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β-Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system

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Abstract

β-Catenin is an essential component of the canonical Wnt signaling system that controls decisive steps in development. We employed here two conditional β-catenin mutant alleles to alter β-catenin signaling in the central nervous system of mice: one allele to ablate β-catenin and the second allele to express a constitutively active β-catenin. The tissue mass of the spinal cord and brain is reduced after ablation of β-catenin, and the neuronal precursor population is not maintained. In contrast, the spinal cord and brain of mice that express activated β-catenin is much enlarged in mass, and the neuronal precursor population is increased in size. β-Catenin signals are thus essential for the maintenance of proliferation of neuronal progenitors, controlling the size of the progenitor pool, and impinging on the decision of neuronal progenitors to proliferate or to differentiate.

Keywords: Wnt signaling; β-Catenin; Neural stem cells; Neuronal progenitors; Progenitor pool; Proliferation versus differentiation

Introduction

Precursor cells that generate the various differentiated cell types of the central nervous system are generally located in defined areas, which frequently correspond to the ventricular zones of the developing nervous system. During much of development, neural precursors proliferate to renew the progenitor pool, and in parallel, give rise to postmitotic cells that move out of the progenitor areas (Temple, 2001). The balance between the generation of precursor and postmitotic cells shifts during development, but has to be tightly controlled to guarantee the formation of the appropriate neuronal cell numbers and the size of the tissue domains.

β-Catenin is a central and essential component of the canonical Wnt signaling pathway and acts downstream of the Frz and LRP receptors (Huelsken and Birchmeier, 2001; Moon et al., 2002; Wodarz and Nusse, 1998). In the presence of Wnt signals, β-catenin is stabilized and translocates to the nucleus, where it interacts with transcription factors of the LEF/TCF family to induce changes in gene expression (Behrens et al., 1996; Willert et al., 2002). In the absence of Wnt signals, the N-terminus of β-catenin is phosphorylated by casein kinase I on serine residue 45; GSK3β then phosphorylates the serine/threonine residues at positions 41, 37, and 33 (Amit et al., 2002; Liu et al., 2002; Schwarz-Romond et al., 2002). Fully phosphorylated β-catenin is subsequently ubiquitinated and degraded by proteosomes (Aberle et al., 1997). Mutations that affect these N-terminal phosphorylation sites have been observed in various tumors and lead to a stabilization of β-catenin and enhanced signaling activity (Bienz and Clevers, 2000; Moon et al., 2002; Polakis, 2000). N-terminal sequences containing these phosphorylation sites are encoded by exon 3 of the β-catenin gene. Using Cre/loxP technology, exon 3 was removed in the mouse intestine, resulting in the expression of activated β-catenin and the formation of tumors

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(Harada et al., 1999). However, only canonical Wnt signals are transmitted by the β-catenin-dependent pathway. Other, noncanonical Wnt signals are mediated by Jun N-terminal kinase (planar cell polarity pathway) or by Ca^{2+} (Boutros et al., 2000; Kuhl et al., 2000; Niehrs, 2001; Tada et al., 2002).

During development, Wnts and their receptors are expressed in complex spatiotemporal patterns throughout the nervous system, which are often overlapping (Parr et al., 1993). Genetic analysis in mice has indicated that signals provided by these factors can control growth and/or patterning in the central nervous system (Brault et al., 2001; Dickinson et al., 1994; Galceran et al., 2000; Ikeya et al., 1997; Lee et al., 2000; McMahon and Bradley, 1990; McMahon et al., 1992; Muroyama et al., 2002; Pinson et al., 2000; Thomas and Capecchi, 1990). For instance, mutation of Wnt1 in mice leads to a loss of cells at the midhindbrain boundary (McMahon et al., 1992). Similarly, conditional mutation of β-catenin in the Wnt1 expression domain by the use of the cre-loxP system leads to an elimination of the cells at the midhindbrain boundary (Brault et al., 2001). In contrast, overexpression of Wnt1 or ectopic expression of activated β-catenin in the developing chick spinal cord or mouse forebrain, respectively, results in an enlarged mass of the neural tissue (Dickinson et al., 1994; Chenn and Walsh, 2002). However, the complexity of the expression patterns of Wnts and their receptors has precluded an assessment of a general function of Wnt signals in development of the nervous system. β-Catenin represents a central and nonredundant signaling component in the canonical Wnt pathway, and its activation or ablation allows therefore a general assessment of canonical Wnt signals. We used here two conditional mutant β-catenin alleles in mice: one allele to express a constitutively active β-catenin and the second allele to ablate β-catenin in the central nervous system (Harada et al., 1999; Huelsken et al., 2001). In mice that express a stabilized β-catenin, the central nervous system is much enlarged, whereas after ablation of β-catenin, the tissue mass is reduced. We demonstrate that β-catenin signals control proliferation of progenitors, and that they impinge on the decision of neuronal progenitors to proliferate or differentiate. In the presence of β-catenin signals, progenitors exit the cell cycle less frequently, and instead, continue to proliferate. It is noteworthy that these changes are observed in various regions of the nervous system, indicating that β-catenin signals can, in general, control the size of the precursor pool.

Materials and methods

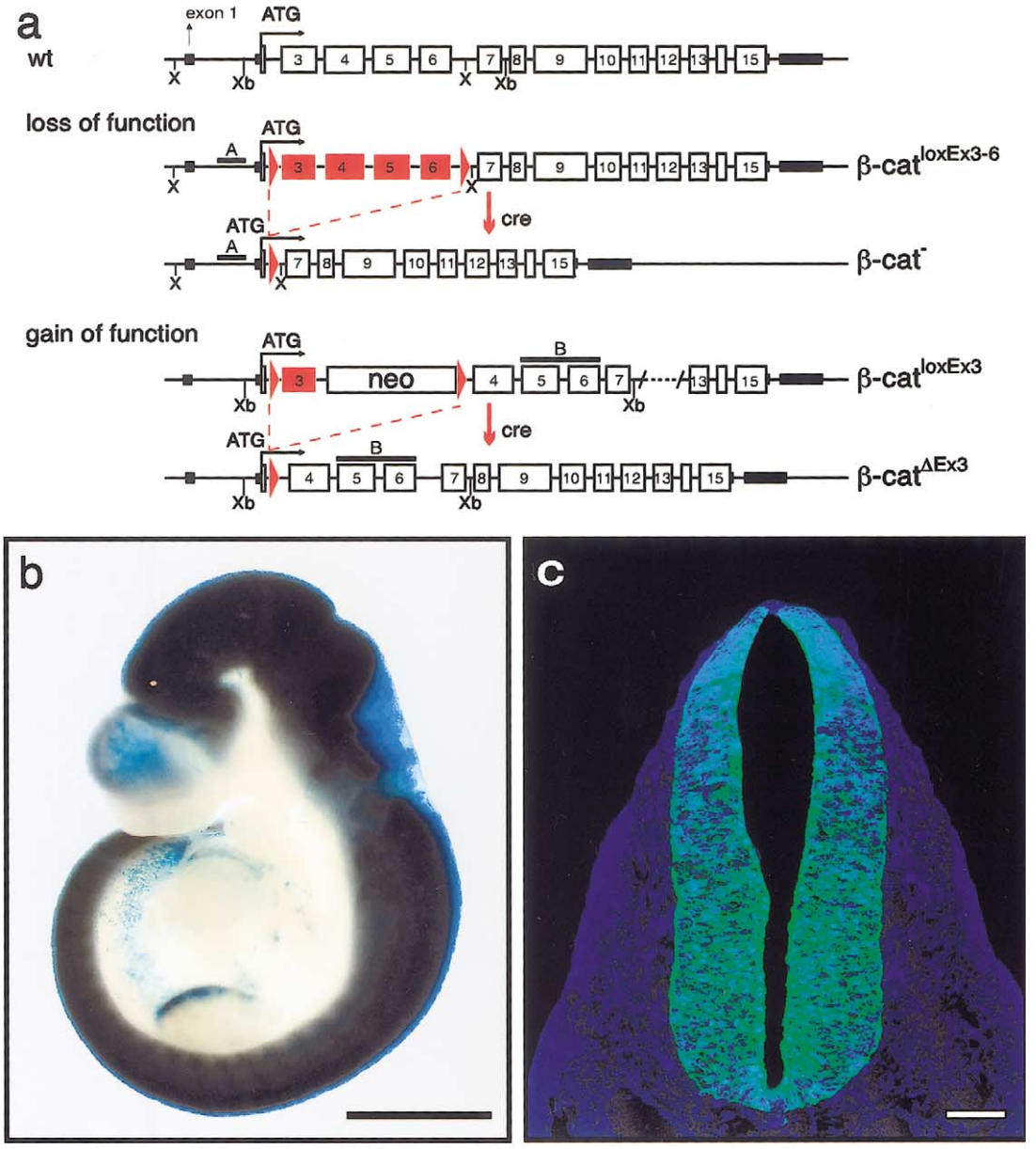
Mouse strains and X-gal staining

Mouse strains harboring the floxed alleles of β-catenin (β-cat$^{loxEx3-6}$; β-cat^{loxEx3}) as well as the transgenic mouse line that expresses cre-recombinase under the control of the Brn4 promoter (*bcre-32* line) have been described (Ahn et al., 2001; Harada et al., 1999; Huelsken et al., 2001). Genotyping was performed by PCR on DNA prepared from the yolk sac. To verify the expression pattern of cre-recombinase, a lacZ reporter line was used (Akagi et al., 1997) and stained with X-gal. Mice with a conditional loss- (Brn4cre; β-cat$^{floxEx3-6/floxEx3-6}$) or gain- (Brn4cre; β-cat$^{+/floxEx3}$) of-function mutation in the nervous system were generated by crossings. To detect β-catenin protein in the tissue of these mutant mice, spinal cords from mouse embryos at day 11.5 of embryonic development were dissected, and the tissue was homogenized. Whole cell lysate was subjected to SDS–PAGE and Western blotting using anti-β-catenin or anti-p120 antibody (Transduction Laboratories, Lexington, KY, USA).

Histology and immunohistology

Embryos were dissected, staged according to somite number, and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight. For frozen sections, fixed embryos were rinsed with PBS, soaked in cold 30% sucrose, and embedded in OCT compound. For paraffin sections, embryos were dehydrated in ethanol, cleared in toluol, and embedded in paraffin. Immunohistochemistry was performed on paraffin- or cryo-sections. Antibodies used were directed against β-galactosidase (Abcam, Cambridge, UK),

Fig. 1. Introduction of conditional loss- and gain-of-function mutations into the mouse β-catenin locus (a) Schematic representation of the wildtype β-catenin locus and of the alleles used for the introduction of loss- and gain-of-function mutations in the nervous system. Coding exons are represented by numbered boxes and noncoding exons by black bars. The loxP sequences are represented as red arrowheads; exons removed after cre-mediated recombination are highlighted in red. Tissue-specific recombination in the β-cat$^{loxEx3-6}$ locus results in deletion of exons 3–6, and no protein is produced from this recombined allele (β-cat$^{-/-}$; cf. Huelsken et al., 2001). Tissue-specific recombination of the β-cat^{loxEx3} locus results in the deletion of exon 3 (β-cat$^{\Delta Ex3}$ allele); from this allele, a shortened form of β-catenin is produced that provides Wnt-independent signals and corresponds thus to a gain-of-function mutation (Harada et al., 1999). Black boxes labeled A and B represent probes used for Southern analysis of recombined β-cat$^{loxEx3-6}$ and β-cat^{loxEx3} alleles, respectively, and X and Xb correspond to Xho1 and Xba1 restriction sites. (b) Brn4cre-mediated recombination of a lacZ reporter gene was visualized by X-gal staining in an E10 embryo; note the pronounced staining in most of the central nervous system. (c) Immunohistochemistry using anti-β-galactosidase antibodies on an E10 embryo that carries Brn4cre and the lacZ reporter gene; the section shows the presence of β-galactosidase in the entire the spinal cord. (d) Southern blot of spinal cord DNA of control and mutant embryos. *Xho*I-digested DNA of β-cat$^{loxEx3-6/loxEx3-6}$ (lane 1) and Brn4cre^{+} β-cat$^{loxEx3-6/loxEx3-6}$ (lane 2) embryos were hybridized by using probe A; *Xba*I-digested DNA of β-cat$^{\Delta Ex3/+}$ (lane 3) and Brn4cre^{+} β-cat$^{\Delta Ex3/+}$ (lane 4) embryos were hybridized by using probe B. (e, f) Western blot analysis of spinal cord extracts of control and conditional mutant E11.5 embryos using anti-β-catenin (d) and anti-p120 (e) antibodies. Shown are spinal cords extracts from control (lane 1), conditional loss-of-function (lane 2), and conditional gain-of-function (lane 3) mutant embryos; note the reduction in the amount of β-catenin in the conditional loss-of-function mutants, and the appearance of a shortened β-catenin variant in the conditional gain-of-function mutant mice. Similar amounts of p120 protein were present in the extracts from control and mutant mice. Bars: b, 1 mm; c, 50 μm.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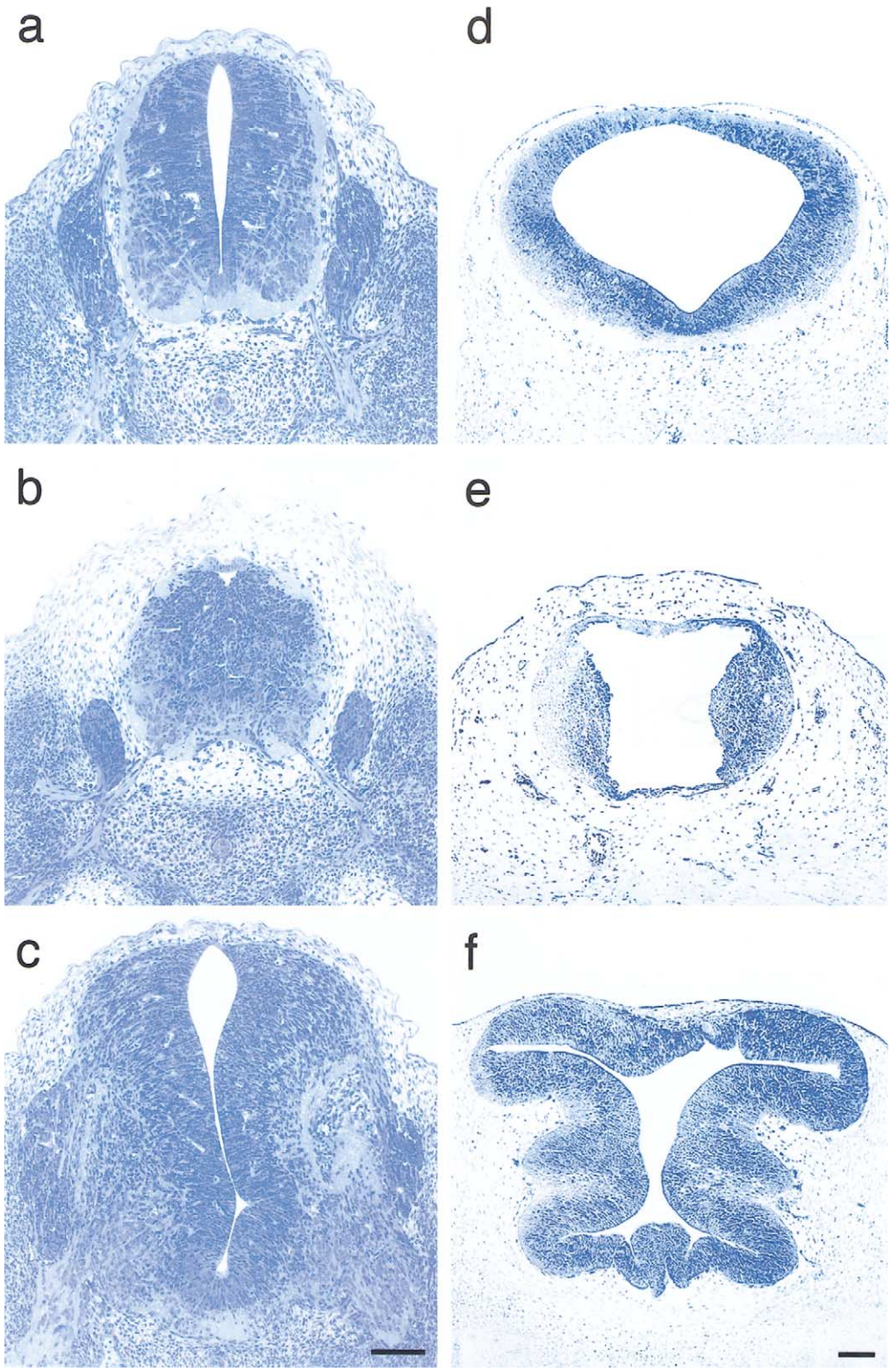


Fig. 2. Changes in the mass of neural tissue in mice with loss- and gain-of-function mutations of β-catenin. (a–c) Sections of the spinal cords at the forelimb level of control (a), loss- (b), and gain- (c) of-function mutant embryos at E12. (d–f) Coronal sections of the midbrain of control (d), loss- (e), and gain- (f) of-function mutant embryos at E11.5. Sections are stained with toluidine blue. Bars, 100 μm.

β-catenin (Hulsken et al., 1994), nestin (Johe et al., 1996), NeuN (Chemicon, Temecula, CA, USA), and neuron-specific class III β-tubulin (TuJ1 antibody) (BAbCO, Richmond, CA, USA); secondary antibodies used were goat anti-mouse or goat anti-rabbit IgG conjugated with Cy3 (Sigma, München, Germany) or Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA). For TUNEL staining, an "in situ cell death kit-fluorescein" was used (Roche Pharma, Mannheim, Germany). To prepare semithin sections, tissues were dehydrated and embedded in Technovit 7100 (Kulzer Histo-Technik, Wehrheim, Germany), and the sections were stained with toluidine blue O.

BrdU (200 μg/g body weight) was injected intraperitoneally into pregnant mice. At various time points after injection, tissues were dissected and fixed overnight in 4% paraformaldehyde in PBS. To detect $BrdU^+$ neurons or progenitor cells on paraffin sections, the cells were first labeled with TuJ1 or anti-NeuN and the appropriate secondary antibodies. Sections were then postfixed in 4% PFA, incubated in 2 N HCl (30 min), and neutralized, and subsequently the $BrdU^+$ nuclei were visualized by the use of rat anti-BrdU antibodies (Abcam, Oxford, UK). Nuclei were stained with 0.5 μg/ml LDS751 (Molecular Probes, Eugene, OR, USA) in PBS. Sections were inspected by a confocal microscope (LSM 510 META; Zeiss, Göttingen, Germany). Images were processed by using Adope Photoshop, and cells and nuclei were counted on such images.

All counts were performed on sections on the forelimb level of embryos at the indicated stages. The number of $BrdU^+$ nuclei in the entire spinal cord was determined. For the determination of the proliferation and apoptosis rates of progenitors, the number of progenitor cells were counted; these correspond to the number of nuclei in the $TuJ1^-$ progenitor area. The proliferation rate corresponds to the number of $BrdU^+$ nuclei in the entire spinal cord divided by the number of progenitor nuclei on the same section. The apoptosis rate corresponds to the number of $TUNEL^+$ nuclei in the $TuJ1^-$ progenitor area divided by the number of progenitor nuclei in the $TuJ1^-$ progenitor area on each section. The percentage of differentiated neurons was defined as the number of $NeuN^+$ cells divided by the number of spinal cord cells on each section. To determine the differentiation rate, $BrdU^+$ nuclei in the spinal cord were counted 24 h after administration of BrdU. The differentiation rate corresponds to BrdU/NeuN double-positive cells divided by the number of $BrdU^+$ nuclei on each section. The displayed numbers are the average determined by counting three to four sections of two to three individual animals.

Results

To investigate the function of β-catenin in the developing central nervous system, we used two distinct mutant alleles to introduce loss- and gain-of-function mutations into the mouse β-catenin gene (Fig. 1a). The first allele contains two loxP sites that surround exons 3–6 ($\beta\text{-cat}^{loxEx3-6}$). Upon recombination, no β-catenin protein is produced, and the recombined allele corresponds thus to a null-mutation (Huelsken et al., 2001). The second allele contains two loxP sites that surround exon 3 and a neighboring neomycin-resistance cassette ($\beta\text{-cat}^{loxEx3}$); after cre-induced deletion of exon 3, activated β-catenin protein ($\beta\text{-cat}^{\Delta Ex3}$) is produced, which lacks N-terminal sequences essential for β-catenin degradation (Harada et al., 1999). To introduce mutations into the developing central nervous system, a transgenic mouse strain that expresses cre under the control of the Brn4 promoter was employed (Ahn et al., 2001). Cre activity in Brn4cre transgenes was assessed with the help of a lacZ indicator strain (Fig. 1b and c, and data not shown). In mouse embryos that carry Brn4cre and a cre-inducible lacZ reporter, cre activity was first detected at E8.5 in the future di- and mesencephalon, and subsequently expanded into other areas of the nervous system. Further caudally, lacZ expression was observed first in the hindbrain and subsequently expanded into the spinal cord. In the spinal cord, LacZ expression initiated in a ventral domain and had spread dorsally by E10. Analysis of β-galactosidase by immunohistology demonstrates that, at E10, the majority of cells in the entire spinal cord had undergone recombination and expressed β-galactosidase in such indicator mice (Fig. 1c). LacZ expression was, however, not detected in neural crest cells and neural crest cell derivatives. Further rostrally, expression extended from the di- and mesencephalon first into the optic stalk and then into the forebrain. In the forebrain, expression was restricted to few cells at E10, but was widespread at subsequent stages.

The Brn4-cre strain was then used to introduce mutations into the β-catenin alleles, and the extent of recombination of the β-catenin loci was tested by Southern hybridization (Fig. 1d). At E11.5, only a minor proportion of the β-catflox DNA had not recombined in the spinal cord of mice with a $\beta\text{-cat}^{loxEx3-6/loxEx3-6}$ genotype by E11.5. Similarly, a large proportion of the $\beta\text{-cat}^{loxEx3}$-allele had recombined in mice with a $\beta\text{-cat}^{loxEx3/+}$ genotype by E11.5. The effect of the recombination on β-catenin protein was assessed by immunohistology and Western blot analysis (Fig. 1e and f, and data not shown). In mice with cre-induced loss-of-function mutation, β-catenin staining intensity in the E10.5 spinal cord was reduced when analyzed by immunohistochemistry, and little β-catenin protein was detected by immunoblotting at E11.5 (lane 2 in Fig. 1e). In mice with cre-induced gain-of-function mutation in the β-catenin locus, the expected shorter variant of β-catenin ($\beta\text{-cat}^{\Delta Ex3}$) was detected (lane 3 in Fig. 1e).

In mice that carry loss- or gain-of-function mutations in β-catenin, major changes in the size and histology of the spinal cord and brain were detected (Fig. 2). The spinal cord of embryos with ablated β-catenin was significantly smaller than that of control embryos, and a well-defined ventricular zone or ventricle was absent at E12 (Fig. 2b; compare with

control in a). At earlier stages, a reduced size of the spinal cord and its ventricular zone were apparent (see Fig. 6b and e, and data not shown). In contrast, the spinal cord and its ventricular zone of the embryos that express the activated β-catenin was enlarged at E10.5 and subsequent stages and appeared undulated at E12 (Fig. 2c). Similar changes were also observed in the brain; for instance, the tissue mass of the midbrain was decreased or enlarged in loss- or gain-of-function mutants, respectively (Fig. 2d–f).

Precursor cells and differentiated neurons were visualized by immunohistochemistry, using anti-nestin (shown in green) and TuJ1 (shown in red) antibodies (Fig. 3). Compared with control mice, the area occupied by progenitors was reduced in size in the spinal cord of loss-of-function mutants (Fig. 3b; compare with a). By E11.5, a clearly defined progenitor area was not apparent ventrally; a dorsal progenitor domain was still discernable at this stage (Fig. 3b), but absent by E12 (see Fig. 7b). Instead, a proportionally larger area of the spinal cord was occupied by differentiated neurons. Similarly, progenitor domains in the midbrain and other brain areas were strongly reduced in size in the loss-of-function mutants at E11.5 (Fig. 3d and e, and data not shown). In contrast, in mice with a gain-of-function β-catenin mutation, the progenitor zone of the spinal cord was enlarged, and a relatively small proportion of the spinal cord area was occupied by differentiated neurons (Fig. 3c). Progenitor domains in the midbrain and other brain areas were also increased in size (Fig. 3d and f, and data not shown). Nrarp, Hes5, and Hes1 are expressed in neuronal progenitors and are genes whose expression is controlled by Notch signaling (Campos-Ortega, 1995; Kageyama and Ohtsuka, 1999; Krebs et al., 2001; Lamar et al., 2001). These genes are expressed at similar intensities in progenitor cells in the spinal cord of control and conditional mutant mice at E11.5 (Fig. 4, and data not shown). However, the spinal cord area occupied by cells that express Hes1, Hes5, or Nrarp was smaller in the loss-of-function mutants (Fig. 4b and e, and data not shown). In gain-of-function mutants, the area occupied by Hes1-, Hes5-, or Nrarp-expressing cells was enlarged (Fig. 4c and f, and data not shown). We conclude that loss of β-catenin function is associated with a decrease of the progenitor pool in the nervous system of mouse embryos. In contrast, gain-of-function mutations in β-catenin result in an increase in the progenitor pool.

Conductin (axin II) is a gene whose expression is induced by canonical Wnt signals in colon carcinoma cells (Lustig et al., 2002). In the spinal cord of control mice at E9.5, *conductin* is expressed in dorsally located progenitors and in a stripe of progenitors cell located in the ventral domain. During subsequent development, only the dorsal domain is maintained, and at E11.5, the most dorsally located progenitors still express *conductin* (Fig. 5a). Expression of *conductin* in the spinal cord is abolished in embryos with a cre-induced loss-of-function mutation (Fig. 5b). Expression is not only increased, but also observable in the entire progenitor domain in embryos with a cre-induced gain-of-function mutation (Fig. 5c). This is consistent with a loss- and gain-of-function in the canonical Wnt signaling pathway by cre-induced recombination of the β-cat$^{loxEx3-6}$ and β-cat^{loxEx3} alleles.

BrdU injections were used to assess proliferation of cells in the spinal cord, and BrdU-positive cells (green) and TuJ1-positive neurons (red) were visualized (Fig. 6a–c). Compared with controls, the number of cells that had incorporated BrdU was reduced in the loss-of-function mutants (Fig. 6a and b). Proliferation of spinal cord cells was quantified at different stages of development (Fig. 6g). Total number of BrdU-positive cells in the spinal cord of control and loss-of-function mutants were similar at E10, but were reduced by 45 and 50% at E11 or E11.5, respectively. Proliferation rates ($BrdU^+$ cells/total number of progenitors) were determined in the spinal cord at E11 (Fig. 6h). Compared with controls, the proliferation rate in the spinal cord of loss-of-function mutants was reduced by 35%. The number of apoptotic cells was assessed by TUNEL staining (Fig. 6d–f), and the rate of progenitor cell death was determined ($TUNEL^+$ progenitor cells/total number of progenitors). Compared with control tissue, the rate of progenitor cell death in the loss-of-function mutant was increased 4.8-fold (Fig. 4i). Decreased proliferation and increased apoptosis were also apparent in the brain of loss-of-function mutants (data not shown). We conclude from these experiments that decreased proliferation as well as increased cell death account for the reduction in the numbers of progenitors and thus the smaller overall size of the neural tissue in the loss-of-function mutants.

In contrast, gain-of-function mutants showed increased proliferation in progenitors of the spinal cord and forebrain, midbrain, as well as hindbrain (Fig. 6c, and data not shown). Compared with the spinal cord of control animals, numbers of BrdU-positive cells in the spinal cord were increased by 110 and 300% at E11 or E11.5, respectively (Fig. 4g). Proliferation rates in the spinal cord was increased 1.4-fold at E11 (Fig. 4h), and the rate of progenitor cell death was increased 6.7-fold (Fig. 4i). Increased apoptosis was also apparent in the brain of these mutant mice. We conclude from these data that enhanced proliferation is an important cause of the net increase in the size of the progenitor pool and neural tissue mass in the β-catenin gain-of-function mutants.

We next assessed to what extent changes in the differentiation capacities of progenitors contribute to the phenotype observed in the loss- or gain-of-function mutants (Fig. 7). The proportion of differentiated neurons in the spinal cord (number of $NeuN^+$ cells/total cell number in the spinal cord) was determined at E12 (Fig. 7d). In the spinal cord of loss-of-function mutants, proportionally more neurons ($NeuN^+$ cells) were present (Fig. 7b and d). This indicated that not only the growth of progenitors, but also their differentiation capacity might be affected by the mutation. To investigate this further, pulse chase experiments were performed by injection of BrdU at E11 and analysis of BrdU-

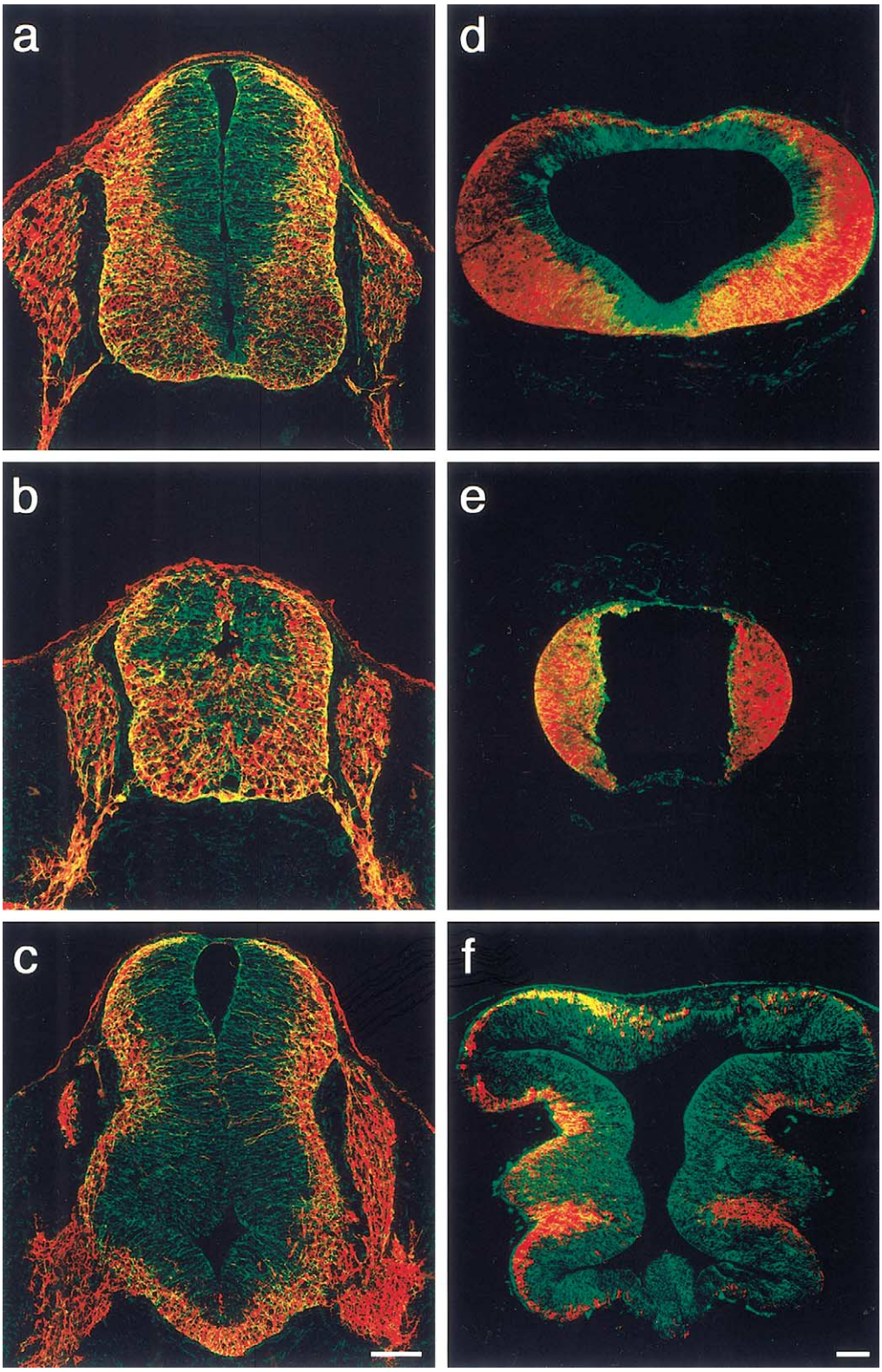


Fig. 3. Changes in size of the neural progenitor pool in β-catenin mutant mice. Neural progenitors and differentiated neurons were visualized by immunohistology, using antibodies directed against the neuron-specific TuJ1 (red) and the progenitor-specific nestin (green) antigens. (a–c) Sections of the spinal cords at the forelimb level of control (a), loss-, (b) and gain- (c) of-function mutant embryos at E11.5. (d–f) Coronal sections of the midbrain of control (d), loss- (e), and gain- (f) of-function mutant embryos at E11.5. Bars, 100 μm.

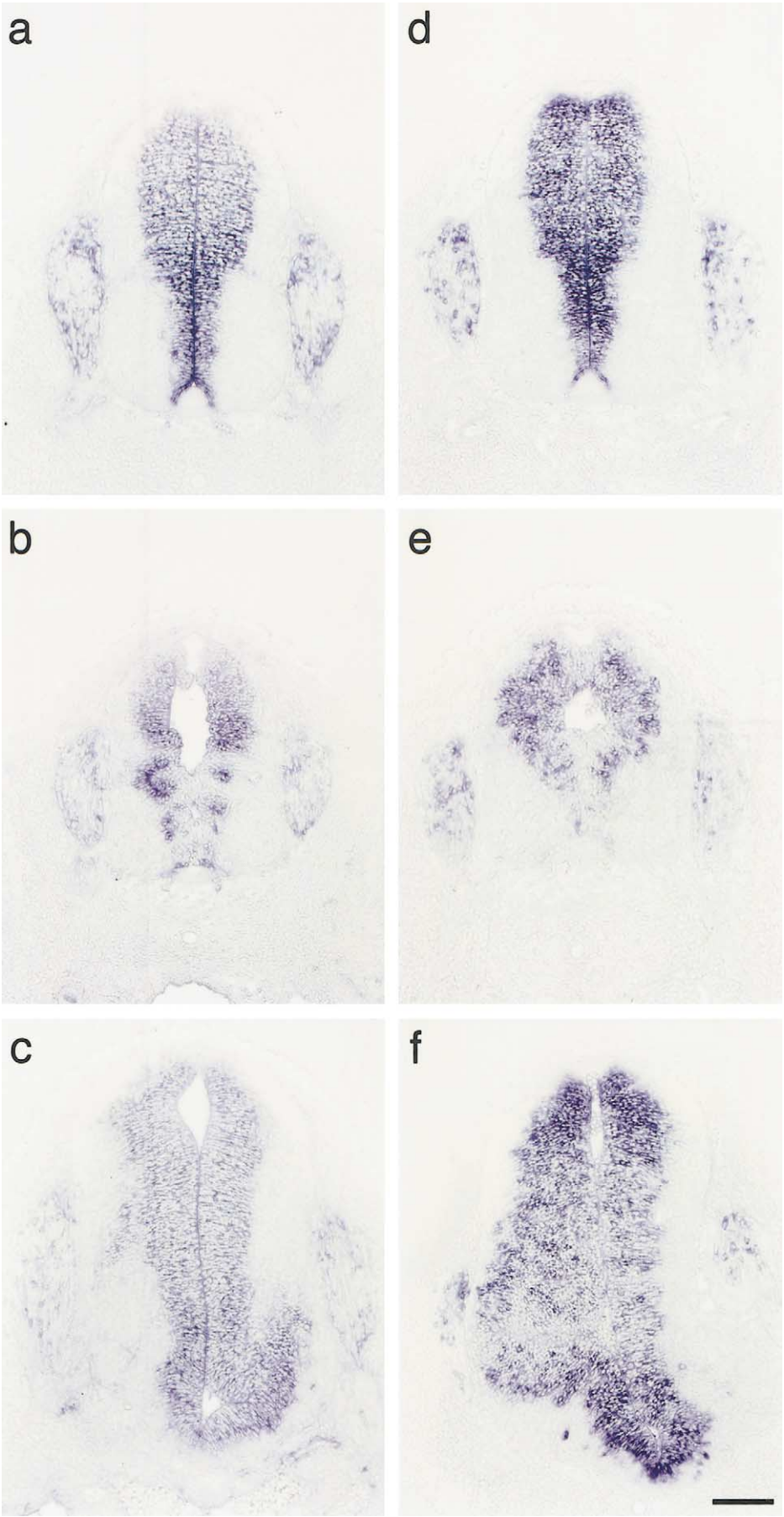
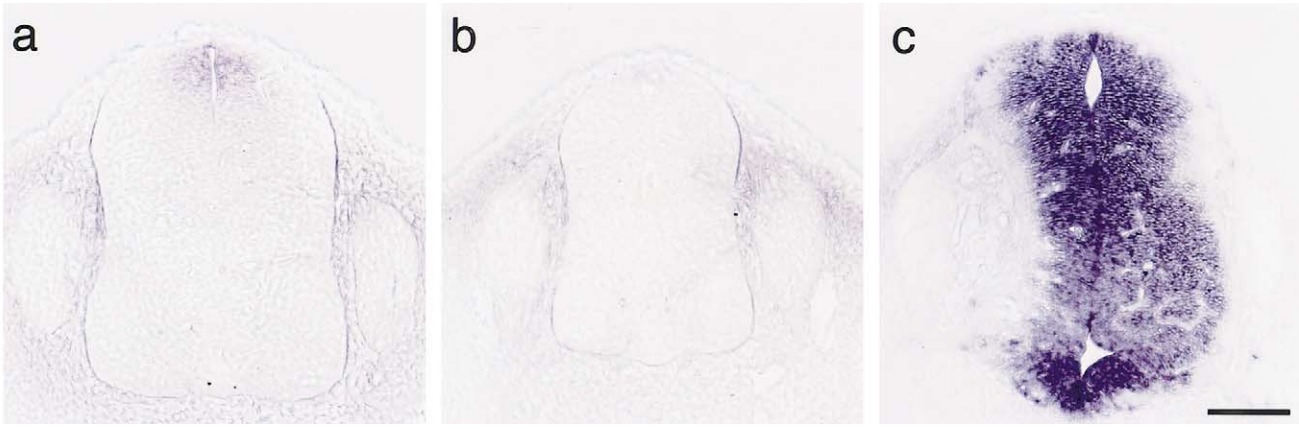


Fig. 4. Expression of *Nrarp* and *Hes5* in β-catenin mutant mice. Expression of *Nrarp* (a–c) and *Hes5* (d–f) was analyzed by in situ hybridizations. Sections of the spinal cord at the forelimb level of control (a, d), loss- (b, e), and gain- (c, f) of-function mutant embryos at E11.5. Bar, 100 μm.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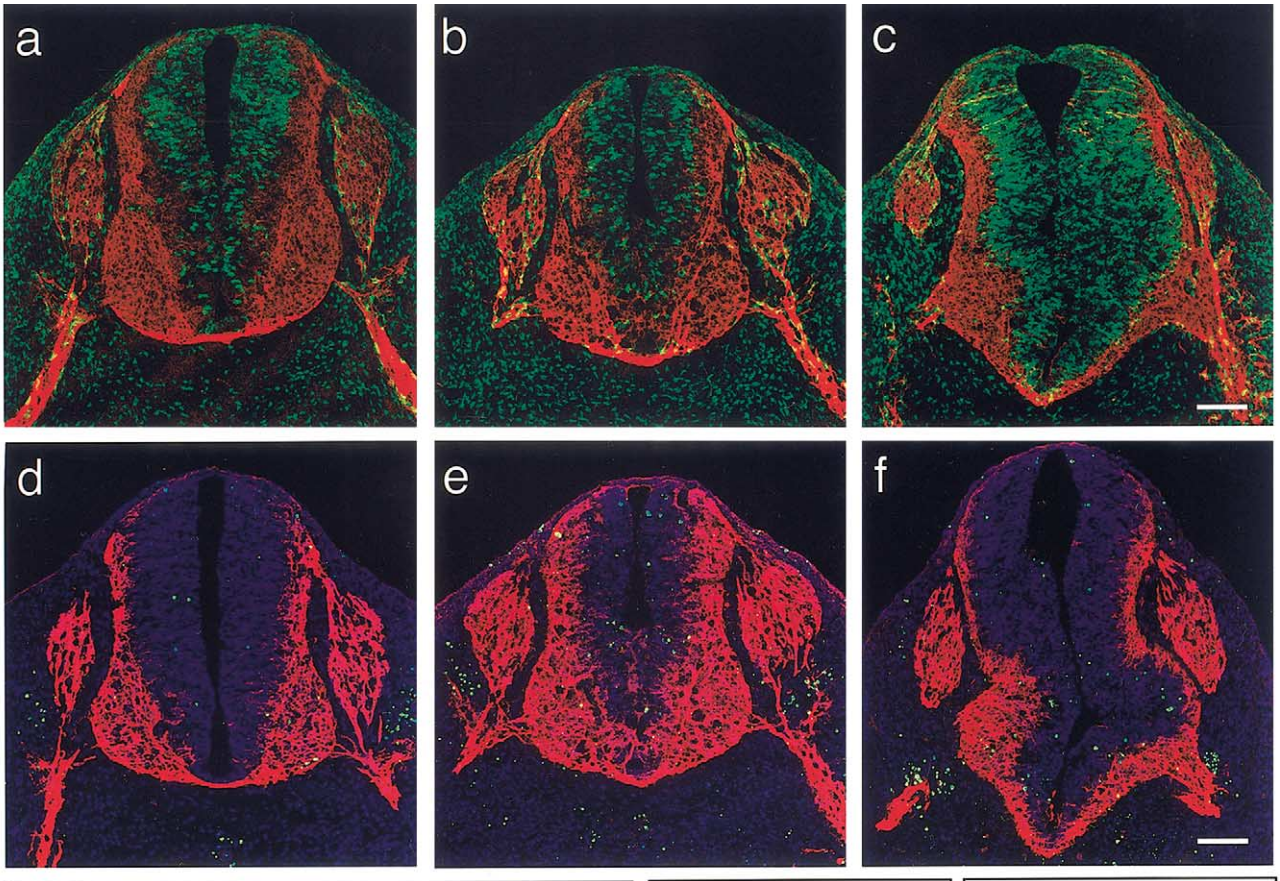
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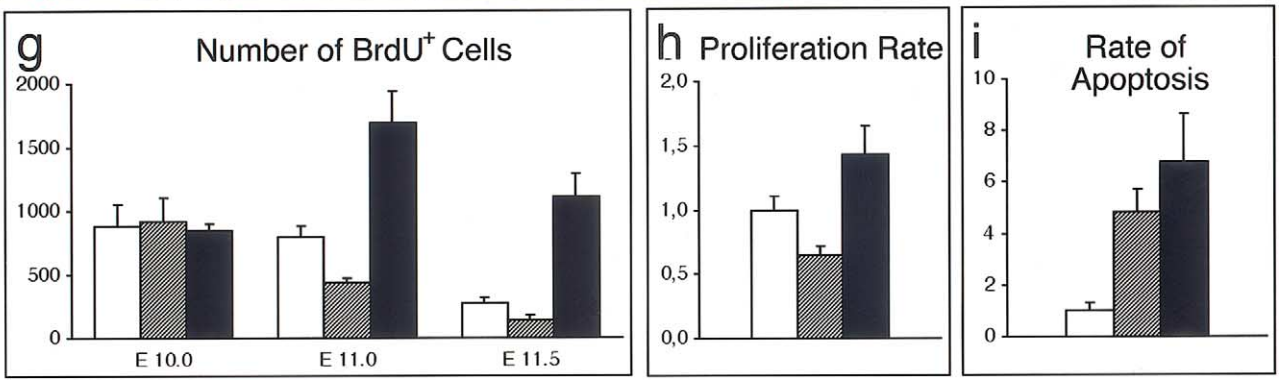
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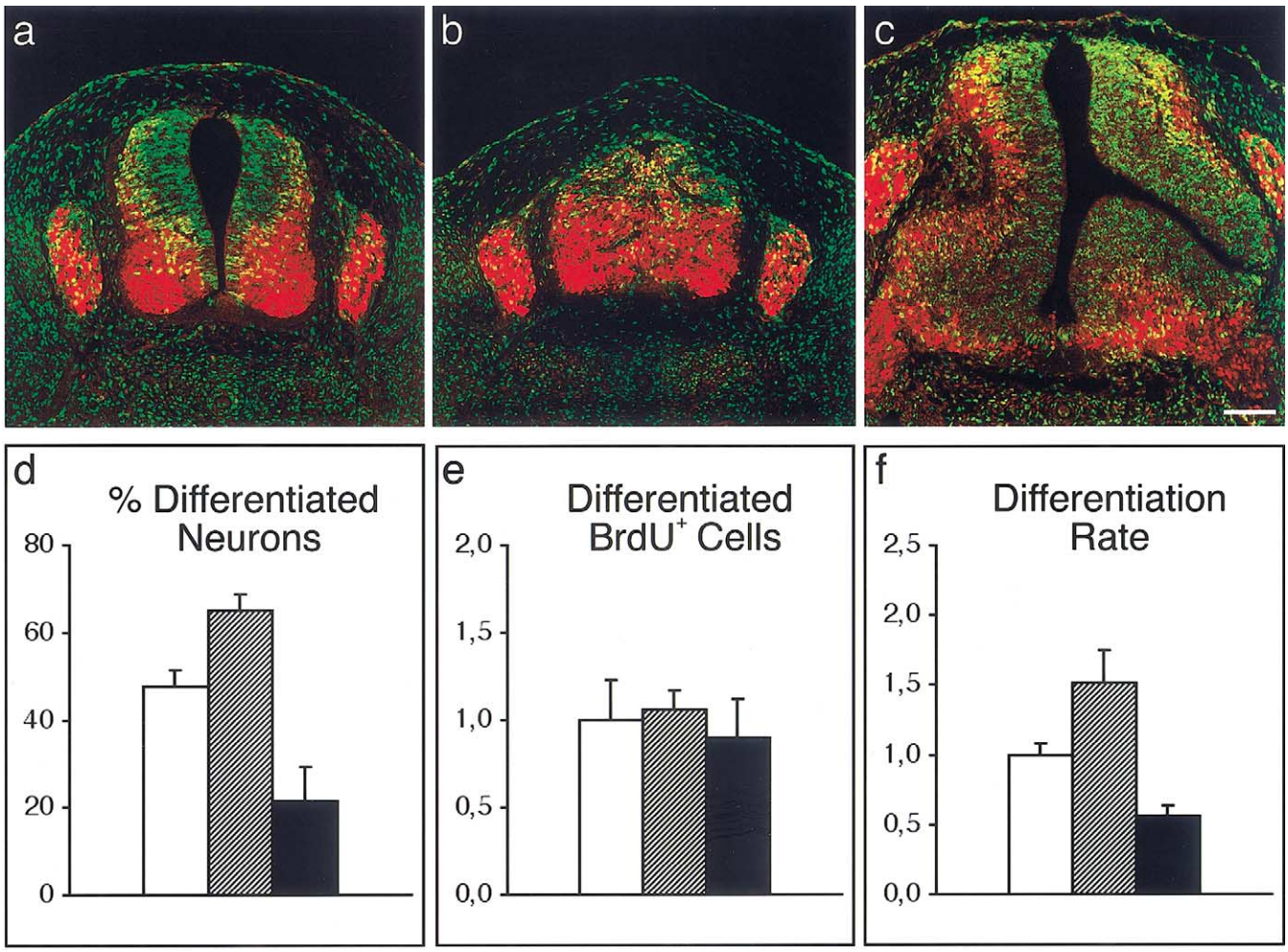

Fig. 7. Changes in the differentiation rate of neuronal progenitors in β-catenin mutant mice. (a–c) BrdU incorporation into neural progenitors and neurons 24 h after BrdU injection at E11 in control (a), loss- (b), and gain- (c) of-function mutant embryos, as assessed by immunohistochemistry using anti-BrdU (green) and anti-NeuN (red) antibodies. (d) Percentage of differentiated $NeuN^+$ neurons in the spinal cord of control (white bar), loss- (hatched bar), and gain-of-function (black bar) mutant embryos at E12, compared with the total cell number in the spinal cord. (e) Number of BrdU/NeuN double-positive cells per section in the spinal cord of control, loss-, and gain-of-function mutant mice observed 24 hrs after BrdU injection. The average number of BrdU/NeuN double-positive cells neurons in control embryos was taken as 1. (f) Differentiation rates of neural progenitors in the spinal cord of control, loss-, and gain-of-function mutant embryos. The differentiation rate was defined as the number of BrdU/NeuN double-positive neurons in the spinal cord/total number of $BrdU^+$ cells in the spinal cord observed 24 h after BrdU injection. The average differentiation rate in control embryos was taken as 1. The displayed numbers are the average determined by counting three to four sections of two to three individual animals. Error bars represent standard deviation. Bar, 100 μm.

Fig. 5. Expression of *conductin* in β-catenin mutant mice. (a–c) Expression of *conductin* was analyzed by in situ hybridisation. Sections of the spinal cord at the forelimb level of control (a), loss- (b), and gain- (c) of-function mutant embryos at E11.5. Bar, 100 μm.

Fig. 6. Changes in proliferation of neural progenitors in β-catenin mutant mice. (a–c) Proliferation of neural progenitors in the spinal cord was assessed by BrdU injection in control (a), loss- (b), and gain- (c) of-function mutant embryos at E11. BrdU-positive nuclei were visualized 2 h after injection by using anti-BrdU antibodies (green), and differentiated neurons using TuJ1 antibodies (red). (d–f) Apoptosis in the spinal cord was assessed by TUNEL assay of control (d), loss- (e), and gain- (f) of-function mutant embryos at E11. TUNEL-positive nuclei are depicted in green, nuclei are depicted in blue, and differentiated neurons were visualized by using TuJ1 antibodies (red). (g) Number of BrdU-positive nuclei in the spinal cords of E10, E11, and E11.5 embryos observed 2 h after BrdU injection. Values for control, loss-, or gain-of-function mutations are depicted by white, hatched, and black bars, respectively. (h) Proliferation rates in the spinal cord of E11 embryos with the indicated genotypes. The proliferation rate was defined as the number of $BrdU^+$ nuclei in the spinal cord/total number of nuclei in the progenitor zone of the spinal cord observed 2 h after BrdU injection. The average proliferation rate in control embryos was taken as 1. (i) Rate of apoptosis in the spinal cord of E11 embryos with the indicated genotypes. The rate of apoptosis was defined as the number of TUNEL-positive cells in the progenitor zone/total number of nuclei in the progenitor zone; the average apoptosis rate of progenitor cells in control mice was taken as 1. The displayed numbers are the average determined by counting three to four sections of two to three individual animals. Error bars represent standard deviation. Bar, 100 μm.

positive neurons at E12. Whereas the absolute number BrdU/NeuN double-positive cells was similar to that observed in control mice, the ratio of proliferating cells that had differentiated into neurons (i.e., the number of BrdU/NeuN double-positive cells/number of BrdU-positive cells) was increased (Fig. 7e and f). Thus, the probability that progenitors differentiate into neurons is higher in the spinal cord of loss-of-function mutants than in control mice.

Differentiation capacity of spinal cord progenitors was also analyzed in gain-of-function mutants. The proportion of neurons among spinal cord cells was smaller in gain-of-function mutants than in control mice (Fig. 7c and d). Pulse chase experiments demonstrated that the ratio of proliferating cells that had differentiated into neurons (i.e., the number of BrdU/NeuN double-positive cells/number of BrdU-positive cells) was decreased (Fig. 7c and f). However, many neuronal $BrdU^+$ cells were detected in the gain-of-function mutant mice, indicating that neuronal differentiation was impaired but not blocked (Fig. 7c and e). Thus, the probability that progenitors differentiate is lower in the presence of activated β-catenin, which contributes to the net increase in the size of the progenitor pool.

Discussion

We report here the ablation of β-catenin and the expression of an activated β-catenin in the nervous system, using conditional mutagenesis in mice. Ablation of β-catenin results in a marked decrease of the overall size of the nervous system. In contrast, the expression of an activated β-catenin causes a large size increase. The underlying cause for these phenotypes is changes in the proliferation rate of neural progenitors, accompanied by a shift in the balance between self renewal and differentiation. This indicates that canonical Wnt signals, which are mediated by β-catenin and its downstream interaction partners, control proliferation and the balance between progenitor expansion and differentiation. We observe these changes in many areas in the developing nervous system, i.e., throughout the entire spinal cord as well as in the brain. Gene ablation studies have previously implicated TCF4, a transcription factor and downstream interaction partner of β-catenin, in the maintenance of a distinct stem cell compartment, the self-renewing progenitor cells in the intestinal crypts (van de Wetering, 2002). The genetic program controlled by β-catenin/TCFs appears thus to control the maintenance of distinct stem cell types, neural and intestinal stem cells.

The balance between proliferation and differentiation of progenitor cells in the nervous system

Notch signaling has been implicated in regulating the balance between neuronal differentiation and progenitor pool expansion in the mammalian central nervous system (Gridely, 1997; Kageyama and Ohtsuka, 1999). Notch activity suppresses neuronal differentiation and keeps cells in the progenitor pool. Ablation of Notch or of the main effectors of Notch signals, Hes1 and Hes5, causes precocious neuronal differentiation (de la Pompa et al., 1997; Lutolf et al., 2002; Nakamura et al., 2000; Ohtsuka et al., 1999). However, remaining progenitors in these mutants do not appear to change their proliferation rate, indicating that Notch signaling affects primarily the decision of progenitor cells to differentiate (Lutolf et al., 2002; Nakamura et al., 2000). This is in contrast to the changes we observed in the nervous system of β-catenin mutant mice, where proliferation of progenitors is affected in a profound manner and soon after the mutations are induced by the cre-recombinase. Not only the differentiation rate, but also the decision between self-renewal and differentiation is changed in the neural tissue of the gain-of-function mutant mice. Notch signaling, as assessed by the induction of the Notch responsive genes Hes5 or Nrarp, can occur in these mutant mice. β-Catenin appears thus to affect the decision of cells to self renew or to differentiate in a Notch-independent manner. However, subtle effects on Notch signaling cannot be excluded at this point. The major changes in proliferation that are observed in these mutants show that β-catenin and Notch signals elicit principally distinct responses in neural stem cells, and they establish β-catenin as potent regulator of proliferation. In addition, apoptosis rates are increased in the nervous system of the gain- and loss-of-function mutant mice, which might appear unexpected and even contradictory. It should, however, be noted that both types of experimental manipulations, which increase or decrease transmission of canonical Wnt signals, induce apoptosis also in other tissues (Hasegawa et al., 2002; Hsu et al., 2001; Ioannidis et al., 2001; Wong et al., 1998). Apoptosis rates in our mutants might be affected by p53 and c-Myc. Accumulation of p53 can be regulated by β-catenin levels, and c-Myc is a known β-catenin target gene. Changes in p53 and c-Myc expression can impinge on apoptosis rates (Damalas et al., 1999; Hsu et al., 2001).

β-Catenin and Wnt signals in the developing nervous system

β-Catenin is an essential component of canonical Wnt signaling, and Wnts are known to be expressed in complex patterns in the nervous system (Parr et al., 1993). Functions of Wnts in the control of tissue size in the nervous system have been described, for instance, in the midbrain or the spinal cord. Ablation of Wnt1 or the Wnt coreceptor LRP6 results in a loss of midbrain and hindbrain tissues (McMahon et al., 1992; Pinson et al., 2000). In Wnt1/Wnt3a compound mutant mice, the dorsal neural tube is reduced in size, and distinct neural crest derivatives are hypoplastic or completely absent (Ikeya et al., 1997). Similar changes in development of neural crest derivatives were observed in mutant mice, in which β-catenin was ablated in the dorsal neural tube (Brault et al., 2001). Moreover, mutations in

LEF1 or Wnt3a result in a marked hypoplasia of the hippocampus (Galceran et al., 2000; Lee et al., 2000). Further experiments in mouse and chick, in which Wnt1 was overexpressed in the spinal cord, resulted in an enlargement of these tissues (Dickinson et al., 1994; Megason and McMahon, 2002). In addition, it was recently reported that overexpression of constitutively active β-catenin in the forebrain increases the cortex size by enhancing progenitor growth and inhibiting neuronal differentiation (Chenn and Walsh, 2002). Our study extends this finding to other regions of the nervous system and provides the analysis of the loss-of-function of β-catenin. In addition to these observations that relate to a role of Wnt signaling in progenitor growth, further phenotypes were attributed to defects in patterning and/or specification of particular domains of the nervous system. For instance, Wnt1 mutants miss midbrain tissue and engrailed 1 expression, and engrailed 1 expression is affected prior to a loss of midbrain tissue (McMahon et al., 1992). In addition, changes in patterning in the spinal cord and the hippocampal field, respectively, were observed in compound Wnt1/Wnt3a and in Lef1 mutant mice (Lee et al., 2000; Muroyama et al., 2002). Despite this prominent role of β-catenin signals in expansion of the progenitor pool, additional more subtle local changes can be detected in the nervous system of our mutant animals. For instance, ablation of β-catenin also has an effect on the size of the choroid plexus that, despite the general brain hypoplasia, is very much enlarged and appears to arise prematurely at the level of the lateral ventricles. This may indicate that β-catenin signals not only control growth, but affect also specification of particular cell types and/or patterning. More work is required to assess possible changes in patterning and cell type specification of the central nervous system in these mutant embryos.

Neuronal stem cells and β-catenin/Wnt signals

Prior to neuronal differentiation, the nervous system is already regionalized, and the developing spinal cord and brain contains many types of progenitors with distinct developmental potentials (Jessell, 2000; Simeone, 2002). However, our β-catenin mutations affect the size of the progenitor pools and tissues in many areas of the spinal cord and brain. β-Catenin and the Wnt pathway can therefore be considered a fundamental regulator of proliferation of neural progenitors. Cell cultures of neuronal progenitors rely typically on the usage of EGF, FGF, or IGF-1 to expand the multipotent cell population (Cameron et al., 1998). Our finding of a prominent and general role of β-catenin/canonical Wnt signal in the expansion of neural progenitors in vivo indicates that Wnts, despite their difficult biochemical properties, might be of use in such in vitro experiments. Progenitor cells in the developing nervous system express similar sets of genes as the stem cells that are found in the adult brain; for instance, nestin marks progenitors in the embryo and stem cells in the adult. Moreover, these two cell types resemble each other with respect to their in vitro differentiation potential and their capacity to repopulate the central nervous system in vivo (Temple, 2001). Whether adult stem cells in the brain respond also to β-catenin or Wnt signals remains to be determined.

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