Conservation of the Notch signalling pathway in mammalian neurogenesis

José Luis de la Pompa1,2,*, Andrew Wakeham1,2, Kristen M. Correia3, Enrique Samper1,2, Stephen Brown4, Renato J. Aguilera4, Toru Nakano5, Tasuku Honjo5, Tak W. Mak1,2, Janet Rossant6,7 and Ronald A. Conlon3

1Amgen Institute, 620 University Avenue, Toronto, Ontario, Canada M5G 2C1
2Ontario Cancer Institute, and Departments of Medical Biophysics and Immunology, University of Toronto, Toronto, Ontario, Canada M5G 2C1
3Department of Genetics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106-4955, USA
4Department of Molecular, Cell, and Developmental Biology, University of California at Los Angeles, Los Angeles, California 40095-1606, USA
5Department of Medical Chemistry, Faculty of Medicine, Kyoto University, Kyoto 606, Japan
6Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario, Canada M5G 1X5
7Departments of Molecular and Medical Genetics, and Obstetrics and Gynecology, University of Toronto

*Author for correspondence (e-mail: Jose.delaPompa@amgen.com)

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 expanded 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,
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 antiseraum (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, NiCl2 (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 (Hybaid N+, Amersham). Full-length Hes-1, Hes-3 and Hes-5 cDNAs were 32P-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.
**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, 15). Decreased RBPJK expression is observed in heterozygous (+/-) E8.5 RBP-Jk embryos (lane 5). No signal is observed in E8.5RBP-Jk homozygous (-/-) mutant embryos (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 Notch-Jk homozygous mutant embryos (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).
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. 3](image1.png)

**Fig. 3.** Hes-5 expression in RBP-Jk 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.

![Fig. 4](image2.png)

**Fig. 4.** Down-regulation of Hes-5 and up-regulation ofDll1 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 DI 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., 2000; 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 an 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 *Notch1* (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 Schweiguth, 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...
In Drosophila, a connection between the up-regulation of E(spl) and the downregulation of Dll, 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). Dll expression is positively regulated by ac-sc, so that reduced ac-sc leads to downregulation of Dll, providing a feedback loop to link the expression of Dll 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 Dll expression, because it is not up-regulated throughout the expanded domain of Dll 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 neurogenesis. 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). In Drosophila, after activation of Notch signalling, the Notch1 receptor does not affect the subcellular localization of the protein during neurogenesis.

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 assessed 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 down-regulate 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.,
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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 epidermblast 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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