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Transgenic mice expressing F3/contactin from the TAG-1 promoter exhibit developmentally regulated changes in the differentiation of cerebellar neurons

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SUMMARY

F3/contactin (CNTN1) and TAG-1 (CNTN2) are closely related axonal glycoproteins that are differentially regulated during development. In the cerebellar cortex TAG-1 is expressed first as granule cell progenitors differentiate in the premigratory zone of the external germinal layer. However, as these cells begin radial migration, TAG-1 is replaced by F3/contactin. To address the significance of this differential regulation, we have generated transgenic mice in which F3/contactin expression is driven by *TAG-1* gene regulatory sequences, which results in premature expression of F3/contactin in granule cells. These animals (*TAG/F3* mice) display a developmentally regulated cerebellar phenotype in which the size of the cerebellum is markedly reduced during the first two postnatal weeks but subsequently recovers. This is due in part to a reduction in the number of granule cells, most evident in the external germinal layer at postnatal day 3 and in the inner granular layer between postnatal days 8 and 11. The reduction in granule cell number is accompanied by a decrease in precursor granule cell proliferation at postnatal day 3, followed by an increase in the number of cycling cells at postnatal day 8. In the same developmental window the size of the molecular layer is markedly reduced and Purkinje cell dendrites fail to elaborate normally. These data are consistent with a model in which deployment of F3/contactin on granule cells affects proliferation and differentiation of these neurons as well as the differentiation of their synaptic partners, the Purkinje cells. Together, these findings indicate that precise spatio-temporal regulation of TAG-1 and F3/contactin expression is critical for normal cerebellar morphogenesis.

Key words: Cerebellar development, Neurite growth, Axonal glycoproteins, F3/contactin, TAG-1, Gene regulation, Cell proliferation, Mouse

INTRODUCTION

The elaboration of the architecture of the cerebellar cortex involves a sequence of specific cell contacts that trigger proliferation, differentiation and migration events. Early in development, granule cell precursors (GCPs) migrate from the rhombic lip to provide the main neuronal population of the cerebellar anlage (Hatten and Heintz, 1995; Alder et al., 1996; Wingate, 2001). These cells then progress from proliferation to tangential and then radial migration to reach their final resting place in the internal granular layer (IGL) (Komuro and Rakic, 1998; Komuro et al., 2001). During this process, contacts among GCPs sustain their proliferation while interaction with glial cells induces cell cycle exit and differentiation (Gao et al., 1991), and provides a substratum for radial migration (Rakic, 1971; Hatten and Heintz, 1995; Hatten, 1999; Komuro and Rakic, 1998; Komuro et al., 2001). In addition, contacts between granule cells (GCs) and Purkinje neurons (PCs) are critical for the survival and differentiation of both cell types (Herrup and Sunter, 1987; Smeyne et al., 1995; Mason et al., 1997).

Recent studies have revealed the involvement of a number of diffusible factors in this process, in particular sonic hedgehog (Dahmane and Ruiz i Altaba, 1999; Wechsler-Reya and Scott, 1999; Ruiz i Altaba et al., 2002), neurotrophins (Schwartz et al., 1997; Morrison and Mason, 1998; Hirai and

Launey, 2000) and fibroblast growth factor (Wechsler-Reya and Scott, 1999). Surprisingly however, although a number of cell adhesion molecules (CAMs) have been implicated in GC axon outgrowth and fasciculation (Walsh et al., 1997; Buttiglione et al., 1998; Meiri et al., 1998; Hillenbrand et al., 1999; Sakurai et al., 2001) and in their migration (Fishell and Hatten, 1991; Hatten, 1999; Adams et al., 2002), few studies have attempted to address the role of such molecules in the cell-cell interactions that control cell differentiation in the cerebellar cortex.

Notable among the adhesion molecules that could mediate such interactions are members of the immunoglobulin-like L1 subfamily, comprising L1-like transmembrane (Kadmon and Altevogdt, 1997; Kamiguchi and Lemmon, 1997) and F3/contactin-like GPI-linked molecules (Ranscht, 1988; Gennarini et al., 1989; Furley et al., 1990; Yoshihara et al., 1994; Yoshihara et al., 1995; Lee et al., 2000; Ogawa et al., 2001), all of which are expressed on cerebellar neurons: L1, NrCAM, CHL1, TAG-1, F3/contactin, NB-2 and BIG-2 on GCs (Yoshihara et al., 1995; Hillebrand et al., 1999; Virgintino et al., 1999; Sakurai et al., 2001; Ogawa et al., 2001) and neurofascin (Zhou et al., 1998), NrCAM (Sakurai et al., 2001), F3/contactin (Virgintino et al., 1999), L1 (Jenkins and Bennet, 2001), BIG-1 (Yoshihara et al., 1994) and NB-3 (Lee et al., 2000) on PCs. Although gene targeting has revealed a critical role for F3/contactin in GC development (Berglund et al., 1999), the effects of deleting other members of the family have been more subtle (Dahme et al., 1997; Fransen et al., 1998; Fukamauchi et al., 2001) (L. Y. and A. F., unpublished) and there is evidence for functional redundancy among these molecules (e.g. L1 and NrCAM) (Sakurai et al., 2001). Nonetheless, specific differences in their function in in vitro assays, and their distinct spatial and temporal expression profiles, suggest that their deployment at specific times in cerebellar development may be important.

A good example of this is provided by the two GPI-linked glycoproteins F3/contactin and TAG-1. These molecules share ~50% amino acid identity (Gennarini et al., 1989; Furley et al., 1990), common binding to L1, NrCAM and neurofascin (Olive et al., 1995; Malhotra et al., 1998; Volkmer et al., 1998; Faivre-Sarrailh et al., 1999; Fitzli et al., 2000) and the ability to stimulate neurite elongation (Furley et al., 1990; Gennarini et al., 1991; Stoeckli et al., 1991) and direct axonal growth (Berglund et al., 1999; Fitzli et al., 2000; Fujita et al., 2000). However, each also has distinct binding capabilities (e.g. F3/contactin-tenascin R, TAG-1-neurocan) (Pesheva et al., 1993; Milev et al., 1996) and, in some contexts, F3/contactin may inhibit neurite outgrowth (Buttiglione et al., 1996; Buttiglione et al., 1998). In the cerebellum, TAG-1 is predominantly expressed on premigratory granule neurons in the external granular layer (EGL) whereas F3/contactin peaks on postmitotic migrating neurons (Faivre-Sarrailh et al., 1992; Wolfer et al., 1994; Wolfer et al., 1998; Stottmann et al., 1998; Virgintino et al., 1999). Since the function of these two molecules may be antagonistic (Buttiglione et al., 1998), their differential expression may be critical to the orderly differentiation and morphogenesis of the cerebellar cortex. To address this possibility, we have deregulated F3/contactin expression in transgenic mice by placing its cDNA under the control of the proximal promoter region of the human TAG-1 gene (*TAX1*). This results in ectopic expression of F3/contactin in the outer EGL and in a developmentally regulated cerebellar phenotype in which neuronal proliferation and differentiation are affected. These data indicate a novel role for these adhesion molecules and that precise regulation of their expression is critical to normal cerebellar differentiation and morphogenesis.

MATERIALS AND METHODS

Transgenic mice

TAX1/*lacZ* construct

An adaptor oligonucleotide (5′ ccATGgggacagccaccaggaggaagCCC 3′) encoding the first 8 amino acids of TAX1 was inserted into the *Sma*I site at the 5′ end of *lacZ* in pMC1871 (Amersham Pharmacia, Biotech, UK), thus creating a start codon *Nco*I site that could be fused in frame to the start codon of *TAX1* using the endogenous *Nco*I site, while preserving the natural Kozak initiation sequence. This was joined to a 16 kb *Not*I/*Nco*I genomic fragment from the human *TAX1* gene, including 3.9 kb of the 5′ flanking region, exon 1, intron 1 (approximately 11 kb) and the non-coding region of *TAX1* exon 2 (Kozlov et al., 1995) and then cloned into pGL3-Basic (Promega Inc., Madison, WI, USA).

TAX1/F3 construct

Using a *Nco*I-*Hin*dIII adaptor oligonucleotide, the same *TAX1* genomic fragment was cloned by three-way ligation into *Not*I/*Xba*I-cut pGL3-Basic, along with a 3.1 kb *Hin*dIII-*Eco*RI fragment containing the full-length F3/contactin cDNA except for the triplet encoding for the most C-terminal amino acid and a TGA opal stop codon (Gennarini et al., 1989). The junction at the 3′ end was achieved by partial filling of *Eco*RI and *Xba*I 5′ overhangs, each with two corresponding nucleotides, resulting in creation of compatible two-letter sticky ends; upon ligation this restored the last F3/contactin triplet followed by a TAG stop codon. The integrity of both constructs was verified by sequencing.

Generation and breeding of transgenic mice

DNA fragments were excised by digestion with *Sal*I, diluted in TE buffer and injected into fertilised oocytes from CBA × C57 Black10 F_1 donor mice (Hogan et al., 1994). Founders carrying the transgene were identified by southern blot and gene-specific PCR and used to establish colonies at the breeding facilities of the Department of Pharmacology and Human Physiology of Bari University. Animal experimentation conformed to the EU directive 86/609 EEC.

Immunohistochemical procedures

F3/contactin immunostaining was performed on paraffin sections from developing mice cerebella perfused with 2% paraformaldeyde and 0.1% glutaraldehyde as described previously (Virgintino et al., 1999). For calbindin immunostaining a mouse monoclonal antibody (Sigma, St Louis, USA) was used on adjacent sections; alternatively, 20 μm cryostat sections from mice cerebella perfused with 4% paraformaldehyde were stained with a rabbit anti-rat calbindin serum (Swant, Bellinzona, Switzerland) and revealed by the Vectastain *elite* ABC kit (Vector Laboratories, Burlingame, CA, USA) using diaminobenzidine as a substrate. To account for individual variations, four different mice from each wild-type and transgenic genotype were processed at each developmental step for both F3/contactin and calbindin immunohistochemistry.

Staining for β-galactosidase was performed on 20 μm cryostat sections from tissues fixed with 2% paraformaldehyde/0.1% glutaraldehyde according to described protocols (Hogan et al., 1994). For double TAG-1/*lacZ* labelling, sections stained for β-galactosidase were extensively washed in phosphate-buffered saline (PBS), incubated with the TAG-1 rabbit antiserum (Dodd et al., 1988) and developed with goat anti-rabbit secondary antibodies. For double

F3/contactin-TAG-1 staining, 20 μm cryostat sections from brains perfused with 4% paraformaldehyde were incubated with the F3/contactin fusion protein antiserum 24III (Gennarini et al., 1991) and the mouse TAG-1 monoclonal antibody 4D7 (Yamamoto et al., 1986). Biotinylated goat anti-rabbit IgG followed by TRITC Avidin D (Vector Laboratories) and fluorescein-conjugated goat anti-mouse IgM (Jackson Laboratories, West Grove, Pennsylvania, USA), respectively were used as secondary antibodies.

Double labelling was performed with polyclonal anti-TAG-1 (Dodd et al., 1988) and monoclonal anti-proliferating cells nuclear antigen (PCNA) (Novocastra Laboratories) antibodies, or monoclonal anti-TAG-1 (4D7) and polyclonal anti-phosphorylated histones H1 and H3 antibodies (Upstate Biotechnology). Pups were perfused with 4% paraformaldehyde in PBS and brains were then further fixed for 2 hours before being processed for cryosectioning (Dodd et al., 1988). Sections on slides were incubated for 12 hours at 54°C before being stained with antibodies as previously described (Dodd et al., 1988) and visualised by standard epifluorescent or confocal (Leica TCS) microscopy.

Cerebellar granule cell cultures and immunocytochemistry

Cerebellar cultures were generated at postnatal day 7 as described (Buttiglione et al., 1996; Buttiglione et al., 1998).

To visualise neurites, after fixation with 4% paraformaldehyde in PBS, aggregate cultures were stained with the 24III serum and dissociated neurons with a rabbit anti-GAP43 serum (Research Diagnostics INC, NY, USA), using Cy3-conjugated goat anti-rabbit immunoglobulins (Jackson laboratories) as secondary antibodies.

Cell proliferation and apoptosis assays

To estimate cell proliferation in vivo, mice received three subcutaneous injections of 5-bromo-2′-deoxyuridine (BrdU; Roche Molecular Biochemicals, Mannheim, Germany; 30 μg/g body weight) at 0, 8, 12 hours and were sacrificed 8 hours after the last injection. Brains fixed by immersion in 3% acetic acid in ethanol were embedded in paraffin wax. Three μm sections were incubated with a nuclease-containing anti-BrdU mouse monoclonal antibody and revealed by alkaline phosphatase-conjugated sheep anti-mouse IgG (Roche).

To measure cell proliferation in vitro, high density primary cerebellar cultures (0.3×10^6 cells/cm^2 on polylysine-coated LabTek slides) were incubated overnight with BrdU (10 μM in medium) either immediately after plating or 16 hours later. Cells were then washed and fixed with 70% ethanol in 15 mM glycine buffer pH 2 before detecting BrdU as above.

Cell death was estimated by using the ApopTag kit (Intergen, NY, USA) on 5 μm paraffin sections from developing mice cerebella or on high density primary cerebellar cultures according to the manufacturer's instructions.

Morphometric analysis

Image acquisition, processing and measurement were performed by the image analyser VIDAS 2.5 (Kontron Elektronik GmbH, Eching, Germany).

Cerebellar cortex

Four cerebella from wild-type and four from transgenic mice were examined at each developmental step (0, 3, 8, 11, 16 and 30 postnatal days). Fifty Toluidine Blue-stained sections from each cerebellum (in the region of the cerebellar vermis, from the nucleus medialis habenulae to the nucleus medialis cerebelli) were digitised. The total area of the cerebellum and of each cortical layer was measured in μm^2 by a custom written semiautomatic sequence of commands (macros); this included a segmentation step by a thresholding method able to automatically identify the cerebellar cortex layers according to the grey level of the individual pixels; mean (M)±standard error of mean (s.e.m.) were calculated and values were expressed as their ratios in transgenic versus wild-type (wt) mice.

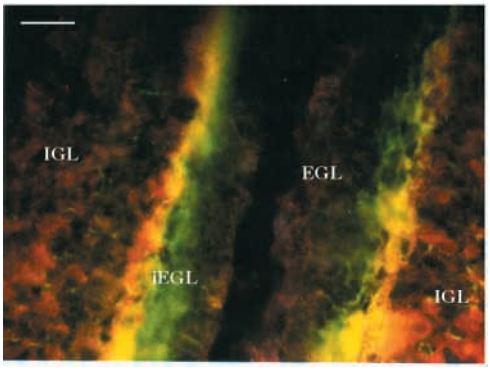

Fig. 1. Expression of F3/contactin (red) and TAG-1 (green) in postnatal day 6 cerebellar cortex. The region of TAG-1 and F3/contactin overlap is in yellow. IGL, inner granular layer; EGL, external germinal layer; iEGL, inner region of the external germinal layer. Scale bar: 40 μm.

Cells density in the IGL and EGL was measured by counting Toluidine Blue-labelled nuclei in random fields of identical size (40× magnification) by using a custom written macro. For BrdU incorporation or cell death the number of labelled cells was estimated using VIDAS 2.5 interactive measurement functions. For measuring the number of PCs and the area of their dendritic tree, calbindin-immunostained sections from postnatal days 8 cerebella were used, acquiring the fields at 5× and 40× magnifications, respectively.

Neuronal cultures

To quantify neurite growth, immunolabelled cultures were photographed under a Leica DRMB microscope connected to a Polaroid DMC camera. The resulting microphotographs were acquired with a CCD/RGB video camera (TK-1070E; JVC, Japan) connected to the VIDAS 2.5 and analysed using a specifically written macro.

For aggregate cultures absolute values for neurite-occupied surface were referred to a total area of 12.9 mm^2. A total of 28 cell aggregates was analysed from both wild-type and transgenic mice. To measure neurite length, photographs of dissociate cultures were acquired at a 40× magnification. A total of 408 and 241 isolated neurons from wild-type and *TAG/F3* mice, respectively, were analysed. Values were expressed as M±s.e.m.

The number of BrdU-labelled or TUNEL-stained cells in high density primary cerebellar cultures was measured in random fields of identical size (77440 μm^2) by using a custom-written macro.

Statistical significance of the morphometric data was assessed by the Student's *t*-test.

RNA analysis

Total RNA was prepared using the Trizol reagent (Life Technologies). For RT-PCR amplification, the Superscript One-Step RT-PCR System (Life Technologies) was used on 3 μg of total RNA. Amplification products were analysed on 2% agarose gels.

RESULTS

F3/contactin and TAG-1 are differentially expressed during cerebellar development

In the cerebellar cortex, TAG-1 and F3/contactin are expressed on premigratory and migrating GCs respectively (Pickford et al., 1989; Yamamoto et al., 1986; Yamamoto et al., 1990;

Faivre-Sarrailh et al., 1992; Kuhar et al., 1993; Virgintino et al., 1999; Berglund et al., 1999). Indeed, as shown in Fig. 1, at postnatal day 6 (P6) TAG-1-positive neurons formed a discrete band within the inner region of the external germinal layer (iEGL), while F3/contactin was found on migrating elements extending through to the IGL. Only a thin band of migrating cells was labelled by both antibodies. Since GCs progressively differentiate during migration from the EGL towards the IGL (Kuhar et al., 1993) this suggests that TAG-1 is expressed earlier than F3/contactin on these cells. We reasoned, therefore, that a *TAG-1* gene promoter element might be an appropriate tool with which to deregulate F3/contactin developmental expression.

A promoter element from the human *TAG-1* (*TAX1*) gene drives expression in premigratory granule cells

To define such a regulatory element, the proximal promoter region of the *TAX1* gene (Kozlov et al., 1995) – including the first exon, the ATG-containing second exon and the intervening first intron – was fused in frame to a *lacZ* reporter (Fig. 2A, see Materials and Methods) and used to generate transgenic mice (designated *TAG*/*lacZ*). In *TAG*/*lacZ* mice, *TAG-1* and *lacZ* expression largely overlapped in the EGL at postnatal day 8 (P8) (Fig. 2B, a-c); however, transgene activation was weaker in the anterior lobes (compare lobules V-VI in f with lobules VIII-IX in i). Similarly to TAG-1 protein (Furley et al., 1990), transgene expression was strongest on premigratory neurons (f,i). However, particularly in lobules VIII-IX, it also extended towards the outermost region of the EGL (oEGL) (h-i) and in the IGL, where GCs expressed the transgene with a similar anteroposterior gradient (b,c). These differences between *TAG-1* and *lacZ* expression may reflect the difference in the sensitivity of the assays for TAG-1 and β-galactosidase, and the fact that the transgene is present in multicopies (data not shown), as low levels of *TAG-1* mRNA are normally detected in both the oEGL and in the IGL (Furley et al., 1990). Moreover, the transgene maintained normal *TAG-1* cell-type specificity and was not expressed, for example, in Purkinje neurons (h) (Stottmann et al., 1998). Thus, these regulatory elements seemed appropriate to induce premature expression of F3/contactin.

Transgenic mice were then generated using a construct in which the same *TAX1* gene 5′ flanking region was fused to a full length F3/contactin cDNA (Fig. 2A). Five different founder mice were obtained and used to establish lines. Reverse transcription polymerase chain reaction (RT/PCR) amplification (see Fig. 3A for details) revealed that the transgene mRNA was expressed in the cerebellum of several lines (Fig. 3B and data not shown), beginning at birth and continuing as late as postnatal day 30 (P30), reflecting normal TAG-1 expression (Wolfer et al., 1994; Wolfer et al., 1998). Immunohistochemical staining at postnatal day 4 (P4; Fig. 3C) confirmed that the transgene directs ectopic expression of F3/contactin protein throughout most of the EGL, as expected from the *TAG*/*lacZ* results (Fig. 2B, h,i), whereas wild-type controls were F3/contactin-negative in this region. Having confirmed the success of this approach, further detailed analysis was performed and representative results from one of these lines (designated *TAG/F3*) are presented below.

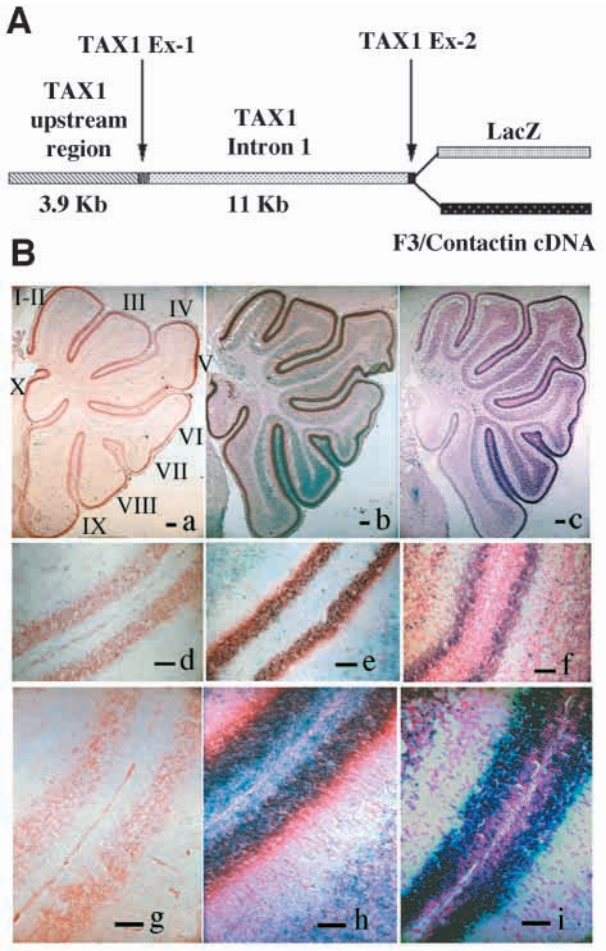

Fig. 2. (A) Promoter/reporter constructs generated by fusing the 5′ region of the human *TAX1* gene to either a *lacZ* reporter or the F3/contactin cDNA. (B) Expression of TAG-1 protein and *lacZ* reporter in the cerebellar cortex of *TAG*/*lacZ* transgenic mice at postnatal day 8. (a-c) TAG-1 immunostaining (a) and X-gal staining (c) of sequential sagittal sections obtained at the level of the vermis. In b a double TAG-1/*lacZ* staining is shown. In a, the lobules are indicated by roman numerals. Scale bars: 200 μm. (d-f) Expression of the TAG-1 protein (d) and of the TAX-1/*lacZ* transgene (f) in lobules V-VI. Double TAG-1/ *lacZ* staining is reported in e. Scale bars: 100 μm. (g-i) TAG-1 (g) and *lacZ* (i) expression in the cerebellar cortex of lobules VIII-IX. h shows a double TAG-1/X-gal staining. Scale bars: 40 μm. Sections c-f-i are counterstained with Fast Red.

Deregulation of F3/contactin expression results in a developmentally regulated reduction in cerebellar size

Cerebellar development was followed using Toluidine Blue-stained sections (Fig. 4). In newborn *TAG/F3* mice (Fig. 4C, see also Fig. 6A,B), cerebellar size and foliation pattern were not significantly different from wild type. However, by postnatal day 3 (P3) the relative size of the transgenic cerebellum was slightly reduced (Fig. 4A, a,b), and by P8 the size of the transgenic cerebellum was markedly smaller than controls (Fig. 4A, c,d). Prominent effects were observed in lobules I to V and VIII-X, while lobules VI-VII displayed comparatively minor changes. The reduction in cerebellar size

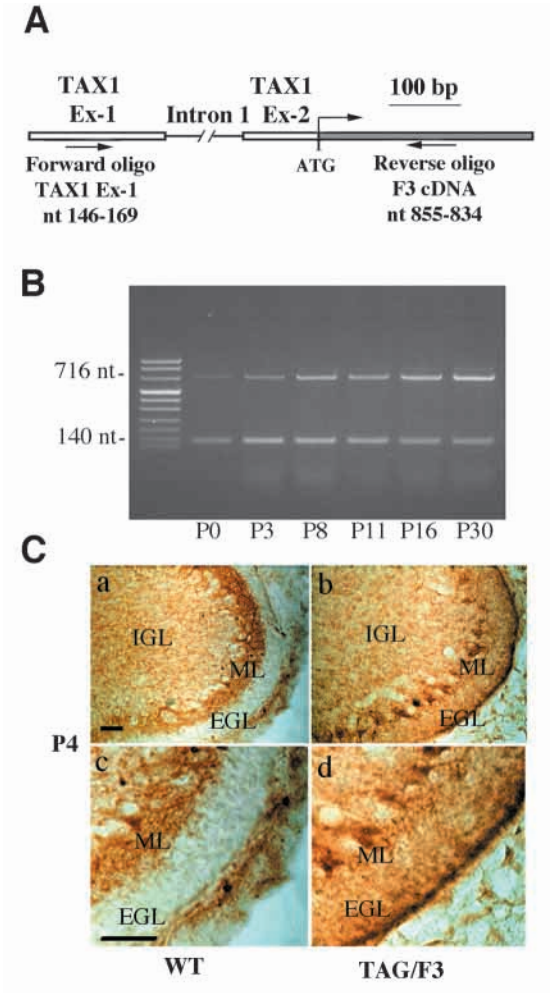

Fig. 3. Expression profile of the *TAX-1/F3* transgene. (A,B) Expression of the *TAX-1/F3* fusion mRNA in transgenic mice cerebellum throughout development, demonstrated by RT/PCR. (A) Amplification scheme. (A) *TAX1* exon 1-derived forward primer (nt 146-169) (Kozlov et al., 1995) was used with an *F3/contactin* exon VII-derived reverse primer (nt 855-834) (Gennarini et al., 1989). (B) A 716 nt band could be detected throughout development. An oligonucleotide pair derived from the mouse *actin* gene generated a 140 nt band we used as an internal control. (C) Expression profile of F3/contactin in the cerebellar cortex of wild-type (a,c) and *TAG/F3* (b,d) mice at postnatal day 4. Scale bars: 40 μm.

persisted at P11; again, lobules I to V and VIII-X were mostly affected (Fig. 4A, e,f). However, by P16, only lobules VIII-IX were still slightly affected (Fig. 4A, g,h), and by P30 the *TAG/F3* cerebellum was indistinguishable from wild-type controls (not shown).

Cerebellar cortical layers are differentially affected by deregulation of F3/contactin as development proceeds

A morphometric analysis was then performed of the overall surface area of the cerebellar sections and of the different cortical layers (see Methods for details). Sample sections are presented in Fig. 4B, and in Fig. 4C the ratios of the absolute values of these parameters in *TAG/F3* versus wild-type mice are plotted. Differences in the surface area of the cerebellar sections, already apparent at P3, become most significant at around P8, followed by a recovery after P16. The areas of both the IGL and the molecular layer (ML) were similarly reduced at P8 to almost half their normal values. By contrast, the EGL, most affected at P3, was already showing signs of recovery by P8, and by P11 had almost completely recovered normal size, even though the IGL and ML were still significantly smaller than normal. This suggested that the primary effect of the transgene was on the number of GCs in the EGL with consequent effects on cell numbers in the IGL. To rule out the alternative possibility that the reduction in the IGL size was due to a reduced development of the surrounding neuropil, we determined the GC density in the IGL at P8. No differences were found between wild-type (198.15±3.47 s.e.m. cells/field) and *TAG/F3* mice (194.78±3.70 s.e.m., P=0.59); similarly, in the EGL, comparable values were obtained for wild-type and *TAG/F3* mice (138.4±4.56 versus 140.04±3.85 cells/field, P=0.8) confirming that changes in the size of the EGL and IGL were due primarily to a reduction in the absolute number of cells. Reduction in the size of the ML will be addressed further below.

F3/contactin overexpression results in changes in granule cell proliferation

The reduction in the number of GCs in the *TAG/F3* cerebellum could depend upon their reduced production in the EGL, their increased death or a combination of both. To test the first possibility, cell proliferation was followed by bromodeoxyuridine (BrdU) incorporation. Significant differences were not detected at P0 (Fig. 5A, a,b; 73.51±1.96 s.e.m. labelled cells in *TAG/F3* versus 70.96±2.21 in wild-type mice/18667 μm^2; P=0.36). However, at P3 (c,d), a lower density of BrdU-labelled cells was apparent in the EGL of the *TAG/F3* mice cerebellum (84.54±2.18 s.e.m. labelled cells/field) versus wild-type mice (103.28±1.58 s.e.m.; P=0.001). Although this difference had disappeared by postnatal day 6 (e,f) (94.68±1.23 for *TAG/F3* versus 96.13±1.19 for wild-type mice, P=0.4), by P8 the situation was reversed with a significantly higher level of BrdU labelling in *TAG/F3* mice (g,h) (89.38±1.15 versus 82.28±1.21 cells/field, P=0.001). This latter effect was also developmentally regulated since by P11 it was no longer evident (i-j) (46.04±1.03 labelled cells/field for *TAG/F3* versus 46.68±1.0 for wild-type mice; P=0.6). Thus, overexpression of F3/contactin under the TAG-1 promoter resulted in developmentally regulated changes in GC proliferation, with a significant decrease in early development followed by an apparently compensatory increase at the end of the first postnatal week.

BrdU incorporation was also measured in high density (0.3×10^6 cells/cm^2) dissociated cultures from P3 cerebellum (Fig. 5B). In these conditions, while comparable values were obtained when the labelling was performed immediately after plating (a,c) (497±30.4 s.e.m. labelled cells/77440 μm^2 in wild-type versus 470±26.2 in *TAG/F3* mice, P=0.33) a consistent difference was observed in cultures labelled 16 hours after plating (b,d) (174±10.3 in wild-type versus 82±4.9 in *TAG/F3* mice, P=0.0001). These data, reported in graphic form in Fig. 5C, indicated that GCs overexpressing F3/contactin drop out of cell cycle more rapidly than normal.

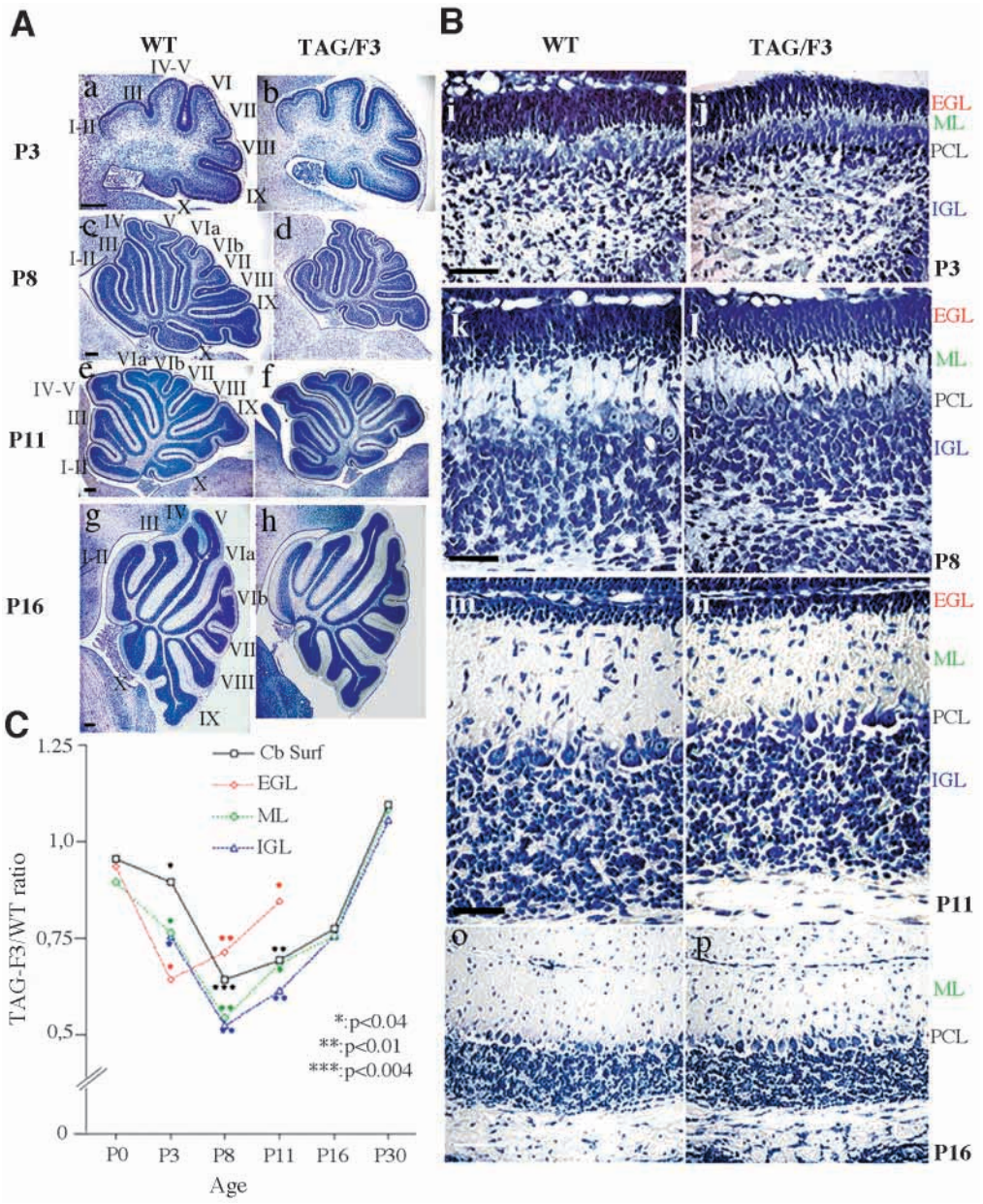

Fig. 4. Cerebellar phenotype of developing wild-type and *TAG/F3* mice shown in sagittal sections stained with Toluidine Blue. (A) Low magnification images of P3 (a,b), P8 (c,d), P11 (e,f) and P16 (g,h) cerebella. In sections from wild-type mice the lobules are indicated by roman numerals. Scale bars: 200 μm. (B) Differences in layer morphology between wild-type (i,k,m,o) and *TAG/F3* (j,l,n,p) mice, shown in lobule VIII at P3 (i,j), P8 (k,l), P11 (m,n), P16 (o,p). The different cortical layers are indicated on the right side. EGL, external germinal layer; ML, molecular layer; PCL, Purkinje cells layer; IGL; inner granular layer. Scale bars: (i-n) 40 μm; (o,p) 100 μm. (C) Morphometric analysis of *TAG/F3* and wild-type mice cerebella. The overall surface areas of the cerebellar sections, the EGL, IGL and ML, from P0 to P30, were expressed as the ratio of their values in transgenic versus wild-type mice. Asterisks refer to statistical significance of the differences estimated in the absolute values of the different parameters.

TAG-1 is expressed on mitotically active granule cells

The observation that expression of F3/contactin from the *TAG-1* promoter affects cell proliferation was unexpected since TAG-1 has usually been described on post-mitotic neurons (e.g. Kuhar et al., 1993). To test the possibility that some TAG-1-expressing cells may still be proliferating in the EGL, we double labelled P8 wild-type cerebellum with antibodies to TAG-1 and to markers of mitotic activity, including proliferating cells nuclear antigen (PCNA) (Ino and Chiba, 2000) and phosphohistones H1 and H3 (Lu et al., 1994; Hendzel and Bazett-Jones, 1997). As shown in Fig. 5D, TAG-1 expression (b) overlapped significantly with both PCNA (a,c) and phosphohistones H1 and H3 (d). This indicated that a substantial proportion of the TAG-1-expressing cells in the iEGL are still mitotically active (~20% (45/215) of TAG-1+ cells also expressed medium to high levels of PCNA) and, therefore, that F3/contactin is likely to be expressed on dividing cells in *TAG/F3* mice.

Minor differences in cell death occur in *TAG/F3* mice

Cell death was estimated in situ by the TUNEL method (see Materials and Methods). In Fig. 6A sample elements undergoing apoptosis are shown at postnatal day 3 in lobule IX of both wild-type and *TAG/F3* mice cerebella and in Fig. 6B the overall density of such elements in the whole cerebellar cortex is reported from P0 to P8. No differences were observed between wild-type and transgenic mice at P0 (78±5.7 versus 74±4.0 cells/mm^2) and P6 (113±6.8 versus 105±7.2 cells/mm^2). However, at P3 the number of apoptotic cells was significantly higher in *TAG/F3* (180±11.9 cells/mm^2) versus wild-type (125±8.3) mice (P=0.001) whereas at P8 the reverse

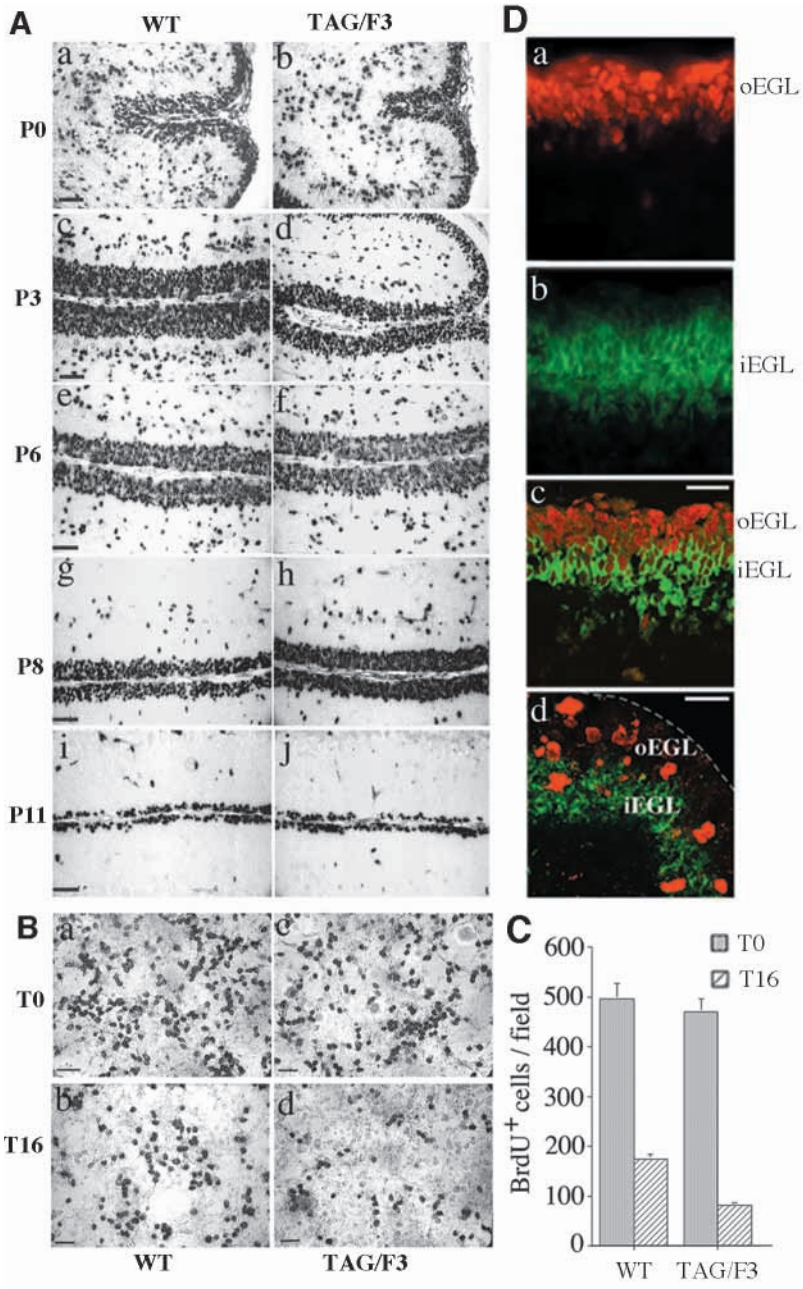

Fig. 5. (A) Analysis of precursor granule cell proliferation in the developing cerebellum of wild-type and *TAG/F3* mice. 5-bromo-2′-deoxyuridine (BrdU) incorporation in lobules VIII-IX of developing cerebellar cortex from wild-type (a,c,e,g,i) and *TAG/F3* (b,d,f,h,j) mice at P0 (a-b), P3 (c-d), P6 (e-f), P8 (g-h) and P11 (i-j). Scale bars: 40 μm. (B) Analysis of cell proliferation in primary cultures from postnatal day 3 cerebellum. BrdU incorporation is shown in primary cerebellar cultures from wild type (a,b) or *TAG/F3* (c,d) mice labelled immediately after plating (T0) or 16 hours later (T16). Scale bars: 40 μm. In C the data are shown in graphic format. (D) Epifluorescent labelling of P8 cerebellar cortex from wild-type mice with an anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (a) and a rabbit anti-TAG-1 serum (b). (c) Confocal optical section of the same field as a and b, showing PCNA (red) and TAG-1 (green) expression. (d) Confocal optical section of P8 cerebellar cortex from wild-type mice labelled with polyclonal antibodies to phosphohistones H1 and H3 (red) and monoclonal anti-TAG-1 antibodies (green). Note that phosphohistones H1 and H3 label mitotic cells most strongly, while other phases are marked by weaker phosphohistone H1 expression alone (Lu et al., 1994); this image was contrast-enhanced to emphasise cells in mitosis thus de-emphasising labelling in other phases of the cycle. The pial surface is marked by a dashed line. Scale bars: 20 μm.

situation was observed (66±1.9 in *TAG/F3* versus 94±3.6 in wild-type mice, P=0.001). Thus, changes in cell death mirror those in cell proliferation, with cell death increasing when proliferation drops and vice versa. However, whereas the changes in cell proliferation were observed in GC within the EGL, changes in cell death mostly affected postmitotic cells in the IGL.

TUNEL staining was also performed in high density primary cultures from P3 cerebellum plated on polylysine. Sample elements undergoing apoptosis are shown in Fig. 6C in both wild-type and *TAG/F3* mice. When their number was estimated in fields of identical size (77440 μm^2), comparable values were obtained in wild-type and *TAG/F3* mice (67±4.2 s.e.m. labelled cells/field in wild-type versus 62±3.1 in *TAG/F3* mice; P=0.44).

Developmentally regulated changes of F3/contactin expression in the *TAG/F3* mice

The above data indicated that ectopically expressed F3/contactin in the EGL of the *TAG/F3* cerebellum transiently inhibits GC proliferation. Accordingly, the increase in cell proliferation at P8 might be expected to correlate with a downregulation of F3/contactin. However, RT-PCR analysis indicated persistent transgene mRNA expression at this time (Fig. 3B), suggesting that overall F3/contactin protein levels might not reflect mRNA profile of the transgene. To test this possibility we performed F3/contactin immunohistochemistry throughout the postnatal period in both wild-type and *TAG/F3* cerebella.

As shown in Fig. 7A, a,b, at P0 consistent F3/contactin

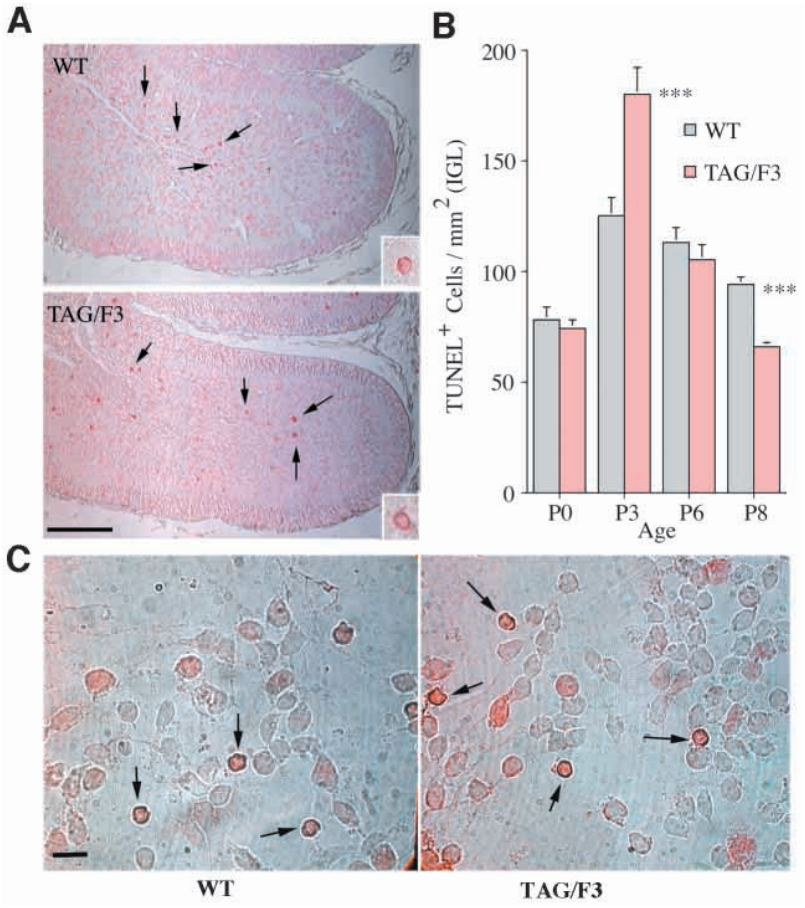

Fig. 6. Changes in cell death in developing wild-type and *TAG/F3* mice cerebella. (A) TUNEL staining in lobule IX of wild-type (WT) and *TAG/F3* mice cerebellum at postnatal day 3. Arrows indicate the TUNEL-positive elements, higher magnification views of which are shown in the insets. Scale bar: 100 μm. (B) Histogram showing the density of apoptotic cells in cerebellar sections from both wild type and *TAG/F3* mice from P0 to P8. Asterisks refer to statistical significance of the differences observed between the two genotypes. ****P*=0.001. (C) Sample of TUNEL-stained elements (arrows) in high density primary cerebellar cultures from postnatal day 3 wild-type and *TAG/F3* mice (Bar=10 μm).

overexpression was observed in the *TAG/F3* mice which, with the exception of lobule X, occurred in both the anterior and posterior lobes. This overexpression was evident on migrating GC within the nascent ML as shown in lobules II-III (Fig. 7B, k,l) and VI-VII (m-n). Comparison of adjacent sections stained for calbindin (Fig. 8A, d) indicated that some of this staining was on PCs (see below). This overexpression was still evident throughout the cerebellar cortex at P3 (Fig. 7A, c,d). This was especially evident for lobules I-IV and X which in wild-type mice express very low levels of F3/contactin (Virgintino et al., 1999). At this stage, F3/contactin is on both the perikarya and forming neurites of migrating GCs and on nascent PC dendrites (Virgintino et al., 1999); in *TAG/F3* mice, F3/contactin was observed on these neurons at clearly elevated levels, as shown for lobules II-III (o,p) and VI-VII (q,r); PCs were identified by comparison with adjacent sections stained with calbindin (Fig. 8A, g,h). These alterations were stronger in lobules VI-VII (q,r), than in II-III (o,p), in agreement with the higher level of transgene activation in the former (see Fig. 2B). Thus, at P0, P3 and P4 (Fig. 3C), levels of F3/contactin protein were elevated in *TAG/F3* cerebellum, as predicted by the levels of transgene mRNA.

In contrast, by P8 F3/contactin protein expression in *TAG/F3* mice (f) appeared, if anything, slightly reduced compared to the controls (e). At higher magnification (s,t) this correlated with markedly reduced width and labelling of the ML, and less elaborate dendrites of PCs whose perikarya were notably smaller than in controls. At P11 (g,h), the level of F3/contactin expression was partially recovered in *TAG/F3* mice. This reflected increased expression on PCs, which by this time had partially recovered their normal morphology, as well as on GC axonal extensions in the ML (u,v). By P16 (i,j,w,y) no differences could be observed among the different cortical layers. F3/contactin immunostaining was restricted to the ML and, as previously reported (Virgintino et al., 1999), no expression could be observed on PCs.

Together, these data suggested that, in contrast to the situation at P0-P3, from P8-P16 there was no evidence of F3/contactin protein overexpression in *TAG/F3* mice, even though the levels of transgene mRNA remained relatively constant throughout this period (Fig. 3B). Indeed, at P8 we detected lower levels of F3/contactin than in wild type which, together with decreased ML width and PC dendrite arborisation, suggested delayed differentiation of granule and Purkinje neurons.

Biphasic effects on Purkinje cells differentiation in *TAG/F3* transgenic mice

The effects of the transgene on PC development were further studied by calbindin immunostaining. Similar to the elevated F3/contactin expression seen at birth (Fig. 7), calbindin expression in *TAG/F3* mice (Fig. 8A, b,d) was also higher than in controls (a,c). This premature expression was restricted to lobules I-V and IX but at P3 was also apparent on lobule X (e,f). Thus, by this criterion, PCs begin to differentiate prematurely in *TAG/F3* mice.

However, there was a reduction in the arborisation of Purkinje cell dendrites at P3 (g,h) in transgenic mice, as suggested by F3/contactin immunostaining (Fig. 7). By P8, this reduction, together with a reduction in PC cell body size, became striking (Fig. 8A, i,j). Similar, though reduced differences were apparent at P11 (k,l), but by P16 (m,n) transgenic PCs took on an essentially normal appearance. Morphometric analysis at P8 indicated an approximately threefold reduction of the overall extension of the PC dendritic tree in *TAG/F3* mice (801.37 μm^2±27.29 s.e.m. versus 273.54 μm^2±8.39/field; *P*=0.007). When studied at higher power in thicker sections (20 μm) from postnatal day 6 cerebellar cortex (Fig. 8B) Purkinje cells dendrites clearly displayed a simplified branch network, suggestive of a delayed growth of the spiny branchlet compartment.

These results are consistent with an apparent delay in PC

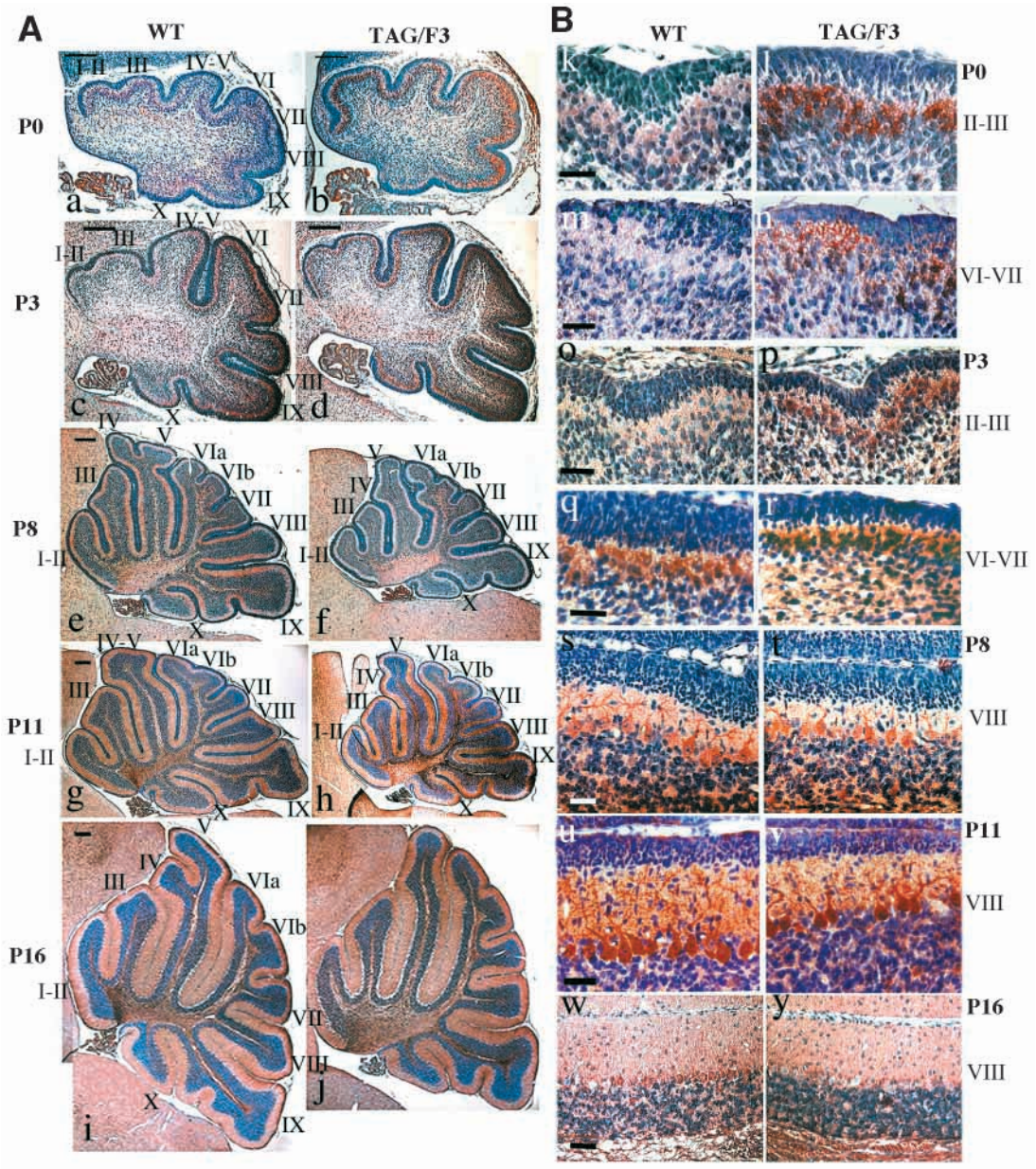

Fig. 7. Developmental profile of F3/contactin expression in the cerebellum of wild-type (a,c,e,g,i,k,m,o,q,s,u,w) and *TAG/F3* (b,d,f,h,j,l,n,p,r,t,v,y) mice shown at postnatal days 0 (a,b; k-n), 3 (c,d; o-r), 8 (e,f; s,t), 11 (g,h; u,v) and 16 (i,j; w,y). (A) Low and (B) high power images. In B, parts k, l, o, p correspond to lobules II and III; parts m, n, q, r correspond to lobules VI and VII. Different levels of TAX-1 promoter activity were observed in these lobules (Fig. 2B). Sections were immunostained with F3/contactin antibodies and counterstained with Hematoxylin (blue). Scale bars: (a-j) 200 μm; (k-v) 40 μm; (w-y) 100 μm.

maturation. However, their overall number per section was not significantly different from wild type (411±44.05 s.e.m. in *TAG/F3* versus 527±58.2; P=0.14), their cell bodies were aligned in a single row and their dendrites were properly oriented along parasagittal planes, indicating that other aspects of their development were normal.

Thus, the data above confirmed that the terminal differentiation of PCs is delayed in *TAG/F3* mice. Paradoxically, however, premature elevation of calbindin expression suggested that aspects of their early differentiation may occur prematurely.

F3/contactin misexpression in granule cells enhances axonal fasciculation and inhibits axonal growth in vitro

The reduced width of the ML and delayed PC dendritic arborisation seen in *TAG/F3* mice from P3-P11 suggested that, in addition to effects on granule cell proliferation, F3/contactin misexpression may affect granule cell axon growth. We tested this in granule cell reaggregate cultures in vitro. In Fig. 9A-D, F3/contactin immunostaining of 2 day-old reaggregate cultures from P7 wild-type (A,C) and *TAG/F3* (B,D) mice cerebella are shown. In *TAG/F3* mice, neurites appeared shorter and the

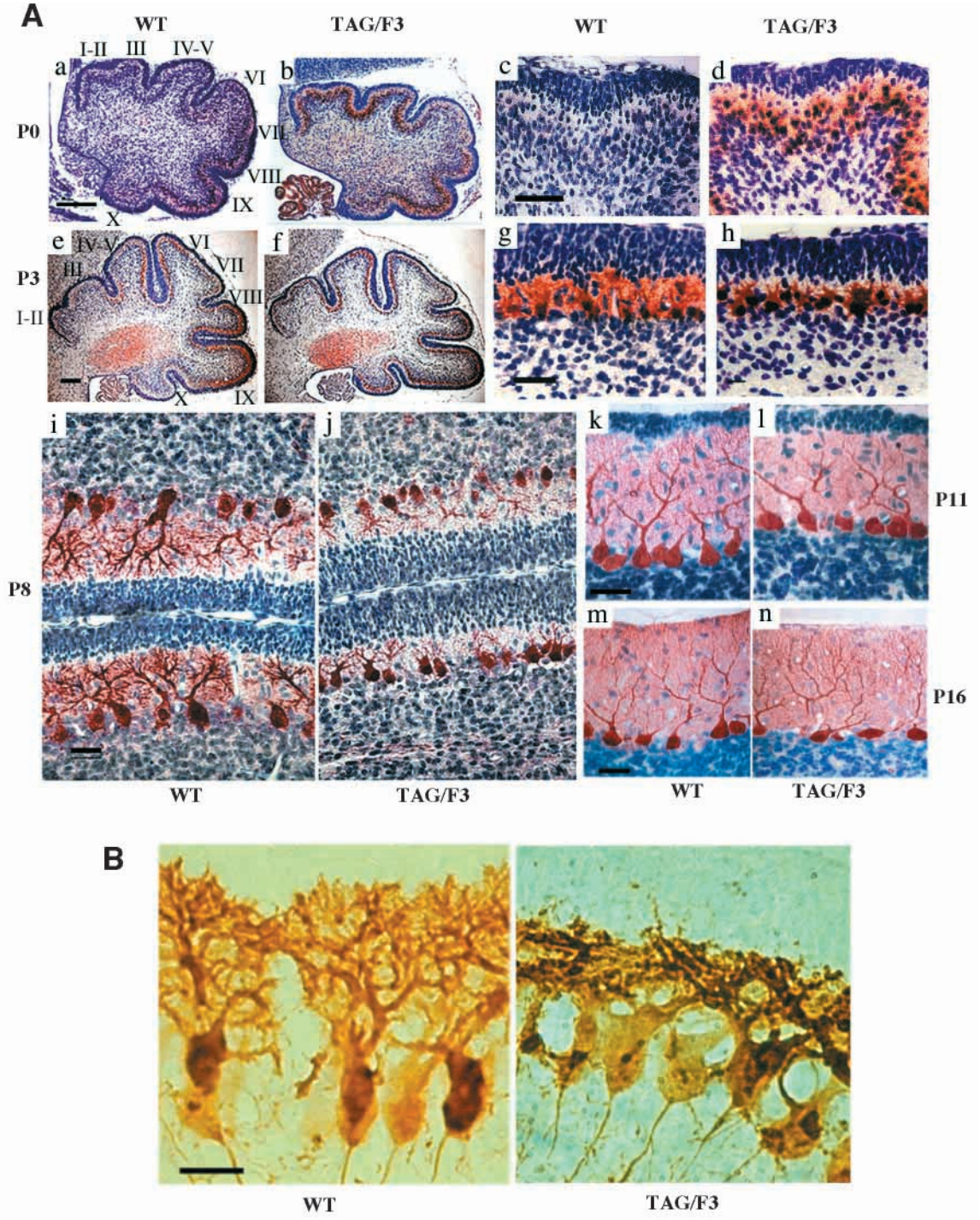

Fig. 8. (A) Developmental changes of Purkinje neurons in wild-type (a,c,e,g,i,k,m) and *TAG/F3* (b,d,f,h,j,l,n) mice as revealed by calbindin immunostaining at postnatal day (P) 0 (a-d), P3 (e-h), P8 (i,j), P11 (k,l) and P16 (m,n) in thin (5 μm) paraffin sections from cerebellar cortex. High-power images in c and d correspond to lobules II and III, and images in g-n correspond to lobule VIII. Scale bars: (a,b,e,f) 200 μm; (c,d,g-n) 40 μm. (B) Phenotype of Purkinje cells dendritic tree shown in thick (20 μm) cryostat sections from wild type (WT) and *TAG/F3* mice cerebella (lobule IX) labelled with calbindin antibodies at postnatal day 6. Bar: 20 μm.

surface area they covered was significantly lower than in controls (1.82 mm^2±0.10 s.e.m. versus 2.74±0.12; P=0.0001). Consistent with this, neurites displayed a strong tendency to fasciculate, noticeably more so than in cultures from wild-type mice (C,D). To discriminate between the effects on neurite fasciculation and growth, dissociated cultures of cerebellar neurons were generated and grown on CHO cells monolayers. In Fig. 9E-F, representative cultures from wild-type (E) and transgenic (F) cerebellum at P7 are shown, stained with GAP-43 antibodies and in G neurite growth is quantified by

cumulative neurite length histogram. Cultures from transgenic mice developed shorter neurites than the controls (60.43±2.9 s.e.m. versus 106.02±3.7 μm; P=0.0014), indicating that, as well as enhancing axonal fasciculation, F3/contactin misexpression also exerted a specific inhibitory effect on neurite growth from cerebellar neurons.

DISCUSSION

In this study we show that deregulation of the expression of the axonal glycoprotein F3/contactin in transgenic mice under the control of *TAG-1* promoter elements transiently disrupts cerebellar development. This indicates for the first time that the precise regulation of this class of neural cell adhesion molecule, evident from several descriptive studies (Faivre-Sarrailh et al., 1992; Wolfer et al., 1994; Wolfer et al., 1998; Stottman et al., 1998; Virgintino et al., 1999) is a critical part of their developmental function. A major cause of the developmental disruption appears to be the effect of F3/contactin expression on cell proliferation, suggesting a novel role for this class of molecule in controlling progression from proliferation to differentiation.

TAG/F3 transgenic mice display a developmentally regulated decrease of the cerebellar size

The most striking effect we observe in *TAG/F3* mice was a decrease in the cerebellar size, predominant at the end of the first postnatal week. This appears to depend primarily upon a reduction in granule cell number, first obvious in the EGL at P3 and in the IGL at P8, and secondly upon a parallel reduction in the growth and fasciculation of their axons in the ML.

F3/contactin expression affects granule cell proliferation

Members of the L1-like family have been proposed to have roles in cell migration, axon growth, fasciculation and guidance, and in synaptic plasticity (Schachner, 1997; Walsh and Doherty, 1997; Kamiguchi and Lemmon, 2000). Here we show that premature expression of F3/contactin in GCs in the EGL from P0 to P3 is accompanied by a nearly 20% reduction in the number of proliferating GCs. The simplest interpretation of our observations is that F3/contactin expression antagonises proliferation-promoting contacts between GCs (Gao et al., 1991), perhaps by stimulating cell repulsion, as has been observed when F3/contactin acts as a receptor for tenascin-R in vitro (Pesheva et al., 1993; Xiao et al., 1996). In fact, in preliminary studies, no changes of the adhesive behaviour of granule cells could be directly demonstrated in primary cultures from *TAG/F3* mice (data not shown); however, the possibility cannot be excluded that more subtle effects may occur in vivo among contacting granule cells, resulting in a reduction of their adhesive strength. Alternatively, the effects on GC proliferation could depend upon changes in PC differentiation, which were evident as early as P0 in *TAG/F3* mice, particularly since it is known that GC proliferation depends on PC-derived signals (Wetts and Herrup, 1983; Smeyne et al., 1995; Baader et al., 1998; Chomez et al., 2000; Dahmane and Ruiz i Altaba, 1999; Wechsler-Reya and Scott, 1999). However, since the *TAX-1* promoter used for transgene generation is not expressed in PCs, it is likely that these changes are anyway initiated through contacts with F3/contactin-overexpressing GCs. Therefore, we favour the hypothesis that F3/contactin expression in granule cells directly modulates proliferation. In support of this, our in vitro experiments show that granule cells from *TAG/F3* mice cultured at high density have substantially reduced proliferative capacity.

An important question is whether this F3/contactin effect has physiological significance. For this to be the case, F3/contactin would be expected to be found on the proliferating cells themselves, or on cells with which they contact. Our data indicate that F3/contactin-expressing GCs normally are separated from the proliferative oEGL by TAG-1-expressing cells (Fig. 1), conventionally thought to be post-mitotic.

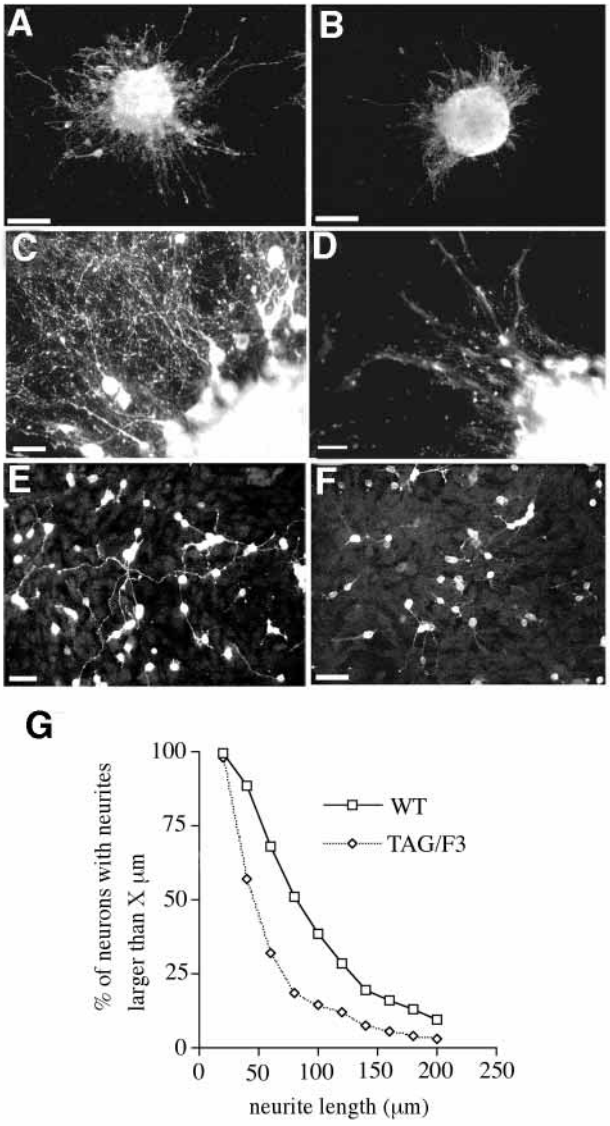

Fig. 9. Primary aggregate (A-D) and dissociated (E-F) cultures from postnatal day 7 cerebella derived from either wild-type (A,C,E) or *TAG/F3* (B,D,F) mice, stained with anti-F3/contactin (A-D) or anti-GAP43 (E-F) rabbit antibodies. Bars: (A,B) 100 μm; (C,D) 10 μm; (E,F) 40 μm. (G) A cumulative neurite length graph in which the percentage of neurons with neurites of a given length (y axis), is plotted against neurite length (in the x axis).

However, as suggested by in vitro studies (Wolf et al., 1997), we have found that a substantial proportion of TAG-1-expressing GCs also express cell proliferation markers (Fig. 5D). Thus, it seems plausible that in normal development, expression of F3/contactin on or close to proliferating TAG-1-expressing cells may contribute to the process of cell cycle exit. Precedent for a similar role for this class of molecule comes from studies in vitro in which soluble NCAM was found to inhibit hippocampal neuronal progenitor proliferation and concurrently induce differentiation (Amoureux et al., 2000).

In several mouse mutants reduced cerebellar size has been attributed in part to increased cell death (Sonmez and Herrup, 1984; Herrup and Sunter, 1987; Smeyne and Goldowitz, 1989; Harrison and Roffler-Tarlov, 1998; Doughty et al., 2000). While we also observed an increase in cell death in the IGL early on, the changes we saw were small (a maximum increase of 40%) compared to, for instance, those seen in the *rev-erbAα* mutant mouse (up to 400% increase) (Chomez et al., 2000) and even in this case the effects on cerebellar size were small. We believe, therefore, that effects on cell death have only a minor role in the phenotype observed in *TAG/F3* animals. In any case the effects we observe are likely to be developmental in nature, triggered by modified interactions among cerebellar neurons as no differences in cell death could be observed in primary cultures.

Effects on neurite growth and fasciculation

While the smaller number of GCs in the IGL may simply reduce axon input to the ML our observations of reduced neurite outgrowth from transgenic GCs in primary culture, accompanied by an increase in axon fasciculation, suggest that F3/contactin misexpression also directly affects axon growth. In previous studies, substrata of F3/contactin were found to exert inhibitory effects on neurite growth and to promote fasciculation in primary cerebellar cultures (Buttiglione et al., 1996; Buttiglione et al., 1998). Moreover, results from our gain-of-function experiment are exactly complementary to those from the F3/contactin loss-of-function mouse insofar as aggregate cultures from those mice exhibited a decrease in neurite fasciculation and, in vivo, their parallel fibres were less compacted (Berglund et al., 1999). Although we have not checked this directly by electron microscopy, the reduction in the width of the ML in *TAG/F3* mice is consistent with an increase in the compaction of parallel fibres. Together these results confirm a direct role for F3/contactin in parallel fibre growth and fasciculation. However, the occurrence of comparable levels of apoptosis in cultured GC from *TAG/F3* and wild-type mice together with the minor differences occurring in vivo exclude the possibility that the effects on neurite growth from GC may reflect differences in their viability.

In other ways, our results were not predicted from previous experiments. Indeed, co-expression of F3/contactin and TAG-1 in CHO cells as a substratum negated the inhibitory effects on GC neurite outgrowth and fasciculation that F3/contactin had when present alone (Buttiglione et al., 1998). In *TAG/F3* mice, however, F3/contactin was overexpressed as a receptor rather than as a substratum, which may invoke different binding partners (Pesheva et al., 1993; Xiao et al., 1996); in addition, evidently higher levels of F3/contactin may have outweighed TAG-1 modulatory effects.

Purkinje neuron terminal differentiation is delayed in *TAG/F3* transgenic mice

Unlike GCs, no significant changes were observed in the number of PCs, consistent with the time course of their proliferation which may be complete before the onset of transgene activation (Altman and Bayer, 1985). However, increased calbindin and F3/contactin expression at P0 suggested that early differentiation events occurred prematurely. Therefore as in the case of peripheral neurons (Gennarini et al., 1991), F3/contactin may positively modulate PC differentiation. However, since the transgene is not activated on Purkinje neurons, these effects are likely to be triggered by F3/contactin expressed at the surface of contacting granule cells or released in soluble form in their microenvironment (see Fig. 7). Paradoxically, however, by P3, and most significantly at P8, the PC morphological differentiation was delayed, as judged by reduced extension and arborisation of their dendrites. Again, these effects seem to be non-autonomous and may reflect the known interactions between granule and Purkinje cells (Hatten and Heintz, 1995; Altman and Bayer, 1997). In particular, they may arise from the delayed development of GC axons since PC dendritic growth can be regulated by interactions with outgrowing parallel fibres (Sotelo, 1978; Baptista et al., 1994; Altman and Bayer, 1997; Morrison and Mason, 1998; Catania et al., 2001).

The effects of F3/contactin misexpression are developmentally regulated

The restoration of normal cerebellar morphology in *TAG/F3* mice after P8 was particularly surprising as, at the mRNA level, the transgene is expressed at high levels until at least P30, reflecting normal TAG-1 expression (Furley, 1990; Wolfer, 1994; Yoshihara, 1995). At least two factors may contribute to this recovery. First, while ectopic expression of the F3/contactin protein is clearly evident at P4 (Fig. 3), by P8 its levels are lower than in controls (Fig. 6 and data not shown), potentially reflecting delayed neuronal differentiation in *TAG/F3* mice. Second, there is evidence to suggest that F3/contactin function may change during the course of the first postnatal week, reflecting changes in the expression profile of some of its binding partners. For instance, tenascin R, whose interaction with F3/contactin is inhibitory for axonal growth (Pesheva et al., 1993), is upregulated on both myelinating cells and neurons at the end of the first postnatal week, when we observe the strongest phenotypic alterations in the ML and IGL, and is downregulated thereafter when we observe recovery (Fuss et al., 1993), suggesting that some aspects of *TAG/F3* phenotype could be accounted for by developmentally regulated interactions between F3/contactin and tenascin R. Indeed, we find that the developmental profile of expression of tenascin R mRNA (estimated by quantitative RT/PCR) in *TAG/F3* mice is similar to those found in wild-type mice (data not shown).

An alternative, but not exclusive possibility is that the mechanism of GC maturation may change with time with respect to F3/contactin function. Indeed, loss of F3/contactin only appears to affect later-differentiating granule cells (Berglund et al., 1999), whereas our gain-of-function experiment seems to affect early-differentiating GCs. Thus, although granule cell differentiation is often assumed to be a uniform process operating throughout the first two postnatal

weeks (e.g. Kuhar et al., 1993), these observations suggest that, at least with regard to F3/contactin function, it may be divided into two phases. The first one operates perinatally and involves the ability of F3/contactin to modulate granule cell precursor proliferation, the second begins towards the end of the first postnatal week and mostly relates to axonal growth control. This differential role during development in turn may depend upon regulated interactions of F3/contactin with its various receptors that are known to have different profiles of developmental activation (Pesheva et al., 1993; Xiao et al., 1996; Buttiglione et al., 1998; Volkmer et al., 1998; Faivre-Sarrailh et al., 1999).

Conclusions

Together, our data strongly support our main hypothesis that the precise regulation of axonal CAMs expression is critical to normal morphogenesis of the cerebellum. In particular, the differential deployment of the antagonistic functions of F3/contactin and TAG-1 may define different stages of cerebellar neuron differentiation. F3/contactin may be important in promoting cell cycle exit, stabilising axon growth and inducing early events of Purkinje cell differentiation. By contrast, the antagonistic activity of TAG-1, suggested by a previous study (Buttiglione et al., 1998), may allow granule cells to continue dividing while extending axons and migrating. Our results also indicate that the emphasis on these differing activities seems to change with time; in the first postnatal week, low F3/contactin activity appears to be critical for the cerebellum to expand appropriately, whereas its upregulation in the second week may be important to stabilise axonal extensions and to take part in the process of shutting down granule cell proliferation.

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