homogeneity by passage over nickel-coated beads (Novagen). Purified protein (1-2 μg/boost) was used to inject a New Zealand white rabbit. The antiserum obtained was evaluated by western blot analysis of crude lymphocyte nuclear extracts (data not shown). For the purification of the antiserum, the technique described by Hall et al. (1984) was used: recombinant RBP protein (500 μg) was separated on a 15% SDS-PAGE gel, transferred to a PVDF membrane and detected by Ponceau Red staining. The RBP strip was excised from the membrane and incubated with 2 ml of antiserum. Anti-RBP-specific antibodies were eluted at pH 2.6 in a glycine buffer and subsequently neutralized with Tris pH 7.5.

Embryo extracts and western blot analysis

Embryos were dissected in ice-cold PBS and frozen immediately. Extracts were prepared using the method described by Lee et al (1988). 25 μg of extract was loaded per lane. Affinity-purified polyclonal anti-RBPJK antibodies were used at a 1/500 dilution. Staphylococcus protein A coupled to horseradish peroxidase (Sigma) was used at a dilution of 1/10,000. Antibody bound to proteins were visualized using the LumiGLO chemiluminescence substrate kit (Kirkegaard and Perry Laboratories) as described by the manufacturer. To verify that equivalent amounts of extracts were loaded in each lane, blots were stripped and reprobed with antiserum (1/2,000) against the ubiquitous protein nucleolin (Miranda et al., 1995).

Immunohistochemistry

Embryos were isolated in ice-cold PBS, fixed in 4% paraformaldehyde for 3 hours, dehydrated, embedded in wax and sectioned at 5 μm. Affinity-purified anti-RBPJK antiserum was used at a 1/50 dilution. A biotinylated secondary antibody against rabbit IgG and avidin-conjugated peroxidase (Vector Laboratories) were used for immunostainings. A protocol described by Trumpp et al. (1992) was used. Briefly, rehydrated sections were incubated for 2 hours at room temperature with the primary antibody, 1 hour with goat anti-rabbit IgG and 1 hour with avidin-peroxidase complex at room temperature. The signal was visualized in 30-60 minutes by an HRP reaction (Vector Laboratories) using diaminobenzidine (DAB, 1 mg/ml in 0.1 M TrisHCl, pH 7.5) and hydrogen peroxide (0.03% final) as substrates. To enhance the signal, $NiCl_2$ (0.04% final) was used in the developer cocktail.

Northern blot analysis

Total RNA was extracted from embryonic day 8.5-9.0 (E8.5-E9.0) whole embryos using Trizol (Life Technologies). 20 μg of total RNA was electrophoresed on a formaldehyde/1% agarose gel and transferred to a nylon membrane (Hybond N^+, Amersham). Full-length *Hes-1*, *Hes-3* and *Hes-5* cDNAs were ^{32}P-labeled and used as probes for hybridization at 65°C in Church and Gilbert buffer. Filters were subsequently stripped and rehybridized with a mouse *β-actin* probe.

Whole-mount in situ hybridization

Embryos were isolated in ice-cold PBS, fixed overnight in 4% paraformaldehyde and processed for whole-mount in situ hybridization following described procedures (Conlon and Hermann, 1993; modified following Koop et al., 1996).

Histology

After whole-mount in situ hybridization embryos were postfixed overnight in 4% paraformaldehyde, dehydrated, cleared in xylene for 15 minutes, embedded in wax and sectioned at 10 μm.

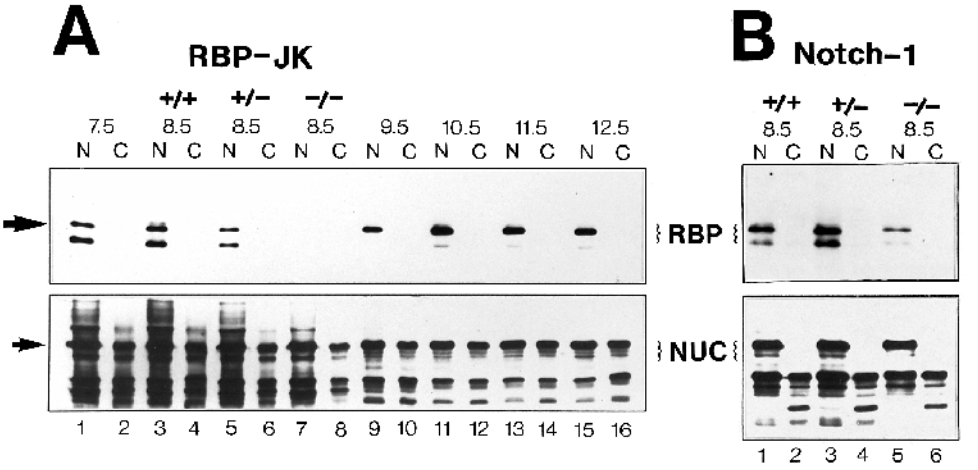

Fig. 1. Nuclear localization of RBPJK throughout embryogenesis. (A) Western blot showing RBPJK expression during embryogenesis (Lanes 1-16). RBPJK is always present in the nuclear fraction (N; lanes 1, 3, 5, 9, 11, 13, 15) and never in the cytoplasmic one (C; lanes 2, 4, 6, 10, 12, 14, 16). Decreased RBPJK expression is observed in heterozygous (+/–) E8.5 *RBP-Jk* embryos (lane 5). No signal is observed in E8.5*RBP-Jk* homozygous (–/–) mutant embryos (Lane 7). (B) Western blot showing nuclear localization of RBPJK in E8.5 wild-type (+/+; lane 1), *Notch1* heterozygous (+/–; lane 3) and *Notch1* homozygous (–/–) mutant embryos (lane 5). The big arrow in the upper panels in A,B indicates full-length RBPJK protein (~60×10^3 M_r; RBP) and the small arrow in the lower panels points to the nucleolin protein (~105×10^3 M_r; NUC). The lower band in the RBPJK blot is most likely the result of alternative splicing of the *RBP-Jk* primary transcript (Kawaichi et al., 1992) or a proteolytic fragment of RBPJK. Nucleolin is known to undergo autoproteolysis and, in addition to being present in the nucleus, is also found in the cytoplasm, mostly in degraded form (as in B; Miranda et al., 1995).

RESULTS

RBPJK is nuclear in wild-type and *Notch1* mutant embryos

To attempt to determine whether nuclear localization of RBPJK varies according to activity of the Notch pathway, polyclonal antibodies raised against RBPJK were used to study the expression and subcellular localization of this protein in the mouse embryo. Western blot analysis reveals that a 60×10^3 M_r RBPJK protein is present in the nucleus throughout embryogenesis and not readily detectable in the cytoplasm component (Fig. 1A; lanes 1, 3, 5, 9, 11, 13 and 15). The specificity of the antiserum is demonstrated by the absence of signal in extracts from *RBP-Jk* mutant embryos (Fig. 1A; lane 7). To determine if the absence of the Notch1 receptor would affect the subcellular localization of RBPJK, we performed western blot analysis of protein extracts from *Notch1* mutant embryos and wild-type littermates. In both wild-type (Fig. 1B; lanes 1, 3) and *Notch1* mutant embryos (Fig. 1B; lane 5), RBPJK is always present in the nucleus.

Immunohistochemical analysis of sections of E8.5 wild-type embryos also revealed widespread RBPJK nuclear staining in the neural tube and paraxial mesoderm (Fig. 2A-C). This staining was absent in *RBP-Jk* mutant embryos (Fig. 2F). Staining of E8.5 *Notch1* embryos also revealed a nuclear localization of RBPJK (Fig. 2D,E). There was no clear change in the nuclear localization of RBPJK in wild-type or *Notch1* mutant embryos that could be related to areas of activity of the Notch pathway. Staining of embryos with the anti-RBPJK monoclonal antibody T6719 (Hamaguchi et al., 1992) supported these results (data not shown).

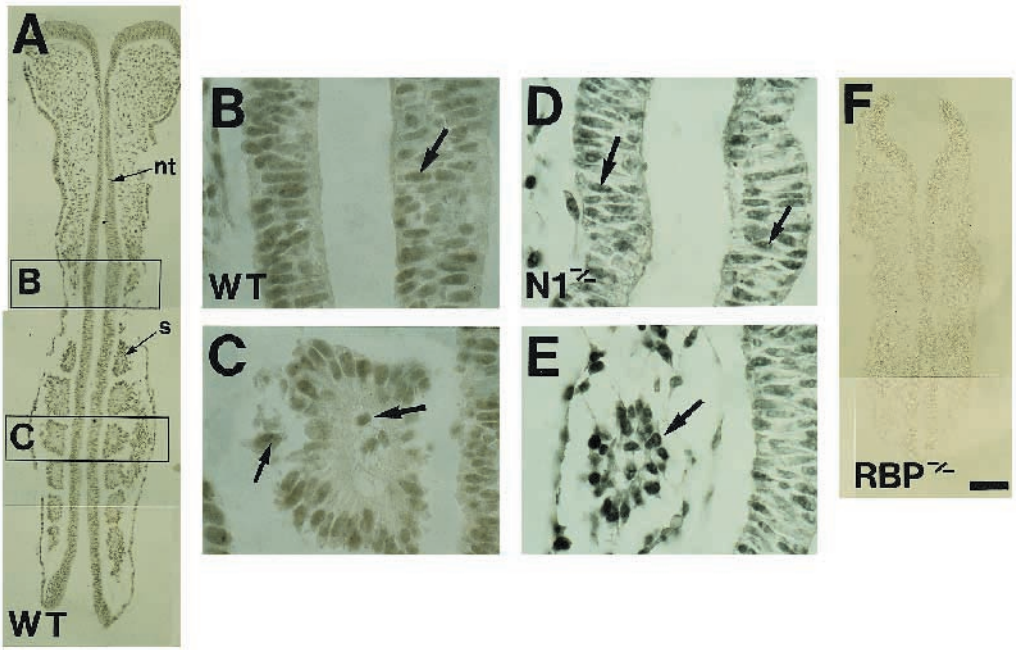

Fig. 2. RBPJK expression in E8.5 embryos. (A) Wild-type embryo, section through the brain, neural tube and somitic region. Nuclei expressing RBPJK appear dark. (B,C) Details showing RBPJK nuclear staining in neural tube (arrow in B) and somite cells (larger arrow in C). (D,E) *Notch1* homozygous mutant embryo. Details showing RBPJK nuclear staining in the neural tube (larger arrow in D) and somite cells (arrow in E). The small arrows in C,D point to the nucleolus that does not express RBPJK. (F) *RBP-Jk* homozygous mutant embryo. No signal is detected. All sections are horizontal. nt, neural tube; s, somites. Bar, 80 μm in A,F; 30 μm in B-E.

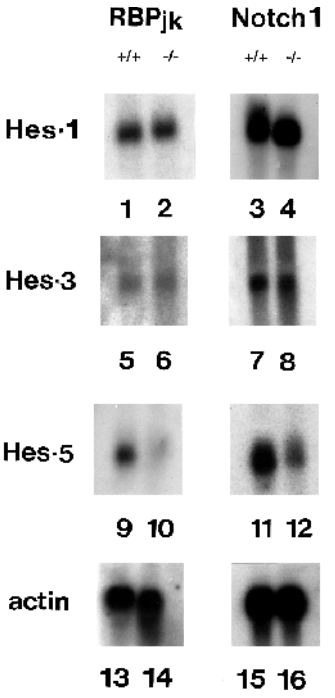

Fig. 3. *Hes-5* expression in *RBPJk* and *Notch1* mutant embryos: northern blot analysis of E9.0 embryos. Total RNA from wild-type (lanes 1, 3, 5, 7, 9, 11, 13, 15), homozygous mutant *RBP-Jk* (lanes 2, 6, 10, 14) and *Notch1* (lanes 4, 8, 12, 16) embryos was hybridized with *Hes-1* (lanes 1-4), *Hes-3* (lanes 5-8) and *Hes-5* (lanes 9-12) probes. Expression of *Hes-5* is strongly down-regulated in *RBP-Jk* mutant embryos (lane 10) and to a lesser extent in *Notch1* mutants (lane 12). A *β-actin* probe (lanes 13-16) was used as a control for sample loading.

Downregulation of *Hes-5* and up-regulation of *Mash-1* expression in *RBP-Jk* and *Notch1* mutant embryos

In *Drosophila*, the spatiotemporal expression pattern of the basic Helix-Loop-Helix (bHLH) transcription factors encoded by the *E(spl)* complex, suggests that they accumulate in response to Notch signalling activity (Jennings et al., 1995). Studies in vertebrates indicate that the related *Hes* genes are candidates to be positively regulated by RBPJK (Jarriault et al., 1995). We examined the expression of *Hes* genes by northern blot and whole-mount in situ hybridization (Figs 3, 4 and data not shown). Only *Hes-1*, *Hes-3* and *Hes-5* are detectably expressed at E8.5-9.0. Levels of *Hes-1* and *Hes-3* expression were not changed in *RBP-Jk* and *Notch1* mutant embryos and the spatial distribution of transcripts was not affected (Fig. 3; lanes 2, 4, 6, 8; and data not shown). In contrast, *Hes-5* expression was almost undetectable in northern blots from *RBP-Jk* mutants (lane 10) and reduced by half in *Notch1* mutants (lane 12). *Hes-5* is normally expressed in a stripe in the midbrain region, two stripes in the hindbrain region and along the neural tube, as well as in the primitive streak, and in two pairs of stripes in the presomitic mesoderm (Fig. 4A). Expression of *Hes-5* was severely reduced in all its expression domains in *RBP-Jk* mutant embryos (Fig. 4B). *Hes-5* expression was also down-regulated in *Notch1* mutants (Fig. 4C), although not so dramatically, in agreement with the

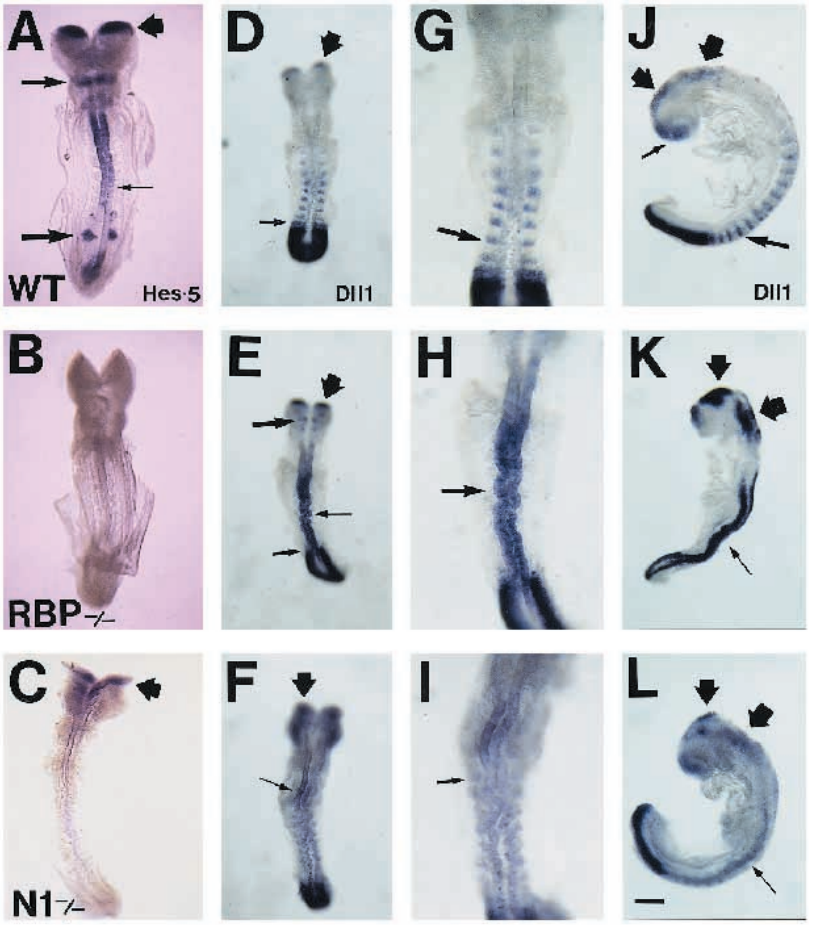

Fig. 4. Down-regulation of *Hes-5* and up-regulation of *Dll1* expression in *RBP-Jk* and *Notch1* mutant embryos. Whole-mount in situ hybridization of E8.5 wild-type (A,D,G); E9.0 wild-type (J); E8.5 *RBP-Jk* (B,E,H); E9.0 *RBP-Jk* (K); E8.5 *Notch1* (C,F,I) and E9.0 *Notch1* (L) embryos. (A-I) Embryos are viewed dorsally, anterior is at the top; (J-L) embryos are oriented laterally. (A) *Hes-5* expression in a wild-type embryo. Signal is detected in midbrain (thick arrow), hindbrain (arrow), neural tube (small arrow) and presomitic mesoderm (big arrow). (B) *Hes-5* expression in a *RBP-Jk* mutant embryo. Signal is strongly down-regulated. (C) *Hes-5* expression in a *Notch1* mutant embryo. Signal is downregulated, although some expression is detected in the midbrain (arrow). (D,G,J) *Dll1* expression in a wild-type embryo. (D) Expression is detected in the midbrain (thick arrow) and presomitic mesoderm (small arrow). (G) The arrow points to expression in the posterior region of a somite. (J) Signal is detected in the forebrain region (small arrow), midbrain (thick arrow), hindbrain (big arrow) and posterior region of the somites (arrow). (E,H,K) *Dll1* expression in a *RBP-Jk* mutant embryo. (E) Expression is detected in the midbrain (thick arrow); one stripe in the hindbrain (arrow), neural tube (thin arrow) and the presomitic mesoderm (small arrow). Signal is strongly up-regulated in the neural tube and brain. Note the striking similarity with the wild-type expression of *Hes-5*, shown in A. (H) The arrow points to up-regulated *Dll1* expression in the neural tube. (K) Up-regulated *Dll1* expression is detected in the midbrain (thick arrow), hindbrain (big arrow) and neural tube (thin arrow). (F,I,L) *Dll1* expression in a *Notch1* mutant embryo. (F) Up-regulated expression is observed in the midbrain (thick arrow) and neural tube (thin arrow). Expression is also observed in the presomitic mesoderm and in the somites (arrow in I). (L) Up-regulated expression is detected in the midbrain (thick arrow), hindbrain (big arrow) and along the neural tube (thin arrow). Bar, 40 μm in G-I; 80 μm in the rest.

northern blot analysis. These results are consistent with *RBP-Jk* and *Notch1* acting in a common pathway to activate *Hes-5* expression in the embryo.

The results obtained above are analogous to what is known of the *Drosophila* Notch pathway. In *Drosophila*, loss of *E(spl)* activity leads to an upregulation of genes of the *achaete-scute* (*ac-sc*) proneural class and, consequently, to an excess of neuroblasts (Skeath and Carroll, 1992). One might predict that loss of *Hes-5* signalling in *RBP-Jk* and *Notch1* mutants would lead to deregulated expression of *Mash-1*, a mouse homologue of *ac-sc* (Guillemot and Joyner, 1993). *Mash-1* is expressed at E8.5 in the anterior region of the neural tube (Fig. 5A), and in scattered cells of the midbrain region from which the first neurons of the CNS emerge (Fig. 5C). In *RBP-Jk* mutant embryos at E8.5, *Mash-1* expression was increased in intensity and extent in the forebrain, midbrain (Fig. 5B) and hindbrain regions (Fig. 5D), and in the anterior part of the neural tube (Fig. 5B,D). At E9.0, wild-type *Mash-1* expression is restricted to the dorsal midbrain and to two patches at either side of the otic vesicle (Fig. 5E,G,I). In the *RBP-Jk* and *Notch1* mutants at E9.0, *Mash-1* midbrain and hindbrain expression was found to be more intense (Fig. 5F,H,J,K), and extended over a larger area of the dorsal and ventral regions of the neural tube (Fig. 5F,H). The latter may correspond to neural-crest-derived precursors of sympathetic ganglia. Taken together, these observations are consistent with *Hes-5* down-regulation of *Mash-1* expression in the midbrain, hindbrain and neural tube.

Histological analysis of E9.0 wild-type and mutant embryos revealed that *Mash-1* expression was restricted to the subventricular zone of the dorsal midbrain in wild-type embryos (Fig. 5L, see also Guillemot and Joyner, 1993). In contrast, *Mash-1* expression domain was expanded to the ventricular zone in the midbrain of *RBP-Jk* and *Notch1* mutant embryos (Fig. 5M,N), suggesting that an excess of committed neuronal precursors were generated in mutant embryos.

Neural tube expression of *Dll1* is increased in *RBP-Jk* and *Notch1* mutants

In lateral inhibition models, Notch ligand expression is responsive to the state of Notch activation (Heitzler and Simpson, 1991; Wilkinson et al., 1994; Heitzler et al., 1996; see Fig. 7A and Discussion). Thus, cells that are stimulated by the ligand down-regulate expression of the ligand itself. Inactivation of the Notch receptor pathway should lead to increased expression of the ligand. To determine if ligand expression is responsive to the Notch pathway in the mouse, *Dll1* expression was examined in *RBP-Jk* and *Notch1* mutant embryos. *Dll1* is normally expressed in the primitive streak and presomitic mesoderm throughout embryogenesis (Bettenhausen et al., 1995). In E8.5 and E9.0 embryos, it is also expressed in the posterior of each somite (Fig. 4D,G,J). In the neural tube, *Dll1* is expressed in individual, isolated cells in a basal position in the neural epithelium, in cells that are thought to be committed neuronal precursors (Bettenhausen et al., 1995; Henrique et al., 1995). In *RBP-Jk* and *Notch1* mutant embryos, the abundance of *Dll1* RNA was not altered as determined by northern blot analysis (not shown). However, there were dramatic changes in the spatial distribution of *Dll1* mRNA as determined by in situ hybridization. In contrast to wild-type embryos, where *Dll1* was expressed in scattered cells in the neural tube, in *RBP-Jk* mutant embryos, *Dll1* was expressed in all cells of the presumptive spinal cord (Fig. 4E,H,K). *Dll1* expression in the neural tube was upregulated in *Notch1* embryos as well, although to not as great an extent (Fig. 4F,I,L). The expression of *Dll1* in *RBP-Jk* mutants was strikingly similar to normal *Hes-5* expression in wild-type embryos (cf Fig. 4A with Fig. 4E), suggesting that *Hes-5* expression represses *Dll1* transcription in these regions. In contrast, *Dll1* expression in the primitive streak and presomitic mesoderm was not changed in the mutant embryos (Fig. 4E,F) and *Dll1* expression in the somites was lost in *RBP-Jk* mutants (Fig. 4H).

Enhanced neuronal differentiation in *RBP-Jk* and *Notch1* mutant embryos

The up-regulated expression of *Mash-1* and *Dll1* in the CNS of *RBP-Jk* and *Notch1* mutants is very reminiscent of events in *Drosophila* neurogenesis where deregulated expression of *Dl* and *E(spl)* in *Notch* mutants leads to an excess of neuroblast differentiation. To determine whether these events led to an excess of neuronal differentiation in the mouse, the expression of three bHLH transcription factors that are expressed in early differentiating neurons, *Math4A*, *neuroD* and *NSCL-1*, was studied. *Math4A* is related to the *Drosophila* proneural gene *atonal* (Gradwohl et al., 1996). In E9.0 wild-type embryos, *Math4A* is expressed in neuronal precursors in the midbrain and ventral spinal cord (Gradwohl et al., 1996; Fig. 6A). In *RBP-Jk* (Fig. 6B), and to a lesser extent in *Notch1* mutant embryos (Fig. 6C), *Math4A* expression was increased in these regions. *neuroD* expression is specifically restricted to the developing trigeminal ganglia at E9.0 (Lee et al., 1995 ; Fig. 6D). In *RBP-Jk* and *Notch1* mutant embryos, *neuroD* was expressed at high levels in the trigeminal ganglion, and was ectopically expressed in the midbrain and the anterior spinal cord (Fig. 6E,F). Interestingly, *neuroD* expression in the midbrain and spinal cord overlapped with that of up-regulated *Mash-1* (compare Figs 5F versus 6E or 5H versus 6F), suggesting that *Mash-1* may positively regulate *neuroD* transcription in these regions. *NSCL-1* also shows a restricted expression and, at E9.0, is transcribed in scattered cells in the anterior midbrain (Begley et al., 1992; Fig. 6G). In *RBP-Jk* mutant embryos, *NSCL-1* expression was increased in the midbrain region, and was ectopically expressed in the trigeminal ganglion and in the caudal neural tube (Fig. 6H). *Notch1* mutant embryos also showed increased and ectopic expression of *NSCL-1* (Fig. 6I), although the increase in expression was less dramatic. Increased expression of these three neuronal differentiation markers in *RBP-Jk* and *Notch1* mutant embryos, confirmed that an excess of committed neuronal precursors were generated at E9.0 in the mutants. This is the first description of a neurogenic phenotype resulting from the disruption of the Notch signalling pathway in the mouse.

DISCUSSION

Target genes of Notch signalling and neurogenesis

In this report, we have provided evidence showing that many changes in gene expression occur in *RBP-Jk* and *Notch1* mutants that reveal striking similarities with the Notch sig-

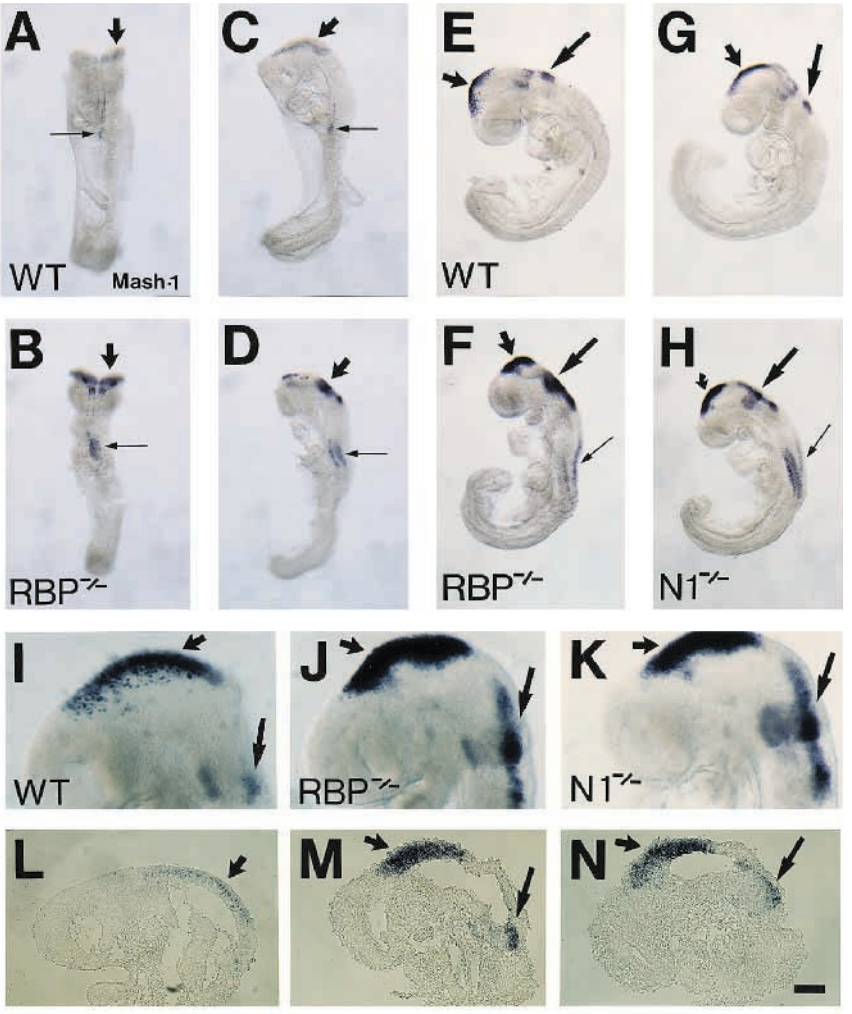

Fig. 5. *Mash-1* expression is up-regulated in *RBP-Jk* and *Notch1* mutant embryos. Whole-mount in situ hybridization of E8.5 wild-type (A,C); E9.0 wild-type (E,G,I,L); E8.5 *RBP-Jk* (B,D); E9.0 *RBP-Jk* (F,J,M) and E9.0 *Notch1* (H,K,N) embryos. (A,B) Embryos are viewed dorsally. (C-N) Embryos are oriented laterally. (A,C) *Mash-1* expression in a E8.5 wild-type embryo. Signal is detected in the anterior neural tube region (thin arrow) and in the midbrain (thick arrow). (B,D) *Mash-1* expression in a E8.5 *RBP-Jk* embryo. Up-regulated expression is observed in the midbrain/hindbrain region and in the anterior neural tube (thick and thin arrows, respectively). (E,G) *Mash-1* expression in an early (E) and more advanced (G) E9.0 wild-type embryo. (E,G) Signal is observed in the midbrain (thick arrow) and in two patches flanking the otic vesicle (arrow points to the posterior patch). (F) *Mash-1* expression in an early E9.0 *RBP-Jk* mutant embryo. Signal is up-regulated in the midbrain (thick arrow), hindbrain (big arrow) and both dorsal and ventral neural tube (thin arrow). (H) *Mash-1* expression in a E9.0 *Notch1* embryo. Stage is comparable to the wild-type embryo shown in G. Up-regulation of signal is noted in the midbrain (bent arrow), hindbrain (big arrow) and neural tube (thin arrow). (I) Detail of *Mash1* expression in the midbrain region (thick arrow) and in a patch posterior to the otic vesicle (arrow) in a E9.0 wild-type embryo. (J,K) Detail of up-regulated *Mash1* expression in the midbrain and hindbrain regions (thick and big arrow, respectively) of E9.0 *RBP-Jk* (J) and *Notch1* (K) mutant embryos. (L-N) Histological sections of the embryos shown in (I-K). (L) *Mash1* expression is restricted to the dorsal most region of the midbrain in a E9.0 wild-type embryo (arrow). (M,N) *Mash1* expression is enhanced and its expression domain expanded dorsoventrally to the ventricular zone in the midbrain (thick arrow) and hindbrain region (big arrow) of *RBP-Jk* (M) and *Notch 1* (N) mutant embryos. Bar, 80 μm in A-H; 8 μm in I-N.

nalling pathway deduced in *Drosophila*. Thus, studies in *Drosophila* have suggested that the genes of the *E(spl)* complex are directly regulated by the Notch pathway (Bailey and Posakony, 1995; Jennings et al., 1995; Lecourtois and Schweisguth, 1995; Furukawa et al., 1995). In mammalian systems, RBPJK has been shown to bind to the regulatory sequences and activate transcription of *Hes-1* in vitro, in combination with the intracellular domain of Notch (Jarriault et al., 1995). However, we could not detect any change in *Hes-1* expression in *RBP-Jk* and *Notch1* mutants, suggesting that the contribution of these genes to the regulation of *Hes-1* in the early embryo must be minimal. In contrast, the expression of another *Hes* family member, *Hes-5*, was reduced in *Notch1* mutants and almost completely eliminated in *RBP-Jk* mutants, indicating that *Hes-5* is highly responsive to Notch signalling. Potential RBPJK-binding sites exist in the promoter of *Hes-5* (data not shown), suggesting that RBPJK may directly regulate the transcription of *Hes-5*. A putative *Hes-5* target is *Mash-1*, whose expression domain is expanded in *RBP-Jk* and *Notch1* mutants. Thus, in *Drosophila*, low levels of *E(spl)* expression imply high *ac-sc* expression (Skeath and Carroll, 1992) and, in the mouse, low *Hes-5* expression correlates with enhanced *Mash-1* expression expanding over a larger CNS region. Interestingly, mutation of *Hes-1* leads also to up-regulation of *Mash-1* (Ishibashi et al., 1995), indicating that alternate pathways for activating *Mash-1* must exist.

Dll1 expression is also highly responsive to mutations in genes of the Notch pathway. In this case, however, the expression of *Dll1* in the neural tube is increased in both *RBP-Jk* and *Notch1* mutants. This result implies that *Dll1* expression is normally repressed by signalling through the Notch pathway.

We have no data that bear on whether this regulation is direct or indirect, but the strength of the response implies that this is an important regulatory interaction in Notch signalling. The negative regulation of the ligand by receptor activation implied by this result is consistent with lateral inhibition models of Notch action in *Drosophila* (Heitzler and Simpson, 1991; Heitzler et al., 1996) and *C. elegans* (Wilkinson et al., 1994). It is also consistent with the fact that *Dll1* expression in the neural tube is restricted to individual, spatially separated cells (Henrique et al., 1995). On the contrary, *Notch1* is normally expressed in all or almost all cells in the same regions (Franco del Amo et al., 1992; Reaume et al., 1992). It is interesting to note, however, that *Dll1* expression in the presomitic mesoderm

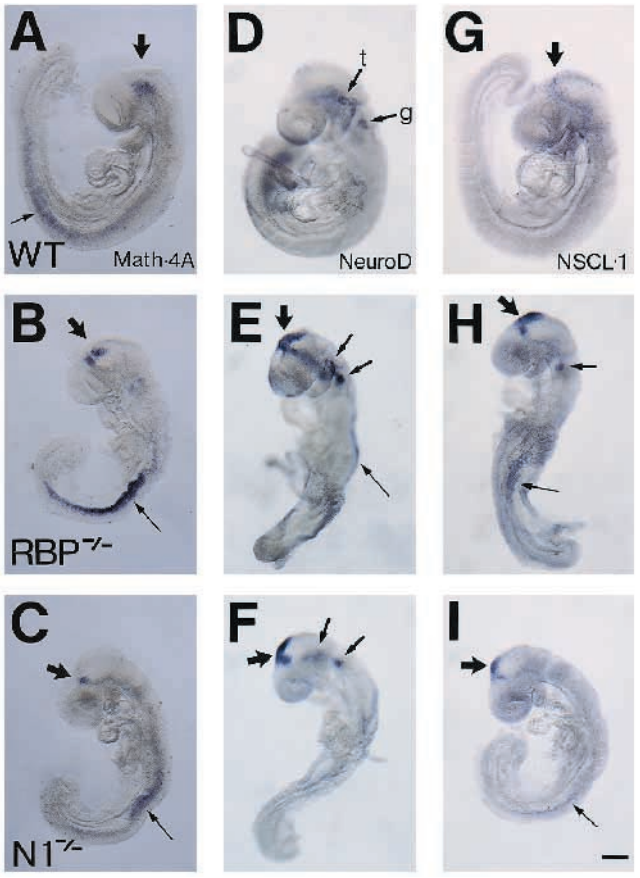

Fig. 6. Increased expression of early neuronal differentiation markers in *RBP-Jk* and *Notch1* mutants. E9.0 wild-type embryo (A,D,G); E9.0 *RBP-Jk* embryo (B,E,H); E9.0 *Notch1* embryo (C,F,I). (A) *Math-4A* expression in a wild-type embryo. Signal localizes to the ventral midbrain (arrow) and along the basal plate of the spinal cord (thin arrow). (B) *Math-4A* expression in a *RBP-Jk* mutant embryo. Signal is strongly up-regulated in the midbrain (arrow) and spinal cord (thin arrow). (C) *Math-4A* expression in a *Notch1* mutant embryo. Signal is up-regulated in the midbrain (arrow) and spinal cord (thin arrow). (D) *Neuro-D* expression in a E9.0 wild-type embryo. Signal is detected in the developing trigeminal (t) and geniculate (g) ganglia. (E) *Neuro-D* expression in a *RBP-Jk* embryo. Up-regulated signal is observed in the midbrain region (thick arrow) and in the developing trigeminal and geniculate ganglia (arrows). Expression in the midbrain overlaps with the region of up-regulated *Mash-1* expression. The dorsal neural tube also shows *neuro-D* signal (thin arrow). (F) *Neuro-D* expression in a *Notch1* embryo. Up-regulated expression is observed in the midbrain (thick arrow), trigeminal and geniculate ganglia (arrows). (G) *NSCL-1* expression in a wild-type embryo. Weak signal is observed in the midbrain region (arrow). (H)*NSCL-1* expression in a *RBP-Jk* mutant embryo. Note up-regulated expression in the midbrain (thick arrow), geniculate placode (small arrow) and in the neural tube (thin arrow). (I) *NSCL-1* expression in a *Notch1* mutant embryo. Up-regulation is observed in the midbrain (thick arrow) and neural tube (thin arrow) regions. Bar, 80 μm.

does not change in the mutants. Moreover, *Dll1* expression in the presomitic mesoderm of wild-type embryos is widespread and relatively homogeneous. This result suggests that the mechanism of action of Notch signalling in the presomitic mesoderm is not by lateral inhibition at the single cell level.

In *Drosophila*, a connection between the up-regulation of *E(spl)* and the downregulation of *Dl*, after activation of Notch signalling, is provided by genes of the *ac-sc* complex (Kunisch et al., 1994; Heitzler et al., 1996). The bHLH transcription factors encoded by *E(spl)* (Klaembt et al., 1987), negatively regulate the expression of the neural phenotype in the receiving cell (Fig. 7A, Heitzler et al., 1996). *Dl* expression is positively regulated by ac-sc, so that reduced ac-sc leads to downregulation of *Dl*, providing a feedback loop to link the expression of *Dl* and *N* (Fig. 7A). In the mouse, the functional equivalents of *ac-sc* are not clear. *Mash-1*, which is a homologue of *ac-sc*, is up-regulated in *RBP-Jk* and *Notch1* mutants, as predicted by the *Drosophila* model (Fig. 7A). However, *Mash1* cannot be the only regulator of *Dll1* expression, because it is not up-regulated throughout the expanded domain of *Dll1* expression in *RBP-Jk* and *Notch1* mutants. Mutational analysis of *Mash1* only revealed a role for the gene in the later differentiation of subsets of neural precursors in the PNS (Guillemot et al., 1993), consistent with the possible existence of additional genes overlapping in function with *Mash-1* in early neurogenesis. The recently described *neurogenin* may be such a candidate (Ma et al., 1996). There is no evidence on whether *Mash-1* or related genes directly regulate *Dll* expression in vertebrates. There is however, some evidence supporting a role for *Hes* genes in downregulating *Mash-1*, both in tissue culture experiments (Sasai et al., 1992) and from targeted mutagenesis in vivo (Ishibashi et al., 1995).

The mechanism by which the Notch signal is transduced to the nucleus is under intense investigation (see Introduction). In this study, we have used an affinity-purified polyclonal antiserum to examine the expression of RBPJK and determine if the subcellular localization of the protein changes during mouse embryogenesis. Western blot analysis indicated that RBPJK is specifically localized in the nucleus throughout development. Immunostainings revealed that RBPJK is widely expressed in the E8.5 mouse embryo and appears to be restricted to the nucleus (Fig. 2B, C). In addition, absence of the Notch1 receptor does not affect the subcellular localization of RBPJK in any region of the embryo, including the somites, where phenotypic effects of the *Notch1* mutation were observed. This indicates that the protein localizes stably in the nucleus, independently of the presence or absence of Notch1. These results would suggest that Notch signalling in the mouse embryo does not require major shifts in the subcellular localization of RBPJK and are consistent with the possibility that it is the movement of a proteolitically cleaved form of Notch to the nucleus that is critical in the signalling process (Jarriault et al., 1995; Tamura et al., 1995; Kopan et al., 1996). An additional possibility is that other Notch receptors in the absence of Notch1, would signal through RBPJK, thus affecting its subcellular localization.

A neurogenic phenotype in the mouse

A major issue in the study of vertebrate Notch signalling has been whether the neurogenic function that the pathway has in *Drosophila* is conserved in vertebrates. Overexpression of wild-type or dominant negative *Delta* constructs has provided support for such a role in primary neurogenesis in *Xenopus* (Chitnis et al., 1995). However, in mice, primary neurons do not form, and the role for Notch signalling in more general regulation on the transition from neural stem cell to committed

neuronal precursor has been unresolved. Loss-of-function mutations are essential tools to test whether the murine Notch pathway has a role in neurogenesis. Initially, the analysis of mutant embryos was inconclusive, since *Notch1* and *RBP-Jk* mutant embryos are developmentally retarded and begin to degenerate at about the time that the first neurons express their mature, differentiated phenotype (Swiatek et al., 1994; Conlon et al., 1995; Oka et al., 1995). The problem of early lethality has been circumvented in the present report through the analysis of markers of neuronal determination and early differentiation at stages before developmental arrest. The increased expression of *Dll-1, Mash-1, Math4A, neuroD* and *NSCL-1* strongly suggests that an excess of committed neuronal precursor cells are generated in the *Notch1* and *RBP-Jk* mutants at E9.0. This may well represent premature neuronal differentiation and a loss of stem cells in the nervous system. However, this cannot be assesed directly, since *both RBP-Jk* and *Notch1* mutants die shortly after E9.5. Thus, activation of the Notch pathway normally suppresses the formation of neurons, as it does in *Drosophila*.

The neural tube early in embryogenesis consists of a relatively homogeneous population of rapidly proliferating cells (Hartenstein, 1989, 1993; McConnell, 1981; Sechrist and Bronner-Fraser, 1991). Neurons are the major differentiated cell type generated by the early neural tube; glial cells arise only at later stages (Maier and Miller, 1995; McConnell, 1995). Thus, most neural tube cells may face a simple binary decision between remaining a neural tube cell or differentiating into a neuron. However, this process must be tightly regulated in time and space. Our results lead us to suggest that the Notch pathway regulates this decision in the mouse, by a feedback mechanism between differentiating neurons and the remaining neural stem cells.

A model for Notch-mediated regulation of murine neurogenesis

From our results and studies in other vertebrate systems in vivo and in vitro, it is possible to draw a tentative model for the role of Notch signalling in vertebrate neurogenesis that can be directly compared to the current *Drosophila* model (Fig. 7A). In this model, prospective neurons express the Notch ligand *Dll1* (Fig. 7B). Binding of Dll1 to Notch proteins on the adjacent cell, causes these cells to proteolytically process Notch (Jarriault et al., 1995; Kopan et al., 1996). The Notch cytoplasmic fragment translocates to the nucleus where it binds to RBPJK (Tamura et al., 1995; Hsieh et al., 1996). Notch/RBPJK complexes stimulate transcription of *Hes-5* by binding to the *Hes-5* promoter. Activation of RBPJK by Notch in the receiving neural tube cell, also leads to the repression of *Mash-1* and other genes involved in neuronal development, including the Notch ligand Dll1. We have shown that mutation of either the receptor Notch1, or its downstream effector RBPJK, leads to downregulation of *Hes-5* and concomitant upregulation of *Dll1* in the usual domain of expression of *Hes-5*. By analogy to *Drosophila*, we propose that Hes-5 does not repress *Dll1* directly, but acts indirectly via repression of *Mash-1* and other genes of the 'proneural' class (gene X: Neurogenin?, Fig. 7B), leading secondarily to repression of *Dll1*. *Mash1* cannot be the only regulator of *Dll1* expression, because it is not up-regulated throughout the expanded domain of *Dll1* expression in *RBP-Jk* and *Notch1* mutants. There is as yet no direct evidence for this part of the pathway, although the expression results are consistent with it. Since the cell that expresses Dll1 inhibits all the neighbors that it contacts from also expressing the ligand, Notch signal transduction does not occur in the prospective neuron. Thus, in the prospective neuron *Mash-1* and *neuroD* are highly expressed, and *Hes-5* is not. Mash-1 could in turn stimulate the expression of *Dll1*, completing the regulatory loop. Subsequently, the prospective neuron would commence its differentiation and would downregulate expression of *Dll1* or migrate away, allowing additional neuronal precursors to form. Although our data and the data of others support direct physical interaction or direct gene regulation from Dll1 to Notch to RBPJK to *Hes-5*, the existing data do not permit us to distinguish direct from indirect interactions in the other steps of our model.

The expression of all genes examined in this study was affected to a lesser extent by the *Notch1* mutation than by the *RBP-Jk* mutation, consistent with the weaker phenotype of *Notch1* mutant embryos (Swiatek et al., 1994; Conlon et al.,

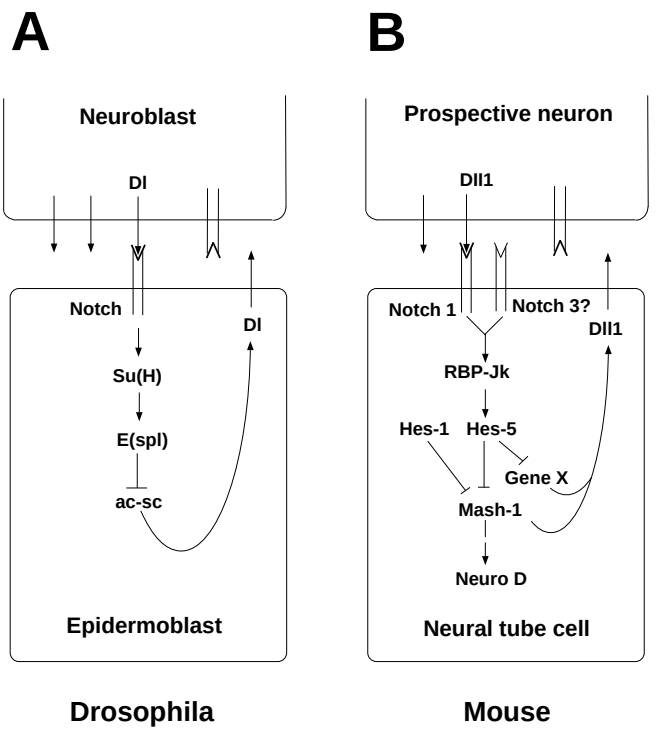

Fig. 7. Conservation of the Notch signalling pathway between *Drosophila* and the mouse. (A) Regulatory loop between Notch and Delta during lateral inhibition in *Drosophila* neurogenesis (modified from Heitzler et al., 1996). All competent cells express *Notch*, *Su(H)*, E(*spl*), *ac-sc* and *Delta*. After binding of Delta to Notch, the inhibitory signal is transduced to the nucleus via Su(H), activating *E(spl)* that represses *ac-sc*. *Delta* expression in the receiving cell probably depends on ac-sc activity. (B) Proposed regulatory loop between Notch1 and Dll1 during neurogenesis in the mouse. A prospective neuron expressing Delta activates Notch1 in a surrounding neural tube cell. The inhibitory signal is transduced to the nucleus via RBPJK that activates *Hes-5* expression, repressing *Mash-1* and probably another, as yet unidentified gene (gene X: Neurogenin?). *Dll1* expression in the receiving neural tube cell would depend on *Mash1* and gene X activity. The final result is the inhibition of neuronal differentiation in the receiving cell.

1995). We suggest that other *Notch* genes, such as *Notch3* (Lardelli et al., 1994), may have overlapping functions with *Notch1*, whereas *RBP-Jk* is required downstream of both genes. Thus, *RBP-Jk* appears to be a non-redundant element of the pathway, although recent analysis indicates the existence of a new *RBP-Jk*-related gene (T. Honjo, unpublished data).

Conclusion: *Drosophila* and mouse neurogenesis

The apparent conservation of function of Notch signaling in neurogenesis is surprising given the fundamental differences between insect and vertebrate neurogenesis. In *Drosophila*, the decision mediated by Notch is one between the epidermoblast and neuroblast lineages, both of which involve subsequent cell division. In the mouse and in other vertebrates, the neurogenic ectoderm is set aside from the surface ectoderm by an inductive interaction with mesoderm, which does not require the activity of the Notch pathway. It is the later decision between postmitotic neuron and proliferating neural epithelium that is regulated by Notch in vertebrates. Further genetic analysis combined with biochemical studies should shed light on how this important signalling pathway works in other aspects of normal development, and how its disruption can lead to tumorigenesis (Ellisen et al., 1991; Girard et al., 1996).

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REFERENCES

Akazawa, C., Sasai, Y., Nakanishi, S. and Kageyama, R. (1992). Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. *J. Biol. Chem.* **267**, 21879-21885.

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signaling. *Science* **268**, 225-232.

Austin, C. P., Feldman, D. E., Ida,J. A. and Cepko, C. L. (1995). Vertebrate retinal ganglion cells are selected from competent progenitors by the action of *Notch*. *Development* **121**, 3637-3650.

Bailey, A. M., and Posakony, J. W. (1995) *Suppressor of Hairless* directly activates transcription of *Enhancer of split* Complex genes in response to Notch receptor activity. *Genes Dev.* **9**, 2609-2622.

Begley, C.G., Lipkowitz, S., Goebel, V., Mahon, K. A., Bertness, V., Green, A. R. and Gough, N. M. and Kirsch, I. R. (1992). Molecular characterization of *NSCL*, a gene encoding a helix-loop-helix protein expressed in the developing nervous system. *Proc. Nat. Acad. Sci. USA* **89**, 38-42.

Bettenhausen, B., de Angelis, M. H., Simon, D., Guenet, J. L. and Gossler, A. (1995) Transient and restricted expression during mouse embryogenesis of *Dll1*, a murine gene closely related to *Drosophila Delta*. *Development* **121**, 2407-2418.

Campos-Ortega, J. (1993). Early neurogenesis in *Drosophila melanogaster*. In *The Development of Drosophila melanogaster* . (ed. pp. 1091-1129). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene *Delta*. *Nature* **375**, 761-766.

Conlon, R.A. and Herrmann, B. G. (1993). Detection of messenger RNA by in situ hybridization to postimplantation embryo whole mounts. *Meths. Enzymol.* **225**, 373-383.

Conlon, R. A., Reaume, A. G. and Rossant, J. (1995). *Notch 1* is required for the coordinate segmentation of somites. *Development* **121**, 1533-1545.

Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D. and Sklar, J. (1991). *TAN-1*, the human homologue of the *Drosophila Notch* gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66**, 649-661.

Fleming, R., Scottgale, T. N., Diederich, R. J. and Artavanis-Tsakonas, S. (1990). The gene *Serrate* encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in *Drosophila melanogaster*.*Genes Dev*.**4**, 2188-2201.

Fortini, M. E. and Artavanas-Tsakonas, S. (1994). The Suppressor of hairless protein participates in Notch receptor signaling. *Cell* **79,** 273-282.

Franco del Amo, F., Smith, D. E., Swiatek, P. J., Gendron-Maguire, M., Greenspan, R.J., McMahon, A. P. and Gridley, T. (1992) Expression pattern of *Motch*, a mouse homolog of *Drosophila Notch*, suggests an important role in early postimplantation mouse development. *Development* **115**, 737-744.

Furukawa, T., Maruyama, S., Kawaichi, M. and Honjo T. (1992). The Drosophila homolog of the immunoglobulin recombination signal-binding protein regulates peripheral nervous system development. *Cell* **69,** 1191-1197.

Furukawa, T., Kobayakawa, Y., Tamura, K., Kimura, K., Kawaichi, M., Tanimura, T. and Honjo, T. (1995). *Suppressor of hairless*, the *Drosophila* homologue of *RBP-J kappa*, transactivates the neurogenic gene *E(spl)m8*. *Jpn. J. Genet.* **70**, 505-24

Gho, M., Lecourtois, M., Geraud, G., Posakony, J. W., Schweisguth, F. (1996). Subcellular localization of Suppressor of Hairless in *Drosophila* sense organ cells during Notch signalling. *Development* **122**, 1673-1682.

Girard, L., Hanna, Z., Beaulieu, N., Hoemann, C. D., Simard, C., Kozak, C. A. and Jolicoeur, P. (1996). Frequent provirus insertional mutagenesis of *Notch1* in thymomas of *MMTV^D/myc* transgenic mice suggests a collaboration of *c-myc* and *Notch1* for oncogenesis. *Genes Dev*. **10**, 1930-1944.

Gradwohl, G., Fode, C. and Guillemot, F. (1996). Restricted expression of a novel murine *atonal*-related bHLH protein to undifferentiated neural precursors. *Dev. Biol.* **180**, 227-241.

Greenwald, I., and Rubin, G. M. (1992). Making a difference: the role of cell-cell interactions in establishing separate identities for equivalent cells. *Cell* **68**, 271-281.

Greenwald, I. (1994). Structure/function studies of lin-12/Notch proteins. *Curr. Opinion Gen. Dev.* **4**, 556-562.

Guillemot, F. and Joyner, A. L. (1993). Dynamic expression of the murine *Achaete-Scute* homologue*Mash-1* in the developing nervous system. *Mech. Dev.* **42**, 171-85.

Guillemot, F., Lo, L.C., Johnson, J. E., Auerbach, A., Anderson, D. J. and Joyner, A.L. (1993)**.** Mammalian *achaete-scute* homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* **75**, 463-476.

Hall, R., Hyde, J. E., Goman, M., Simmons, D.L., Hope, I. A., Mackay, M., Scaife, J., Merkli, B., Richle, R. and Stocker, J. (1984). Major surface antigen gene of a human malaria parasite cloned and expressed in bacteria. *Nature* **311**, 379-382.

Hamaguchi, Y., Yamamoto, Y., Iwanari, H., Maruyama, S., Furukawa, T., Matsunami, N. and Honjo T. (1992). Biochemical and immunological characterization of the DNA binding protein (RBP-JK) to mouse Jk recombination signal sequence. *J. Biochem.* **112,** 314-320.

Hartenstein, V. (1989). Early neurogenesis in *Xenopus*: the spatio-temporal pattern of proliferation and cell lineages in the embryonic spinal cord. *Neuron* **3**, 399-411.

Hartenstein, V. (1993). Early pattern of neuronal differentiation in the *Xenopus* embryonic brainstem and spinal cord. *J. comp. Neurol.* **328**, 213-231.

Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of *Drosophila*. *Cell* **64**, 1083-1092.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C. and Simpson, P. (1996). Genes of the *Enhancer of split* and *achaete-scute* complexes are required for a regulatory loop between Notch and Delta during lateral signalling in *Drosophila*. *Development* **122** 161-171.

Henrique, D., Adam, J., Myat, A. Chitnis, A., Lewis, J. and Ish-Horowicz, D (1995). Expression of a *Delta* homologue in prospective neurons in the chick. *Nature* **375**, 787-790.

Hsieh, J. J., Henkel, T., Salmon, P., Robey, E., Peterson, M. G. and Hayward, S. D. (1996). Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. *Mol. Cell. Biol.* **16**, 952-259.

Ishibashi, M., Ang, S. L., Shiota, K., Nakanishi, S.,Kageyama, R. and Guillemot, F. (1995). Targeted disruption of mammalian *hairy and Enhancer of split homolog-1*(*HES-1*) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. *Genes Dev*. **9**, 3136-3148.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R. and Israël, A. (1995). Signalling downstream of activated mammalian Notch. *Nature* **377**, 355-358.

Jennings, B., de Celis, J., Delidakis, C., Preiss, A. and Bray, S. (1995). Role of *Notch* and *achaete-scute* complex in the expression of Enhancer of split bHLH proteins. *Development* **121**, 3745-3752.

Kawaichi, M., Oka, C., Shibayama, S., Koromilas, A. E., Matsunami, N. Hamaguchi, Y. and Honjo-T. (1992). Genomic organization of mouse J kappa recombination signal binding protein (*RBP-J kappa*) gene. *J. Biol. Chem.* **267**, 4016-4022.

Klaembt, C., Knust, E., Tietze, K., Campos-Ortega, J. A. (1989). Closely related transcripts encoded by the neurogenic gene complex *enhancer of split of Drosophila* melanogaster. *EMBO J.* **8,** 203-210

Koop, K. E., MacDonald, L. M. and Lobe, C.G. (1996). Transcripts of *Grg-4*, a murine *groucho*-related gene, are detected in adjacent tissues to other neurogenic gene homologous during embryonic development. *Mech. Dev.* (in press).

Kopan, R. E. H., Schoerer, H. and Weintraub, H. (1996). Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracelular domain. *Proc. Natl. Acad. Sci. USA* **93**, 1683-1688.

Kunisch, M., Haenlin, M. Campos-Ortega, J. A. (1994). Lateral inhibition mediated by the *Drosophila* neurogenic gene *Delta* is enhanced by proneural proteins. *Proc. Natl. Acad. Sci. USA* **91,** 10139-10143.

Lardelli, M., Dahlstrand, J. and Lendahl, U. (1994). The novel *Notch* homologue mouse *Notch 3* lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. *Mech. Dev.* **46**, 123-136.

Lecourtois, M., and Schweisguth, F. (1995). The neurogenic Suppressor of Hairless DNA-binding protein mediates the transcriptional activation of the *Enhancer of split* Complex genes triggered by Notch signaling. *Genes Dev*. **9**, 2698-2608.

Lee, K. A., Bindereif, A. and Green, M. R. (1988). A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. *Gene Analysis Techniques* **5**, 22-31.

Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N. and Weintraub, H. (1995). Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* **268**, 836-844.

Lehmann, R., Dietrich, U., Jimenez, F. and Campos-Ortega, J. A. (1981). Mutations of early neurogenesis in *Drosophila*. *Roux's Arch. Dev. Biol*. **190**, 226-229.

Lindsell, C.E., Shawber, C. J., Boulter, J. and Weinmaster, G. (1995). Jagged: a mammalian ligand that activates Notch1. *Cell* **80**, 909-917.

Ma, Q., Kintner, C. and anderson, D. (1996). Identification of *neurogenin*, a vertebrate neuronal determination gene. *Cell* **87**, 43-52.

Maier, C. E., and Miller, R. H (1995). Development of glial cytoarchitecture in the frog spinal cord. *Dev. Neurosci.* **17**, 149-159.

McConnell, J. A. (1981). Identification of early neurons in the brainstem and spinal cord. II: An autoradiographic study in the mouse. *J. comp. Neurol.* **200**, 273-288.

McConnell, S. K. (1995). Constructing the cerebral cortex: neurogenesis and fate determination. *Neuron* **15**, 761-768.

Miranda, G. A., Chokler, I. and Aguilera, R. J. (1995). The murine nucleolin protein is an inducible DNA and ATP binding protein which is readily detected in nuclear extracts of lipopolysaccharide-treated splenocytes. *Exp. Cell Res.* **217**, 294-308.

Myat, A., Henrique, D. Ish-Horowicz,D. and Lewis, J. (1996). A chick homologue of *Serrate* and its relationship with *Notch* and *Delta* homologues during central neurogenesis. *Dev. Biol*. **174**, 233-247.

Nye, J. S., R. Kopan, and Axel, R. (1994). An activated Notch suppresses neurogenesis and myogenesis but not gliogenesis in mammalian cells. *Development* **120**, 2421-2430.

Oka, C., Nakano, T., Wakeham, A. de la Pompa, J. L., Mori, C., Sakai, T., Okazaki, S., Kawaichi, M., Shiota, K., Mak, T. W. and Honjo, T. (1995). Disruption of the mouse *RBP-Jk* gene results in early embryonic death. *Development* **121**, 3291-3301.

Reaume, A. G., Conlon, R. A., Zirngibl, R., Yamaguchi, T. P. and Rossant, J. (1992). Expression analysis of a *Notch* homologue in the mouse embryo. *Dev. Biol*. **154**, 377-387.

Sasai, Y., Kageyama, R., Tagawa, Shigemoto, R. and Nakanishi, S. (1992). Two mammalian helix-loop-helix factors structurally related to *Drosophila hairy* and *Enhancer of split*.. *Genes Dev*. **6**, 2620-2634.

Schweisguth, F. and Posakony, J. W. (1992). Suppressor of Hairless, the Drosophila homolog of the mouse recombination signal-binding protein gene, controls sensory organ cell fates. *Cell* **69,** 1199-1212.

Sechrist, J. and Bronner-Fraser, M. (1991). Birth and differentiation of reticular neurons in the chick hindbrain: ontogeny of the first neuronal population. *Neuron* **7**, 947-963.

Skeath, J. B. and Carroll, S. B. (1992). Regulation of proneural gene expression and cell fate during neuroblast segregation in the *Drosophila* embryo. *Development* **114,** 939-946.

Swiatek, P. J., Lindsell, C. E., del Amo, F. F., Weinmaster, G. and Gridley, T. (1994). *Notch 1* is essential for postimplantation development in mice. *Genes and Dev.* **8,** 707-719.

Takebayashi, K., Akazawa, C., Nakanishi, S. and Kageyama, R. (1995). Structure and promoter analysis of the gene encoding the mouse helix-loop-helix factor *HES-5*. Identification of the neural precursor cell-specific promoter element. *J. Biol. Chem.* **270**, 1342-1349.

Tamura, K., Taniguchi, Y., Minoguchi, S., Sakai, T., Tun, T., Furukawa, T., and Honjo, T. (1995). Physical interaction between a novel domain of the receptor Notch and the transcription factor RBPJK/Su(H). *Curr. Biol.* **5**, 1416-1423.

Technau, G. and Campos-Ortega, J. (1986). Lineage analysis of transplanted individual cells in embryos of *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* **195**, 445-454.

Trumpp, A., Blundell, P. A., de la Pompa, J. L. and Zeller, R. (1992). The chicken *limb deformity* gene encodes nuclear proteins expressed in specific cell types during morphogenesis. *Genes Dev.* **6**, 14-28.

Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q., Sassoon, D. and Kitajewski, J. (1996). *Notch4/int-3*, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. *Development* **122**, 2251-2259.

Vässin, H. and Campos-Ortega, J. A. (1987). The neurogenic gene *Delta* of *Drosophila melanogaster* is expressed in neurogenic territories and encodes a putative transmembrane protein with EGF-like repeats. *EMBO J.* **6**, 3431-3440.

Weinmaster, G., Roberts, V. and Lemke, G. (1991). A homolog of *Drosophila Notch* expressed during mammalian development. *Development* **113**, 199-205.

Weinmaster, G., Roberts, V. J. and Lemke, G. (1992). *Notch 2*: A second mammalian *Notch* gene. *Development* **116,** 931-941.

Wharton, K. A., Johansen, K. M., Xu, T., Artavanis-Tsakonas, S. (1985). Nucleotide sequence from the neurogenic locus *Notch* implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* **43**, 567-581.

Wilkinson, H. A., Fitzgerald, K. and Greenwald, I. (1994) Reciprocal changes in expression of the receptor lin-12 and its ligand lag-2 prior to commitment in a *C. elegans* cell fate decision. *Cell* **79**, 1187-1198.

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