

Nuclear Notch1 signaling and the regulation of dendritic development

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To understand the function of Notch in the mammalian brain, we examined Notch1 signaling and its cellular consequences in developing cortical neurons. We found that the cytoplasmic domain of endogenous Notch1 translocated to the nucleus during neuronal differentiation. Notch1 cytoplasmic domain constructs transfected into cortical neurons were present in multiple phosphorylated forms, localized to the nucleus and could induce CBF1-mediated transactivation. Molecular perturbation experiments suggested that Notch1 signaling in cortical neurons promoted dendritic branching and inhibited dendritic growth. These observations show that Notch1 signaling to the nucleus exerts an important regulatory influence on the specification of dendritic morphology in neurons.

Although the role of cell–cell interactions in the regulation of mammalian neural development is not well understood, it has become increasingly apparent from studies in invertebrates that such interactions can exert an important regulatory influence on differentiation. The role of cell–cell interactions has been extensively studied in the context of lateral specification in invertebrates. Such interactions among developmentally equivalent cells allow a single cell within the group to adopt a cell fate distinct from the neighboring cells and have an important role in neuroblast specification^{1,2}. Molecular and genetic studies indicate that the cell-surface protein Notch is a central mediator of lateral specification in invertebrates^{3–5}.

Notch is a type I cell-surface protein, approximately 300 kDa in size, which functions as a receptor. Proteolytic processing of full-length Notch generates two fragments that seem to associate at the membrane to form a receptor complex^{6–9}. One of the fragments (p180) contains most of the extracellular domain, and the other fragment (p120) contains the transmembrane and cytoplasmic domains. An important intracellular target of Notch signaling in *Drosophila* is the transcription factor, Suppressor of Hairless [Su(H)]^{10,11}. There is also considerable evidence that the mammalian homolog of Su(H), called CBF1, is a target of Notch signaling in mammalian cells^{12–15}. Although the mechanisms by which Notch signaling leads to transactivation via Su(H)/CBF1 are poorly understood, Notch-receptor activation seems to involve cleavage and nuclear translocation of the cytoplasmic domain of the receptor^{16–19}.

Several observations suggest that vertebrate Notch proteins are involved in regulating neural development in nonmammalian vertebrates. For example, overexpression of the Notch ligand Delta or a constitutively active form of Notch inhibits primary neurogenesis in *Xenopus*^{20–22}. Similarly, Notch-mediated interactions seem to negatively regulate neuronal differentiation in *Xenopus* and chick retina^{23,24}. It is likely that Notch is also involved in regulating neural development in mammals.

Four mammalian homologs of Notch (Notch1, Notch2, Notch3 and Notch4) are expressed in the developing nervous system^{25–29}. Mammalian homologs of the Notch ligands Delta and Serrate are expressed in the developing brain and spinal cord^{30–33}. Mice lacking Notch1 die embryonically, before the period of neuronal differentiation, and show a high occurrence of cell death in the central nervous system^{34,35}. In addition, transgenic mice overexpressing Notch3 have neural tube defects³⁶. These observations suggest that, as in other vertebrates, mammalian Notch homologs regulate early neural development.

Protein localization studies also suggest a role for mammalian Notch in neuronal differentiation. In the ventricular zone of developing ferret cortex, Notch1 is localized to the basal pole of dividing progenitor cells³⁷. Because the basal daughter is thought to become postmitotic in asymmetric cell divisions, this observation suggests that Notch1 is preferentially inherited by the postmitotic cell. This study suggests that Notch may continue to function in differentiated neurons after cell fate has been decided.

To explore the role of Notch in mammalian neural development, we examined the molecular and cellular consequences of Notch signaling in developing cortical neurons. We report that neuronal Notch1 signals to the nucleus during differentiation and regulates the dendritic development of cortical neurons.

RESULTS

Developmental regulation of Notch1 localization

To characterize expression of Notch1 in cortical tissues, we used affinity-purified antibodies generated against the intracellular domain of Notch1 (α -Notch.IC). These antibodies recognize a truncated Notch1 construct when expressed in COS cells, and this interaction can be specifically blocked by preincubation of the affinity-purified antibodies with recombinant Notch1 protein (Fig. 1a). In addition, these antibodies recognize full-length Notch1 but not Notch2, Notch3 or Notch4 in western blots of

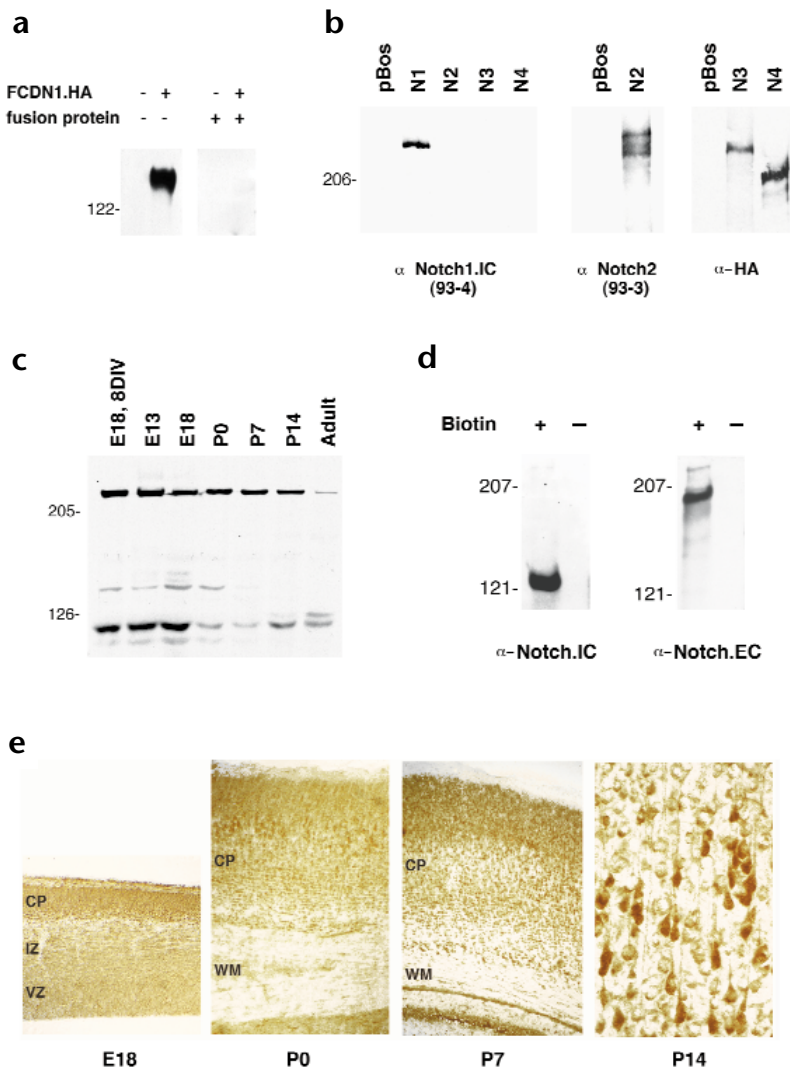


Fig. 1. Notch1 expression in the developing cortex. **(a, b)** Characterization of α -Notch1.IC (93-4). **(a)** On western blots, α -Notch1.IC recognizes FCDN1.HA expressed in COS cells and immunoprecipitated using anti-HA. Staining is blocked by preincubation of α -Notch1.IC with the intracellular domain Notch1 fusion protein. **(b)** α -Notch1.IC recognizes Notch1, but not Notch2, 3 or 4, expressed in 293 cells. Expression of Notch2, Notch3 and Notch4 in the 293 cells was confirmed by western blotting with anti-Notch2 (93-3) or with anti-HA (to detect tagged Notch3 and Notch4). **(c)** Western blot of proteins isolated from cortical neurons in culture at E18 + 8 DIV and from cortical tissue from animals at ages indicated, probed with α -Notch1.IC. The major forms of Notch1 detected by the intracellular-domain antibody are the full-length protein (p300) and a cleaved form of the protein (p120). **(d)** Characterization of the cell-surface forms of Notch1 in cortical neurons. Biotinylated cell-surface proteins on cortical neurons were separated by polyacrylamide gel electrophoresis (PAGE) and probed with antibodies to the intracellular (Notch1.IC) or extracellular (Notch1.EC) domains of Notch1. **(e)** Immunohistochemical localization of Notch1 using α -Notch1.IC in cortical coronal sections taken from E18, P0, P7 and P14 rats. The P14 image is taken from the cortical plate. Notch1 immunoreactivity is present both in the ventricular zone (which contains proliferating cells) and in the cortical plate (which contains postmitotic neurons) at all ages shown. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone; WM, white matter.

recombinant proteins (Fig. 1b). Western blots showed that the two previously described forms of Notch1, full-length p300 and cleaved p120, were present in cortical neurons at all ages (Fig. 1c). Proteins migrating at about 140 kDa and 100 kDa were found at embryonic and early postnatal ages, but not at later ages. This pattern suggests that Notch1 might regulate developmental events both during and after neurogenesis.

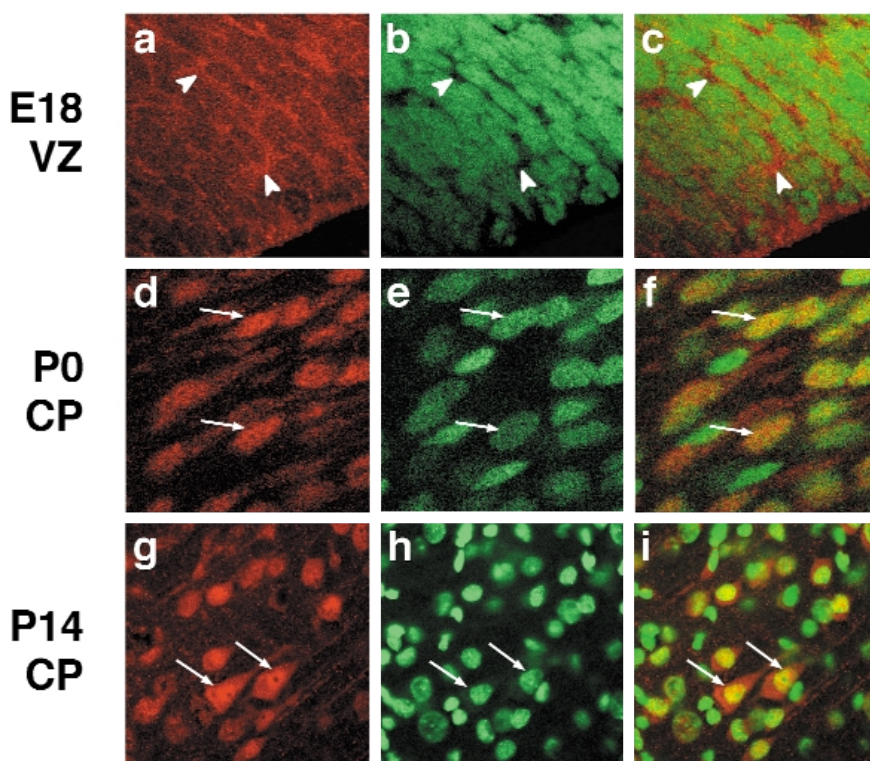
To define the cell-surface form of Notch1 in developing cortical neurons, we did surface biotinylation experiments in E18 cortical neurons in culture. After surface biotin labeling, biotinylated proteins were precipitated using streptavidin-agarose and separated by SDS-PAGE. Western blots using antibodies to the intracellular and extracellular domains of Notch1 indicated that the p120 form of the Notch1 protein was the dominant cell-surface form of the receptor recognized by the intracellular-domain antibody, and the p180 form of the protein was the dominant cell-surface form recognized by the extracellular-domain antibody (Fig. 1d). This is consistent with a model in which the cell-surface Notch1 receptor is a heterodimer of p120 and p180 forms. We also found a high-molecular-weight form (Fig. 1d) that may represent uncleaved, full-length Notch1 at the cell surface.

To determine Notch1 protein localization in developing cortex, we performed immunohistochemical staining with α -Notch1.IC on cortical sections from late embryonic and early postnatal rats. In agreement with previous studies^{25,30,37}, we detected Notch1 protein in cells of the ventricular zone of E18 cortex (Fig. 1e). In addition, at both embryonic and postnatal ages, we detected significant levels of Notch1 immunoreactivity in the cortical plate, suggesting that Notch1 may regulate the development of postmitotic neurons.

We performed confocal microscopy on immunofluorescently labeled sections through the developing cortex to determine the subcellular localization of Notch1 in cortical cells. In the ventricular zone, Notch1 was concentrated near the cell membranes and seemed largely excluded from the nucleus (Fig. 2a-c). By contrast, Notch1 was localized predominantly in the nucleus in cortical plate neurons (Fig. 2d-i). Because cells migrate from the ventricular zone to the cortical plate once they become postmitotic, these observations suggest that neuronal differentiation is accompanied by a translocation of Notch1 to the nucleus.

To examine the relationship between Notch1 localization and neuronal differentiation more carefully, we carried out experiments in E18 dissociated cortical cell cultures, in which the transition from

Fig. 2. The subcellular localization of Notch1 changes as cells migrate from the ventricular zone to the cortical plate. Confocal images of sections taken from the ventricular zone at E18 (**a–c**), the cortical plate at P0 (**d–f**) and the cortical plate at P14 (**g–i**). The sections were labeled with α -Notch1C (red; **a, d, g**) and with Hoechst 33258 (Sigma) to reveal the nucleus (green; **b, e, h**). (**c, f, i**) Merged images showing localization of Notch1 immunofluorescence with respect to the nucleus. Notch1 is largely excluded from the nucleus of ventricular zone cells (**a–c**; arrowheads), but is predominantly nuclear in postmitotic neurons in the cortical plate (**d–g**; arrows).



a dividing to postmitotic state could be examined closely. Western blots indicated that the same forms of Notch1 were expressed *in vitro* as *in vivo* (Fig. 1c). To determine if α -Notch1C could be used to detect Notch1 distribution in dissociated neurons, we transfected E18 cortical neurons with a hemagglutinin (HA)-tagged, truncated Notch1 construct (FCDN1.HA). Transfected neurons were strongly immunopositive for Notch1 (Fig. 3a and b). Notch1 immunofluorescence was eliminated when α -Notch1C was preincubated with recombinant Notch1 (Fig. 3c and d). Immunofluorescence for endogenous Notch1 revealed that many cortical neurons in culture had high nuclear levels of Notch1 (Fig. 3e and f). Endogenous staining was blocked by preincubating the antibody with recombinant Notch1, indicating that the immunofluorescence signal reflects the localization of a Notch1 epitope (Fig. 3; compare e with g).

To examine the relationship between subcellular Notch1 localization and the differentiation state of individual neurons, we labeled E18 cortical cultures with α -Notch1C and differentiation stage-specific antibodies (Fig. 4). In dividing cells (immunopositive for PCNA), Notch1 immunofluorescence was primarily localized to cytoplasmic and membrane compartments, indicating that Notch1 is largely excluded from the nucleus in undifferentiated cells (Fig. 4a–c). In contrast, Notch1 was detected mainly in the nucleus in TuJ1-positive (postmitotic) neurons (Fig. 4d–f). To further analyze the distribution of Notch1 with respect to cell cycle withdrawal, E18 cells were labeled with BrdU for various durations before being fixed and processed for immunofluorescence. In cultures labeled for 6 hours, all BrdU-labeled cells should be actively dividing, and in cultures labeled for 24 hours (longer than the cell-cycle duration), all BrdU-negative cells should be postmitotic. Immunofluorescence using anti-BrdU and α -Notch1C indicated that Notch1 was excluded from the nucleus in actively dividing cells, whereas Notch1 was concentrated in the nucleus in postmitotic neurons (Fig. 4g–i). This relationship was also confirmed by con-

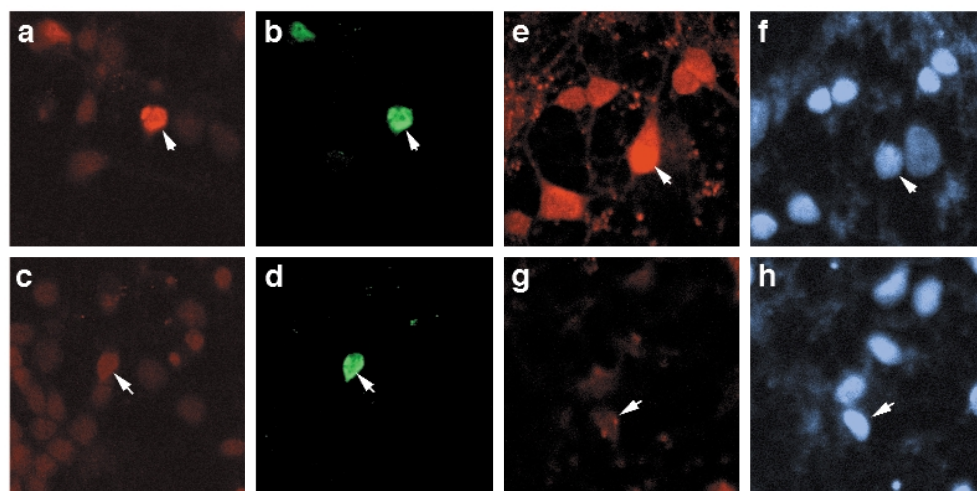
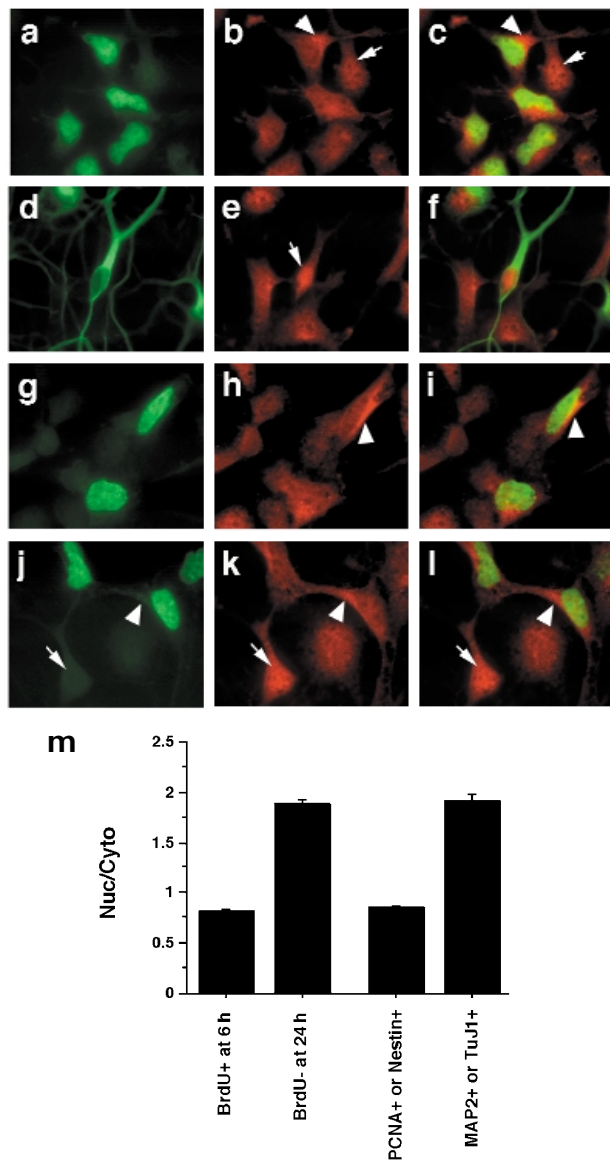


Fig. 3. Distribution of Notch1 in cortical cells in culture. (**a–d**) Characterization of α -Notch1C for immunofluorescence. Cortical neurons were transfected with HA-tagged FCDN1 and processed for immunofluorescence using α -Notch1C (**a, c**) and anti-HA (**b, d**). A strong nuclear Notch1 signal is detected in transfected neurons (**a**) that is blocked by preadsorption of α -Notch1C with Notch1 fusion protein (**c**). FCDN1.HA expression is detected in both conditions with anti-HA (**b, d**). (**e–h**) Distribution of endogenous Notch1 in cortical cultures visualized using α -Notch1C (**e**). Notch1 immunofluorescence is detected in the nucleus (revealed by Hoechst staining in **f, h**), and is blocked by preadsorption of the antibody with Notch1 fusion protein (**g**). Images of cultures with or without preadsorption were obtained under identical confocal settings.

firmation by con-



focal microscopy (data not shown). The relative levels of Notch1 immunofluorescence measured in the nuclei and cytoplasm of dividing cells and postmitotic neurons revealed that neuronal differentiation was associated with an increase in nuclear Notch 1 levels (Fig. 4m).

As nuclear localization of endogenous Notch 1 is difficult to demonstrate, we also determined the subcellular localization of endogenous Notch 1 by biochemical cell fractionation. Tissue extracts from P3 and adult cortex were separated into cytoplasmic and nuclear components on a sucrose-density gradient, separated by SDS-PAGE, blotted onto nitrocellulose and probed with α -Notch1.IC. These western blots revealed two bands with approximate molecular weights of 120 kDa and 100 kDa in the nuclear extracts (Fig. 5a). Probing the same blots with an antibody to the β -1 subunit of Na^+/K^+ ATPase (an integral cell-membrane protein) verified that the nuclear Notch1-immunoreactive bands did not represent contamination by membrane proteins (Fig. 5a). (During development, some Na^+/K^+ ATPase activity is present on intracellular organelles³⁸, which probably accounts for the Na^+/K^+ ATPase signal in the

Fig. 4. Neuronal differentiation is associated with a relative increase in nuclear Notch1 immunofluorescence. E18 cortical neurons in culture were double labeled for expression of Notch1 in red (**b, e, h, k**) and PCNA (**a**), TuJ1 (**d**) or BrdU (**g, j**) in green. (**c, f, i, l**) Merged images. (**a–c**) Notch1 immunofluorescence is predominantly cytoplasmic in PCNA-positive undifferentiated cortical cells (arrowheads) and predominately nuclear in PCNA-negative cells (arrows). (**d–f**) Notch1 immunofluorescence is predominantly nuclear in TuJ1-positive postmitotic cortical neurons (arrow). (**g–i**) Following BrdU labeling for 6 h, Notch1 immunofluorescence is predominantly extranuclear in BrdU-positive (dividing) cells (arrowheads). (**j–l**) Following BrdU labeling for 24 h, Notch1 immunofluorescence is predominantly extranuclear in BrdU-positive cells (arrowheads), and predominantly nuclear in BrdU-negative (postmitotic) neurons (arrows). (**m**) Quantification of the relative intensity of Notch1 immunofluorescence in the nucleus versus the cytoplasm in dividing (BrdU-positive after 6-h labeling) and postmitotic (BrdU-negative after 24-h labeling) cells in E18 cortical cultures. Dividing cells identified by either PCNA or Nestin immunoreactivity and postmitotic neurons identified by either TuJ1 or MAP2 immunoreactivity also demonstrate a predominance of Notch1 in the nucleus of postmitotic neurons.

P3 cytosolic fraction.) These results strengthen the conclusion that the intracellular domain of endogenous Notch1 is present in the nucleus of cortical neurons.

To characterize these two Notch1-immunoreactive proteins, we tagged truncated Notch1 constructs (Fig. 6a) with a hemagglutinin epitope, transfected them into cortical neurons, immunoprecipitated with anti-HA and loaded them alongside a P3 nuclear extract for western blots. ZEDN1.HA and FCDN1.HA were about the same size as p120 and p100 bands detected in P3 nuclear extract, whereas CDN1.HA had a smaller molecular weight than either band (Fig. 5b). (Although the ZEDN1 construct is larger than FCDN1, ZEDN1 protein is cleaved intracellularly to generate a cytosolic fragment the size of FCDN1). Comigration of nuclear Notch1 and FCDN1.HA bands suggests that endogenous nuclear Notch1 probably corresponds to the cytoplasmic domain of Notch1 (cleaved at about aa1744)¹⁸.

Detection of p120 and p100 proteins in cortical nuclear extracts and in ZEDN1- and FCDN1-transfected cells suggested that the proteins might represent differentially phosphorylated forms of the cleaved intracellular domain of Notch1. To examine this possibility, FCDN1.HA and ZEDN1.HA immunoprecipitates were treated with λ phosphatase (a nonspecific phosphatase), separated by SDS-PAGE, and probed with α -Notch1.IC (Fig. 5c). For both constructs, phosphatase treatment led to a decrease in the presence of the p120 band and an increase in the p100 form, suggesting that the p120 and p100 forms represent differentially phosphorylated forms of Notch intracellular domain. This mobility shift was attenuated by the phosphatase inhibitor sodium orthovanadate. To directly determine if p120 was a phosphorylated form of the intracellular domain of Notch1, we transfected E18 cortical cultures with FCDN1.HA and metabolically labeled them with [³²P]orthophosphate. FCDN1-HA was then immunoprecipitated with anti-HA, separated by PAGE and transferred to nitrocellulose. This blot was first exposed to film to detect phosphoproteins and then incubated with α -Notch1.IC for western blotting. Anti-HA antibodies immunoprecipitated only one [³²P]-labeled protein from FCDN1.HA-transfected neurons (Fig. 5d). Western analysis with α -Notch1.IC revealed that the phosphorylated band corresponded to p120 (Fig. 5d, left blot). Thus, the intracellular domain of Notch1 can exist in two forms, p120 and p100, the larger of which is phosphorylated.

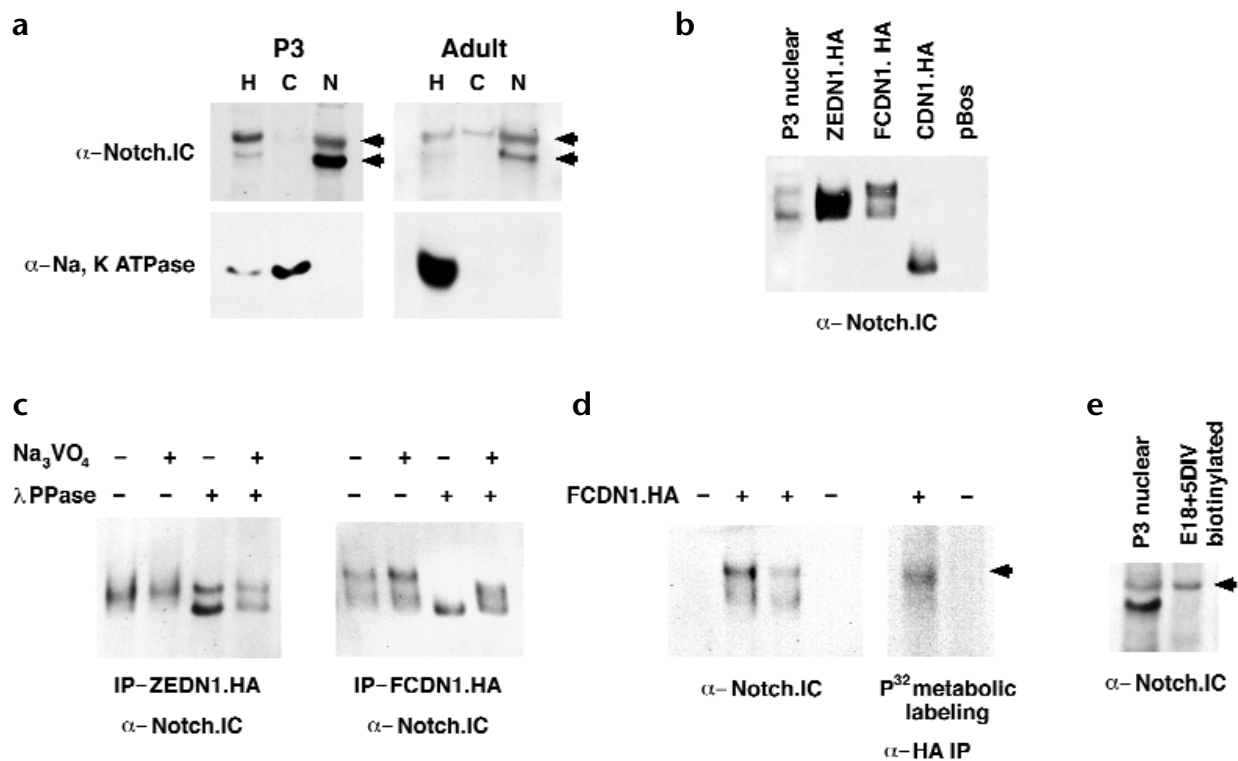


Fig. 5. Characterization of nuclear forms of Notch I. (a) The distribution of Notch I in whole tissue homogenates (H), the cytosolic fraction (C) and the nuclear fraction (N) in extracts prepared from P3 and adult cortex as determined by western blotting using α -Notch.IC. The nuclear extract contains two Notch I-immunoreactive proteins that migrate at 120 kDa and 100 kDa (arrows). Probing the same blot with an antibody to a membrane protein (Na^+/K^+ ATPase) does not give a signal in the nuclear fraction, indicating that the nuclear Notch I signal does not represent membrane-associated Notch I. (b) Western blot of nuclear fraction from P3 cortex loaded alongside immunoprecipitates of HA-tagged truncated Notch I constructs and probed with α -Notch.IC. The upper and lower bands present in P3 nuclear fraction comigrate with the bands found with ZEDN1.HA and FCDN1.HA. CDN1.HA lacks sequences in FCDN1 and migrates lower than the two bands in the P3 nuclear fraction. (c) Phosphatase treatment of ZEDN1.HA and FCDN1.HA immunoprecipitates indicates that the p120 form of these proteins is phosphorylated. E18 cortical neurons were transfected with either ZEDN1.HA or FCDN1.HA, immunoprecipitated with anti-HA, treated with λ PPase or 10 mM Na_3VO_4 and separated by PAGE. The western blot was then probed with α -Notch.IC. Treatment with λ PPase causes a reduction in the p120 band compared to the p100 band. The residual p120 signal in the ZEDN1.HA-transfected cells may correspond to the p120 transmembrane form of Notch I that has not yet undergone cleavage in the intracellular domain. (d) Metabolic labeling of FCDN1.HA-transfected neurons with [^{32}P]orthophosphate followed by immunoprecipitation with HA antibodies identifies the p120 form of FCDN1.HA as a phosphorylated protein. Immunoprecipitates from [^{32}P]-labeled FCDN1.HA (+) and control (-) transfected neurons were separated by PAGE, transferred to nitrocellulose and exposed to film to detect [^{32}P] radioactive signal (right). Subsequently, the blot was probed with α -Notch.IC to reveal the p100 and p120 forms of FCDN1 (left). Lanes 3 and 4 in the western blot (left) are the same two lanes shown in the film exposure (right). Comparison of the two indicates that the p120 form of FCDN1 is phosphorylated (arrow). (e) Relative mobility of nuclear Notch I bands (P3 nuclear) and the biotinylated transmembrane p120 form of Notch I (E18 + 5DIV biotinylated). The upper (phosphorylated) nuclear band comigrates with the transmembrane p120 form of Notch I.

To determine if the phosphorylated form of nuclear Notch I was distinguishable from the truncated transmembrane form by size, we ran cortical nuclear extracts next to biotinylated cell-surface proteins and probed the blot with α -Notch.IC. The upper nuclear Notch I band comigrated with p120 transmembrane Notch I (Fig. 5d). Thus, neurons had two processed forms of Notch I that migrated at about 120 kDa: the truncated transmembrane receptor and the phosphorylated intracellular domain of Notch I. These two forms could not be distinguished by western blots of whole-cell lysates, and their identification required surface biotinylation and cell-fraction experiments.

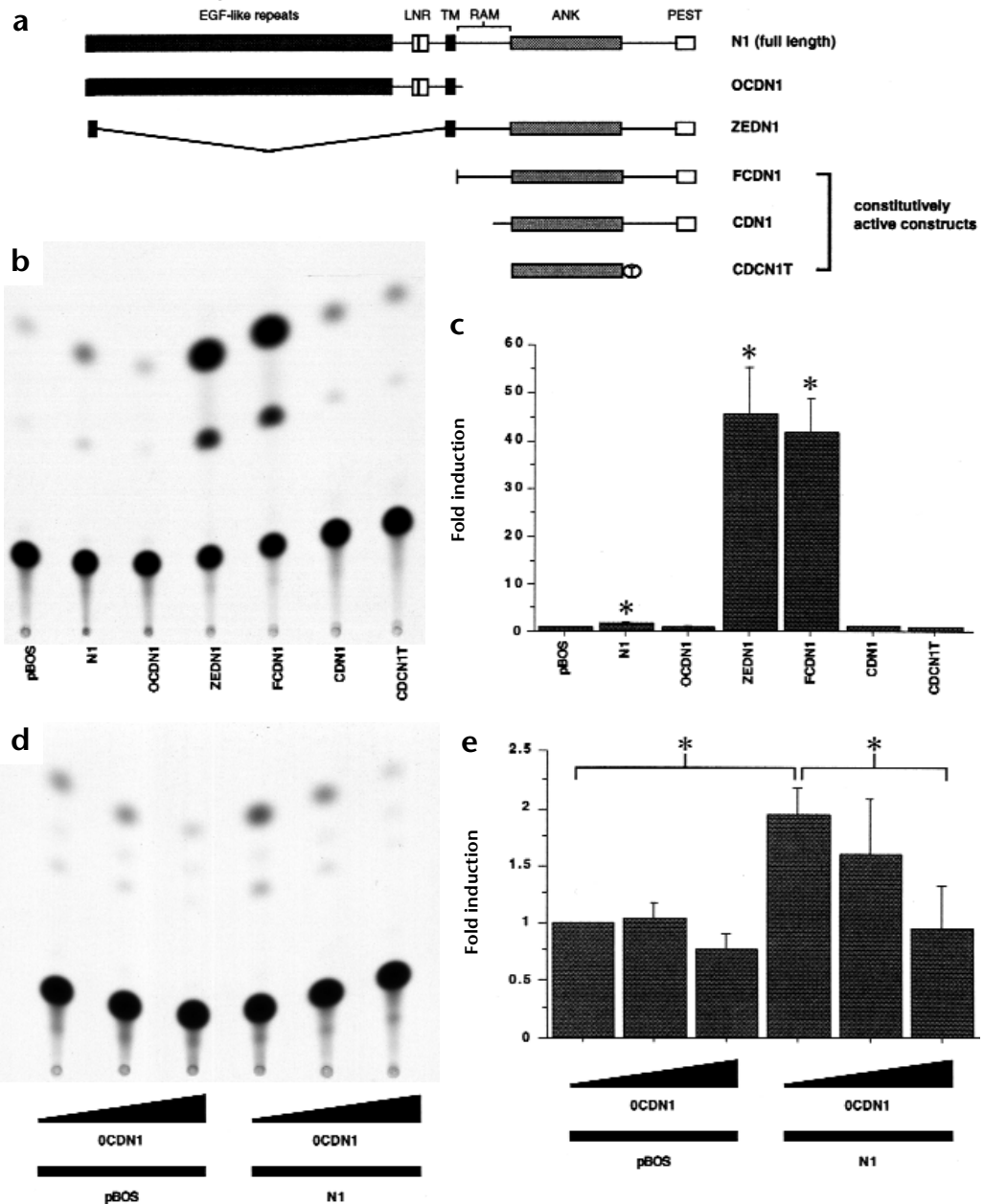
Regulation of transcription by nuclear Notch I

In non-neuronal cell lines, the cytoplasmic domain of Notch I can translocate to the nucleus and induce CBF1-mediated transactivation. To determine whether this signaling pathway is functional

in postmitotic cortical neurons, we transfected a series of Notch I-deletion constructs into cortical neurons (Fig. 6a) together with a reporter driven by CBF1 binding sites¹⁴ (CBF1-CAT). By immunofluorescence, transfected full-length Notch I was excluded from the nucleus and was present mainly in intracellular organelles (and perhaps at the cell surface, to some extent). Transfected 0CDN1 was present mainly on the cell surface and in the cytoplasm. In contrast, CDN1 and CDCN1T were present both in the cytoplasm and in the nucleus, and ZEDN1 and FCDN1 were present mainly in the nucleus (Fig. 3 and data not shown).

In neurons transfected with reporter together with the parent vector (pBos), there was a low basal level of reporter activity (Fig. 6b and c). Transfection with the various Notch I constructs led to markedly different levels of transactivation (Fig. 6b and c). Whereas transfection with full-length Notch I led to a weak but significant activation of the reporter, trans-

Fig. 6. Notch1 can induce transactivation via CBF1 binding sites in cortical neurons. (a) Full-length and truncated Notch1 cDNA constructs used in transfection experiments. The location of a HA-tag in the CDCN1T construct is indicated by a 'T'. (b, c) Relative CAT activity in E18 cortical cultures transfected with indicated Notch1 constructs together with CBF1-CAT. (d, e) Relative CAT activity in E18 cortical cultures transfected with vector (pBos) or full-length Notch1 (N1) together with CBF1-CAT and increasing amounts of 0CDN1. 0CDN1 attenuates Notch1 activation of CBF1-CAT.



fection with ZEDN1 and FCDN1 led to robust activation of the reporter (Fig. 6b and c). Transactivation was only weakly activated by CDN1, a construct lacking the RAM domain, a region between the transmembrane domain and the ankyrin repeats. Thus the cytoplasmic domain of Notch1 can strongly activate CBF1-mediated transcription in cortical neurons, and Notch1 activation of CBF1-mediated transcription requires the RAM domain.

A form of Notch lacking the intracellular domain (0CDN1) can act as a dominant-negative receptor³⁹. When expressed in cortical neurons, 0CDN1 did not activate the CBF1-CAT reporter and inhibited activation of CBF1-CAT by full-length Notch1, indicating that it could inhibit Notch1 signaling to CBF1 (Fig. 6d and e).

We next asked if Notch1 signaling could regulate neuron-specific gene expression. The neuron-specific enolase (NSE) gene is expressed exclusively in terminally differentiated neurons and neuroendocrine cells⁴⁰, and an 1800-bp fragment of the promoter can drive neuron-specific expression of a LacZ reporter in transgenic mice⁴¹. A CAT-reporter construct driven by this 1800-bp fragment (NSE-CAT) transfected into cortical neurons gave a low level of reporter activity (Fig. 7). Cotransfection of full-length Notch1 led to increased reporter expression, indicating that Notch1 signaling can lead to activation of the NSE promoter

(Fig. 7). In this experiment, Notch1 was likely activated by ligands expressed by the cultured cortical neurons. In contrast to the CBF1-CAT reporter, which is strongly activated by ZEDN1 and FCDN1, the NSE-CAT reporter was not activated by truncated Notch1 constructs. The failure of truncated Notch1 constructs to activate NSE-CAT suggests that Notch1 signaling to NSE-CAT may be CBF1 independent. This is not entirely unexpected, as alternate Notch1 signaling pathways have been described both in *Drosophila* development and in myogenesis⁴².

Notch1 signaling regulates dendritic morphology

The Notch1 localization and signaling experiments suggested that Notch1 might regulate aspects of neuronal differentiation. We thus examined the effects of inhibiting Notch1 signaling on one of the clearest manifestations of neuronal differentiation, the development of dendrites. The dendritic morphology of cul-

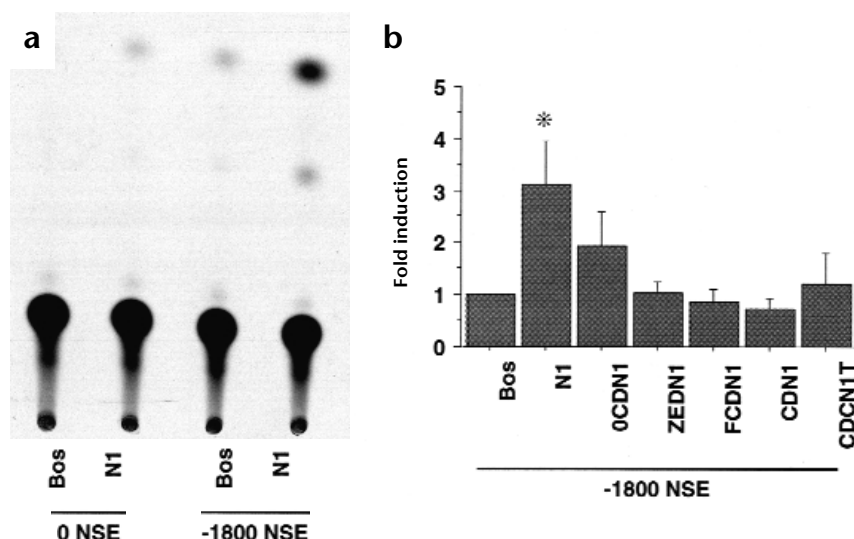


Fig. 7. Notch1 induces gene expression via the neuron-specific enolase (NSE) promoter. (a) Relative CAT activity in E18 cortical cultures transfected with vector (Bos) or full-length Notch1 (N1) together with either the parent vector (0NSE) or an NSE-CAT construct containing 1800 bp of the NSE promoter (-1800 NSE). (b) Relative CAT activity in E18 cortical cultures transfected with indicated Notch constructs and the -1800 NSE reporter.

tured neurons can be clearly visualized by transfecting them with β -galactosidase followed by immunocytochemistry for β -galactosidase⁴⁹. We transfected cortical cultures with β -galactosidase and Bos (parent vector), 0CDN1 (dominant negative receptor) or ZEDN1 (constitutively active receptor) at two days *in vitro* (DIV) and examined the morphology of transfected neurons three days later.

Bos-transfected and ZEDN1-transfected neurons had complex dendritic morphologies characterized by several primary processes and highly branched dendritic trees (Fig. 8a and c). In contrast, 0CDN1-transfected neurons had noticeably simpler dendritic morphologies (Fig. 8b). Processes were confirmed to be dendrites by MAP2 immunofluorescence (data not shown). Quantitative analysis indicated that Notch1 signaling had distinct effects on process number, dendritic length and dendritic branching. Inhibiting Notch1 signaling decreased the number of processes and the number of dendritic branch points per neuron (Fig. 8d and e). In contrast, increasing Notch1 signaling reduced average dendritic length (Fig. 8f). Although constitutively active Notch1 did not affect the total number of branches, it increased the dendritic branching index (number of branches per unit dendritic length; Fig. 8g).

As an alternative method to evaluate the role of Notch1 signaling in dendritic development, we inhibited Notch1 by treating cortical cultures with previously characterized Notch1-antisense oligonucleotides²⁴ (Methods). Antisense treatment decreased Notch1 protein levels approximately 30% compared to sense oligonucleotide-treated controls (data not shown). Cultures were transfected with β -galactosidase one day before oligonucleotide treatment, and transfected neurons were visualized by β -galactosidase immunocytochemistry after two days of oligonucleotide treatment. Whereas neurons treated with sense (control) oligonucleotides had complex dendritic morphologies, neurons treated with either of the two Notch1 antisense oligonucleotides had markedly simpler morphologies (Fig. 9a and b). Like dominant-negative Notch1 transfections, antisense treatments decreased

both process number (Fig. 9c) and dendritic branching (Fig. 9d and f). Notch1 antisense treatments also increased average dendritic length (Fig. 9e). To demonstrate the specificity of the antisense treatments, we rescued the effects by overexpressing an activated Notch1 construct (FCDN1). The effects of antisense treatment on various dendritic parameters were partially or completely reversed by overexpressing FCDN1 (Fig. 9g–j). These observations support the results of the transfection experiments, and suggest that Notch1 signaling exerts a positive effect on dendritic branching and a negative effect on dendritic growth.

DISCUSSION

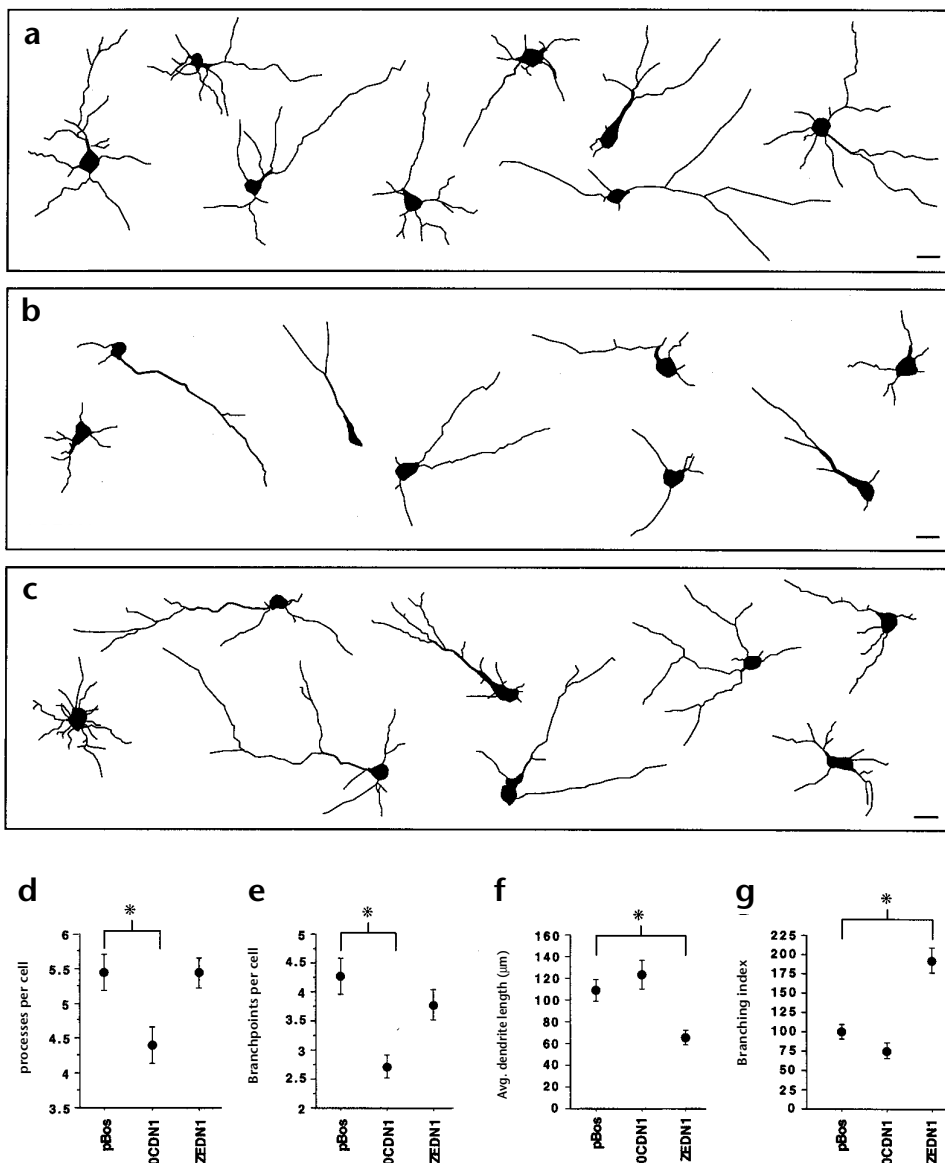
Our experiments demonstrate an important regulatory role for Notch1 signaling in the differentiation of newly postmitotic neurons. We found that neuronal differentiation was associated with a change in the subcellular localization of Notch1 from extranuclear compartments to the nucleus. In cortical neurons, the intracellular domain of Notch1 translocated to the nucleus and could induce transactivation of a CBF1-driven reporter construct. Attenuation of Notch1 signaling, either by expression of a dominant-negative construct or by treatment with Notch1 antisense oligonucleotides, reduced both the number of primary processes and dendritic branching. Increasing Notch1 signaling decreased average dendritic length. These observations indicate that, in addition to previously identified roles in cell-fate specification, mammalian Notch1 continues to function in postmitotic neurons, and is important in the specification of dendritic morphology.

Although Notch1 is largely excluded from the nucleus in undifferentiated cells, it is present in the nucleus of postmitotic neurons both *in vivo* and *in vitro*. A possible regulatory mechanism in this process is receptor activation at the onset of cortical differentiation. As the cytoplasmic domain of Notch1 seems to translocate to the nucleus once neurons reach the cortical plate, it is possible that a Notch1 ligand present in the cortical plate induces intracellular cleavage of the receptor. Although we have not yet identified the relevant Notch ligand, Delta is a likely candidate, as it is expressed in the cortical plate (L.R. and A.G., unpublished observation).

Although there is evidence that Notch signaling might involve nuclear translocation of the cytoplasmic domain, there has been controversy about such a mechanism because of a lack of evidence for endogenous Notch in the nucleus *in vivo*. Our immunofluorescence and cell-fractionation experiments clearly indicated the presence of the cytoplasmic domain of endogenous Notch1 in the nucleus of postmitotic neurons. We detected two forms of nuclear Notch1, p120 and p100, which correspond to differentially phosphorylated forms of the cleaved intracellular domain. A major nuclear target of Notch1 is CBF1, and it will be interesting to determine if phosphorylation affects Notch1–CBF1 interactions or Notch1-induced transcription in neurons.

Our experiments suggest that Notch1 signaling influences dendritic development in cortical neurons. We found that Notch1 signaling exerts a positive effect on dendritic branch-

Fig. 8. Effects of altering Notch1 signaling on dendritic morphology. (**a–c**) Video camera-lucida drawings of the dendritic tree of cortical neurons transfected with β -galactosidase together with pBos (parent vector; **a**), 0CDN1 (**b**) or ZEDN1 (**c**) at 2 DIV, and processed for β -galactosidase immunoreactivity at 5 DIV. Scale bar is 20 μ m. (**d**) Number of primary processes per cell in pBos-, 0CDN1- and ZEDN1-transfected neurons. (**e**) Number of dendritic branch points per cell in pBos-, 0CDN1- and ZEDN1-transfected neurons. (**f**) Average dendrite length (total dendrite length/number of primary dendrites) per cell in pBos-, 0CDN1- and ZEDN1-transfected neurons. (**g**) Branching index (see Methods) in pBos-, 0CDN1-, and ZEDN1-transfected neurons.



ing and a negative effect on dendritic growth. The effects we report are consistent with findings of two other groups^{43–45}. Activation of Notch1 is associated with a decrease in total neurite length⁴⁵, similar to effects we see on dendritic length. We also saw a positive effect of Notch1 signaling on dendritic branching. Although dendritic growth and branching might be mechanistically linked, the branching effects were probably not secondary to an effect on dendritic growth, as inhibition of Notch1 had opposite effects on dendritic length and dendritic branching, and certain perturbations (such as 0CDN1 expression) affected the number of dendritic branches without affecting dendritic length. We should also note that our results do not rule out the possibility that Notch1 signaling favored differentiation of neurons into a subtype characterized by reduced dendritic length and greater dendritic branching. However, such a role of Notch1 in postmitotic neurons seems unlikely, as most cell-fate decisions in the cortex are made before the final cell division.

Despite the dominant view that Notch signaling functions to inhibit differentiation and restrict cell fates, observations in *Drosophila* development suggest a role for Notch in regulating developmental decisions subsequent to specification of cell fate. For example, Notch is present on the growth cones of developing axons, and mutations in Notch affect axonal growth^{46–47}. Similarly, Kuzbanian (a protein implicated in Notch and Delta processing) is required for axon extension in *Drosophila*⁴⁸. These observations, together with our observa-

tions supporting a role for Notch1 in dendritic development, suggest that Notch-mediated interactions may be critical in regulating the patterning of connections well after cell fate has been decided.

METHODS

Immunohistochemistry and immunofluorescence. Animals were anesthetized and perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. The brains were removed, embedded in gelatin, cryoprotected in 4% paraformaldehyde/30% sucrose in PBS and sectioned at 20–40 μ m. Cultures were fixed with 4% paraformaldehyde/4% sucrose in PBS and washed with PBS. Following fixation, tissue sections and cultures were blocked with 3% bovine serum albumin, 3% goat serum and 0.3% Triton X-100 in PBS and incubated in the primary antibody (diluted in blocking solution) overnight. For BrdU immunostaining, cultures were postfixed in 70% ethanol, additionally permeabilized with 0.4% Triton X-100 and 2 N HCl and neutralized with 0.1 M Na₂B₂O₇ before incubation in blocking solution. After incubation in primary antibody, tissue sections and cultures were incubated in peroxidase-conjugated (ABC Elite, Vector Labs, Burlingame, California) or fluorescent (Cy-2 and Cy-3, Jackson Immunoresearch, West Grove, Pennsylvania; Pacific blue and Oregon green, Molecular

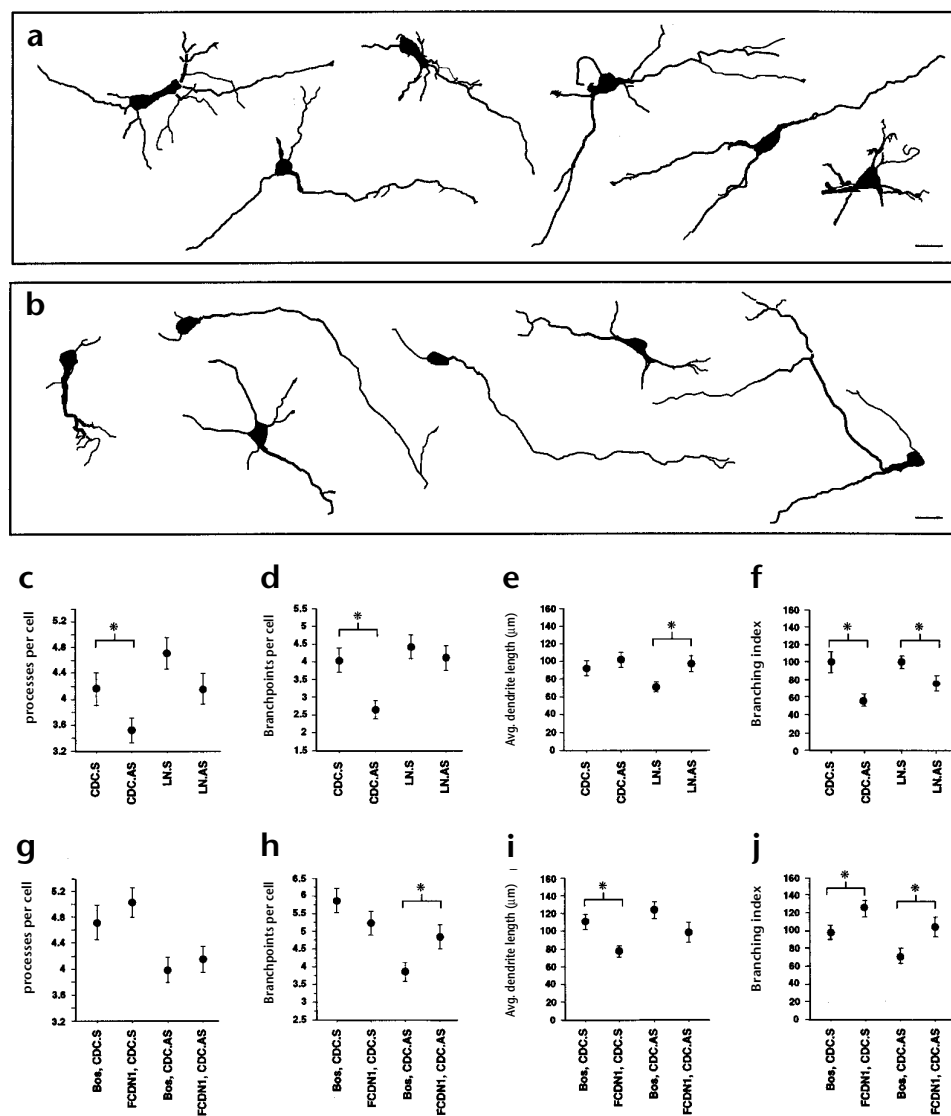


Fig. 9. Effects of Notch1 antisense oligonucleotide treatment on dendritic morphology. (**a, b**) Video camera-lucida drawings of cortical neurons transfected with β -galactosidase at 2 DIV, treated with either 2 μ M CDC sense (**a**) or 2 μ M CDC antisense (**b**) oligonucleotides at 3 DIV, and processed for β -galactosidase immunoreactivity at 5 DIV. Scale bar, 20 μ m. (**c–f**) Number of primary processes (**c**), number of dendritic branch points (**d**), average dendritic length (**e**) and branching index (**f**) in cortical neurons treated with 2 μ M Notch1 sense (CDC.S, LN.S) or antisense (CDC.AS, LN.AS) oligonucleotides. (**g–j**) Effects of FCDN1 expression on number of primary processes (**g**), number of dendritic branch points (**h**), average dendrite length (**i**) and branching index (**j**) in Notch1 sense-(CDC.S) and antisense-(CDC.AS) treated neurons.

Probes, Eugene, Oregon) secondary antibodies. Samples processed for immunocytochemistry without primary antibody incubation did not show detectable staining (data not shown).

The Notch1 antibodies used in this study were the affinity-purified rabbit polyclonal antiserum 93-4, generated against the cytoplasmic domain of Notch1, and affinity-purified rabbit polyclonal antiserum 5261, generated against the extracellular domain of Notch1 (ref. 42). Notch1-glutathione S-transferase (GST) used for affinity purification was engineered and purified using Pharmacia protocols as described⁴². The 93-4 affinity-purified antiserum was used in all of the Notch1 immunofluorescence and immunocytochemistry experiments. In control experiments, 93-4 affinity-purified antiserum was preadsorbed with an excess (200 μ g per ml) of Notch1 fusion protein at 4°C before incubation with cells. We also used the following additional antibodies: mouse anti- β -galactosidase (Promega, Madison, Wisconsin); rabbit anti- β -galactosidase (5′–3′, Boulder, Colorado); mouse anti-Nestin (Pharminogen, San Diego, California); mouse anti-TuJ1 (Sigma); mouse anti-MAP2 (Sigma); mouse anti-BrdU (Becton Dickinson); mouse anti-HA (clone 12CA5, Boehringer Mannheim) and mouse anti-PCNA (Boehringer Mannheim). Confocal images of tissue sections and cultured cells were obtained on a Zeiss LSM410 or Zeiss LSM510 microscope equipped with argon (red), HeNe (green) and UV (blue) lasers.

Primary cell cultures. E18 cortical cells from Long-Evans rats were cultured as described⁴⁹. Dissociated cortical cells were plated onto

poly-D-lysine- and laminin-coated dishes at 1×10^6 cells per well (12-well dishes), at 1.5×10^6 cells per well (6-well and 35-mm dishes), or at 3×10^6 cells per 60-mm dish in glutamine-free Basal Medium Eagle (Sigma), 1 mM glutamine, 1% N2, and fetal bovine serum (Gibco) and maintained at 37°C in 95%/5% O₂/CO₂. The double-labeling experiments shown in Fig. 4 were performed in 35-mm glass bottom dishes, and bFGF (10 ng per ml) was added at the time of plating. For bromodeoxyuridine (BrdU) labeling, 10 μ M BrdU was added to the cultures 6 h or 24 h before fixation.

Constructs and transfections. Wild-type and truncated Notch1 cDNA sequences were engineered in the mammalian expression vector pEF1 α -BOS or pcDNA3 to encode the following amino acids: N1, 1–2531; 0CDN1, 1–1759; ZEDN1, 1–24, 1712–2531; FCDN1, 1741–2531; CDN1, 1848–2531; CDCN1T, 1873–2078. All constructs were confirmed by DNA sequencing⁴². Additional ZEDN1, FCDN1 and CDN1 constructs were engineered with a 3′ HA tag in pEF1 α -BOS. The CBF1-CAT reporters, with four copies of the wild-type or mutant CBF1 binding elements cloned upstream of a chloramphenicol acetyltransferase (CAT) gene in the mammalian expression vector pSG5, were a gift from S. D. Hayward (Johns Hopkins). The previously described NSE-CAT reporter⁴⁰ contains 1800 bp of NSE promoter sequences upstream of a CAT reporter. The control reporter (0-NSE-CAT) was generated by removing the NSE promoter sequences between the *SacI* and *HindIII* sites of NSE-CAT and religating the plasmid.

Cells were transfected by a modified calcium phosphate procedure as described⁴⁹. This procedure exclusively transfects postmitotic neurons. The expression of various Notch1 constructs was confirmed by western blots of cell lysates from cultures of transfected cortical cells and 293T cells, and also by immunofluorescence of transfected cortical neurons. To demonstrate attenuation of full-length Notch1 signaling by 0CDN1, we transfected cultures with CBF1-CAT, either full-length Notch1 or

pBos and either 0CDN1 or pBos at molar ratios of 1:1:1 and 1:1:3. The final amount of DNA transfected was equalized with pBluescript for each condition. For morphological analysis, E18 cortical cultures were transfected at 2 DIV with RSV- β -galactosidase together with the parent expression vector (control), the 0CDN1 expression plasmid or the ZEDN1 expression plasmid in a molar ratio of 1:2 (β gal construct) and were fixed and processed for β -galactosidase immunocytochemistry at 5 DIV before morphology of transfected neurons was analyzed (see Analysis below).

Western blots and cell fractionation. We used 10 μ g of Notch1, Notch2, Notch3-HA, Notch4-HA or parent vector (pBos) to transfect 293 cells using a calcium phosphate procedure. Cells collected from the same transfection were lysed in RIPA buffer, pooled and then split for immunoprecipitation. Lysates were immunoprecipitated with 1:100 of rabbit polyclonal antibodies to either Notch1 (93-4) or Notch2 (93-3) or with monoclonal antibody to HA (12CA5) and captured with Protein-A agarose. Immunoprecipitates were analyzed by western blot using 93-4 (1:5000) to detect Notch1, 93-3 (1:3000) to detect Notch2 and 12CA5 (1:1000) monoclonal to detect the HA tag present on Notch3 and Notch4. Antibody-protein interactions were detected by ECL.

For tissue westerns, cortices were dissected from embryonic (E13 and E18) and postnatal rats (P0, P7, P14 and adult). Tissues were homogenized with a dounce homogenizer in lysis buffer (137 mM NaCl, 20 mM Tris, 1% NP40, 10% glycerol, 1 mM PMSE, 10 μ g per ml aprotinin, 1 μ g per ml pepstatin A and 1 μ g per ml leupeptin at pH 8.0). To disrupt nuclei, homogenates were sonicated with 2 bursts (15 s), homogenized with pestle B and mixed for 1 h at 4°C. E18 cultured cells were rinsed in TBS, harvested and homogenized as for whole tissue. BCA Protein Assay (Pierce, Rockford, Illinois) was used to determine protein concentration, and 10 μ g of each homogenate was separated by PAGE. Proteins were transferred to nitrocellulose using a Genie Electrobloater system (Idea Scientific, Minneapolis, Minnesota). Blots were blocked with 4% BSA in TBST and incubated with α -Notch.IC (93-4) diluted 1:10,000 in blocking solution overnight at 4°C. In control experiments, α -Notch.IC was incubated at 1:15,000 with an excess of Notch1 fusion protein (20 μ g per ml) overnight at 4°C before incubation with blots of FCDN1.HA immunoprecipitates. Blots were incubated with peroxidase-conjugated anti-rabbit secondary antibody (Amersham) diluted 1:20,000 in blocking solution and visualized using a chemiluminescent detection system (SuperSignal Substrate, Pierce).

Cell fractionation and isolation of nuclear proteins was carried out as described⁵⁰. Cortices from P3 and adult rats were minced and homogenized by two-component dounce homogenization, and nuclei were separated on a sucrose density gradient. Isolated nuclei were resuspended in lysis buffer and disrupted by sonication. Protein concentrations were determined by BCA protein assay, and 10 μ g of each fraction—homogenate, cytoplasmic and nuclear—were separated by PAGE and transferred to nitrocellulose. Notch1 was detected using α -Notch.IC as described above. Parallel blots were blocked with 3% nonfat dry milk in PBS and incubated overnight with an antibody to the β -1 subunit of the Na⁺/K⁺ ATPase (Upstate Biotechnology) at 1:1000 in blocking solution at 4°C. Blots were incubated with peroxidase-conjugated anti-rabbit secondary antibody (Amersham) diluted 1:10,000 in blocking solution and were visualized by chemiluminescence, as above.

Immunoprecipitation, metabolic labeling and cell-surface biotinylation. E18 cortical cultures in 60-mm dishes were transfected with 6 μ g per dish of ZEDN1.HA, FCDN1.HA or CDN1.HA. Three days after transfection, cells were lysed in lysis buffer. Lysed cells were homogenized with pestle B of a dounce homogenizer. Lysis supernatants were separated from fragmented cells by centrifugation, incubated with anti-HA antibodies and Gamma Bind G Sepharose (Pharmacia), washed and separated by PAGE. For the analysis of protein phosphorylation, cells from the same transfection condition were lysed as above, pooled and then split for immunoprecipitation. After binding to Gamma Bind G Sepharose, immunoprecipitates were incubated for 1 hour at 30°C with 50 U λ Phosphatase (NEB) or 10 mM sodium orthovanadate (Na₃VO₄) in 50 mM TrisHCl, 5 mM DTT, 2 mM MnCl₂, 100 μ g per ml BSA and protease inhibitors as above at pH 7.8 and washed and separated by PAGE. After separation by PAGE, proteins were transferred to nitrocellulose and detected as above. Metabolic labeling was performed by incubating con-

trol and FCDN1.HA-transfected cultures with 200 μ Ci per 60-mm dish of [³²P]orthophosphate (H₃PO₄; ICN) in phosphate-free DMEM (Gibco) for 4 h at 37°C. Cells were lysed, immunoprecipitated with anti-HA and transferred to nitrocellulose as above. The blot was first exposed to film to detect radioactivity and then immunoprecipitated. FCDN1.HA was detected with α -Notch.IC as described above.

For the biotinylation experiments, E18 cortical cultures in 60-mm dishes were washed with dextrose (1 mg per ml) in PBS, then incubated at 4°C in 2 mM EZ link Sulfo-NHS-LC-Biotin (Pierce) in dextrose (1 mg per ml) in PBS for 30 min. Cells were rinsed multiple times with dextrose (1 mg per ml) in PBS and lysed as above for immunoprecipitation. Lysis supernatants were incubated with immobilized Streptavidin (Pierce) overnight at 4°C, washed and separated by PAGE. Proteins were transferred to nitrocellulose and incubated with α -Notch.IC (93-4) or an extracellular-domain antibody (5261) and detected as above.

CAT assays. Cells were harvested 1 (NSE-CAT) or 2 (CBF1-CAT) days after transfection in isotonic TNE (10 mM Tris, 150 mM NaCl and 1 mM EDTA at pH 7.8). Cells were spun down gently and subjected to three cycles of freeze-thaw lysis. Lysis supernatants were incubated with 0.5 μ Ci [¹⁴C]-labeled chloramphenicol (Amersham) and 0.8 mmol acetyl CoA (Boehringer Mannheim) at pH 7.8 and at 37°C for 1 h. Reaction mixtures were extracted with ethyl acetate, speed-vacuumed, resuspended in chloroform, spotted onto thin-layer chromatography (TLC) plates (J.T. Baker) and separated by ascending chromatography for 2 h (95% chloroform, 5% methanol). To normalize for transfection efficiency, equal amounts of a RSV- β -galactosidase plasmid were cotransfected in all reporter experiments, and β -galactosidase activity was used for normalization. Data from any single histogram were quantified from experiments on cells that were simultaneously cultured, transfected and assayed to minimize variability due to subtle differences in experimental procedure. Fold induction was calculated relative to control transfected cultures. Shown are representative examples of experiments performed three times. For measurements of relative CAT activity, levels of [¹⁴C] emissions on TLC plates were quantified by phosphorimager scans. Statistically significant differences (Student's *t*-test, *p* < 0.05) in reporter activity are indicated by an asterisk.

Antisense oligonucleotides. Oligonucleotides were designed against two distinct regions of rat Notch1 as reported²⁴, a portion of the 5' intracellular cdc10/ankyrin repeat region (CDC) and the extracellular lin12/Notch repeat region (LN). The CDC antisense sequence, 5'-CCTC-CGCTGCAGGAGGCAATCAT-3', and the LN antisense sequence, 5'-CCAGCACTGCAGGGACTGAGTGC-3', were used. For each antisense oligonucleotide, corresponding sense oligonucleotides were synthesized and used in parallel in each experiment. All oligonucleotides were 23 bp in length, with phosphothioate linkages added between all bases. Oligonucleotides were synthesized at the Johns Hopkins School of Hygiene and Public Health DNA Synthesis Core Facility. Optical density (OD₂₆₀) readings were taken to determine concentration. For antisense treatment experiments, E18 cortical neurons were cultured as described on 12-well tissue culture plates. Cells were transfected with RSV- β -galactosidase at 2 DIV. The day after transfection, sense and antisense oligonucleotides were added to a final concentration of 2 μ M and maintained for an additional two days before fixation. The morphology of the transfected neurons was revealed by β -galactosidase immunocytochemistry and analyzed as described below.

Analysis. Transfection experiments were carried out in duplicate wells, and all of the experiments described here were repeated multiple times in several independent experiments with comparable results. To analyze the effects of antisense treatment, ZEDN1 and 0CDN1 expression on dendritic morphology, images of β -galactosidase-transfected neurons were captured using a digital CCD camera (Dage, MTI, Michigan City, Indiana) attached to the side port of a Nikon Eclipse TE300 inverted microscope driven by IP Lab Spectrum 3.1.1 image-acquisition software (Scanalytics, Fairfax, Virginia). Captured cells were then traced using ClarisDraw software. To assess the effects of ZEDN1 and 0CDN1 expression on dendritic morphology, the dendritic trees of at least 50 Bos-(control), ZEDN1- and 0CDN1-transfected neurons were reconstructed and scored for number

of primary processes, dendritic branch points, average dendritic length and branching index (number of branch points divided by average dendritic length, expressed as percent of control). For quantitative analysis of the effects of oligonucleotide treatments on dendritic morphology, the dendritic trees of at least 100 transfected neurons from each treatment condition per experiment were reconstructed and scored for various dendritic parameters. Independent blind analysis of dendritic morphology under various experimental conditions showed similar results. Data are shown as mean \pm standard error. Statistically significant differences (Student's *t*-test; $p < 0.05$) are indicated by an asterisk. To analyze the relative amounts of intracellular domain Notch1 in the nucleus and cytoplasm of double-labeled cells (Fig. 4m), we captured images of Notch1-immunostained neurons as above. The intensity of Notch1 immunofluorescence was then measured in identical volumes of the nucleus and cytoplasm from the same cell using IP Lab Spectrum 3.1.1 image software.

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