INTRODUCTION

Correct development of the central nervous system (CNS) requires the migration, proliferation and survival of precursor cells for neurones and glia. Extracellular matrix (ECM) molecules and their integrin cell surface receptors are important regulators of these aspects of cell behaviour, and genetic studies using naturally occurring mutants or transgenic mice have revealed essential roles in development for some of the ECM glycoproteins and integrins expressed in the CNS. Mutations in reelin (D'Arcangelo et al., 1995) and anosmin 1 (Ballabio and Camerino, 1992), as well as in the laminin receptor alpha 3 integrin (Anton et al., 1999), cause abnormalities of neuronal migration and cortical lamination. Mutations in another laminin receptor, alpha 6 integrin (Georges-Labouesse et al., 1998), cause excessive numbers of neurones in ectopias on the cortical surface. With other ECM glycoproteins expressed in the CNS, however, studies of knockout mice have been surprising in that they have not revealed obvious roles in development. A good example is provided by tenascin-C (TN-C), a glycoprotein assembled from six monomers via disulphide bridges to form a hexameric protein (Erickson and Inglesias, 1984). TN-C is widely expressed at early stages of CNS development and is then downregulated during further differentiation (Bartsch et al., 1992; Bartsch et al., 1994; Crossin, 1996; Crossin et al., 1989; Gotz et al., 1997; Joester and Faissner, 1999; Mitrovic et al., 1994). In the adult CNS, expression remains in a few specific cell populations, including those adjacent to areas of active neurogenesis, such as the hippocampus, the borders of the subventricular zone and the rostral migratory stream used by olfactory neurone precursors en route to the olfactory bulb (Bartsch et al., 1992; Bartsch et al., 1994; Gates et al., 1995; Jankovski and Sotelo, 1996; Miragall et al., 1990). TN-C is also re-expressed in the adult brain at sites of injury (Crossin, 1996; Deller et al., 1997; Gates et al., 1996; Laywell et al., 1992). These expression patterns suggest important roles for TN-C in the modulation of cell behaviour during periods of active CNS modelling and plasticity. In support of this, studies using either neurones or glia have revealed that TN-C can significantly alter CNS cell behaviour, often in a cell type-specific manner. TN-C has been reported to deflect growth cones of embryonic mesencephalic neurones, embryonic hippocampal neurones and postnatal cerebellar neurones (Crossin et al., 1990; Faissner and Kruse, 1990; Faissner and Steindler, 1995; Taylor et al., 1993), as well as inhibiting the migration of oligodendrocyte precursor (OP) cells (Frost et al., 1996; Kiernan et al., 1996). At the same time, TN-C promotes...
neurite outgrowth (Crossin et al., 1990; Faissner and Steindler, 1995; Husmann et al., 1992; Lochter and Schachner, 1993; Lochter et al., 1991; Taylor et al., 1993) and stimulates granule cell migration (Chuong et al., 1987; Husmann et al., 1992).

Given the pattern of expression and the multiplicity of effects in cell culture, the observation that TN-C-null transgenic mice show no obvious abnormalities (Forsberg et al., 1996; Saga et al., 1992) was unexpected. More recent studies have revealed behavioural abnormalities, alterations in neurotransmitter levels in the adult CNS (Fukumauchi et al., 1996; Kiernan et al., 1999) and alterations in the pattern of glomerulonephritis that occurs after administration of renal toxins (Nakao et al., 1998); all these are dependent on the genetic background of the mice. Changes in the architecture of the neuromuscular junction have also been described (Cifuentes-Diaz et al., 1998), although these were not confirmed in a separate study (Moscoso et al., 1998), which may also reflect genetic background effects (see Kiernan et al., 1999 for discussion). However, the phenotype as currently described includes none of the CNS abnormalities that might be expected from previous work. There are two possible explanations for this result. Either TN-C is a largely redundant protein, which seems unlikely given the degree of evolutionary conservation (Erickson, 1993a; Erickson, 1993b; Fassler et al., 1999). Alternatively, TN-C does have specific and important roles during early stages of development but later developmental processes can correct for the loss of TN-C function. An example of such developmental correction is provided by transgenic mice that express high levels of platelet-derived growth factor (PDGF). These mice show increased OP proliferation that increases precursor cell number well above normal levels, although final oligodendrocyte numbers are normal as a result of increased cell death (Calver et al., 1998). It follows that defining a role for potentially important ECM glycoproteins within the CNS may require developmental studies of knockout mice, in which one is able to analyse different aspects of precursor cell behaviour within the intact CNS, rather than an examination of the adult phenotype. We now report such an analysis for TN-C knockout mice. To examine precursor cell behaviour we focused on the OP cells that give rise to myelin-forming oligodendrocytes. These cells arise within the germinal zones and then show phases of migration, proliferation and target-dependent programmed cell death that are characteristic of precursor cells in the developing CNS (Calver et al., 1998; Levison et al., 1993; Levison and Goldman, 1993; Levison and Goldman, 1997; Levison et al., 1999). This developmental analysis has revealed contributions of TN-C that are not apparent from studies of the adult mouse. We have confirmed the role in OP cell migration suggested by the cell culture studies, have demonstrated a novel role in the regulation of neural precursor cell proliferation and provide evidence that reduced levels of programmed cell death provide a corrective mechanism that explains the normality of the final phenotype.

MATERIALS AND METHODS

Animals

TN-C-null mice were derived in a C57Bl/6J and CBA background from the original stock described by Saga et al. (Saga et al., 1992) and age-matched wild-type mice were C57Bl/6J × CBA F1. For littermate experiments heterozygous and homozygous TN-C-null mice were obtained from a cross between heterozygous and homozygous TN-C-null breeding pairs with either a C57Bl/6J/CBA background or a 129 background, as described previously (Kiernan et al., 1999). The genotype was determined by PCR analysis of genomic DNA from tails digested in lysis buffer (10 mM Tris, pH 8.0, 50 mM NaCl, 100 mM EDTA, 0.5% SDS, 0.4 mg/ml proteinase K) for 14-18 hours at 56°C. Genomic DNA was isolated and amplified by PCR using the following primers: 5′-GAG GTG ACT AGA AAC TAG TGG ACA ACT C-3′ and 5′-AGG ATG CCT GGC AGT AGC CAG GTC AC-3′ (directed against the gene for TN-C); and 5′-CTC CAT GCT TGG AAC AAC GAG CGC AGC-3′ (corresponding to the lacZ sequence). lacZ expression in sections from the TN-C transgenic mice was assayed by β-galactosidase activity detected by X-gal staining solution (containing 2 mM MgCl2, 5 mM K3Fe(CN)6, and 5 mM K4Fe(CN)6, in PBS 1X pH 7.4, plus 0.01% sodium deoxycholate, 0.02% NP-40, and 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-D-galactoside)).

PDGFαR in situ hybridisation

Optic nerves were removed from heads immersed in 4% paraformaldehyde (PFA) in phosphate buffer saline pH 7.4 (PBS), embedded in Tissue-Tek OCT compound (Agar Scientific), frozen on dry ice and stored at −80°C until use. Coronal serial cryostat sections (15 µm) were obtained by cutting the optic nerves from the retinal to the chiasmal end. In situ hybridisation using a 1637 bp EcoRI cDNA fragment encoding most of the extracellular domain of mouse PDGF α receptor (PDGFαR) cloned into Bluescript KS (a kind gift from W. Richardson, University College, London) was then performed as described (Kiernan et al., 1999). Average cell numbers/section were calculated at different distances from the retina. This method will slightly overestimate the actual cell numbers, as discussed by Guillery and Herrup (Guillery and Herrup, 1997), but does allow a direct comparison between wild-type and knockout animals that can be used to determine the position of the leading edge of cell migration along the nerve.

Cell migration assays

Cell migration studies using rat oligodendrocyte precursors migrating out of agarose drops on astroglial matrix derived from either wild-type or TN-C-deficient mice were performed as described previously (Milner et al., 1996).

BrdU incorporation in vivo and immunohistochemistry

Animals received two injections of BrdU (100 µg/g body weight) at 1 hour intervals. One hour after the last injection, animals were sacrificed, the brains snap frozen in isopentane cooled by liquid nitrogen (~30°C to ~40°C) and cryosections (10 µm) collected. Frozen sections were thawed and fixed in ethanol 95%/acetic acid 5% (v/v) for 15 minutes, then washed in PBS and incubated for 1 hour in blocking solution containing 10% normal goat serum (Sigma) and 0.2% Triton X-100 (30°C) then washed in PBS and incubated for 1 hour in blocking solution containing 10% normal goat serum (Sigma) and 0.2% gelatin (Sigma) in PBS. For BrdU immunostaining, a BrdU detection kit (Roche) was used, with BrdU-labelled cells revealed using a mouse monoclonal anti-BrdU antibody followed by an anti-mouse FITC-conjugated antibody. For BrdU/NG2 double staining experiments, fixed and blocked sections were first stained for NG2; tissues were incubated with a rabbit polyclonal anti-NG2 antibody (Chemicon, 1/200 dilution in 0.2% gelatin overnight at 4°C), followed by a biotin-conjugated anti-rabbit antibody (Vector, 1/100 dilution in 0.2% gelatin for 1 hour at room temperature) and finally incubated with TRITC-conjugated streptavidin (Amersham, 1/100 in PBS for 1 hour at room temperature), with PBS washes between each step. All slides were finally mounted under coverslip in ImmunoFlourescent mounting medium (ICN). For quantitation of BrdU and NG2 labelling, BrdU-positive and NG2-positive cells were counted in seven adjacent fields of 500 µm x 500 µm each in the subventricular
zone (SVZ). For the corpus callosum, the whole structure in one or both hemispheres was counted. For the cortex, a region of at least seven adjacent fields of 500 μm × 500 μm at the external edge of both cerebral hemispheres was counted. For the striatum, an area of seven fields of 500 μm × 500 μm each was counted.

Cell culture
Purified oligodendrocyte precursors from mouse and rat were obtained by the mechanical dissociation method from cultures of cerebral cortex as originally described (McCarthy and Vellis, 1980) with minor modifications (Milner and ffrench-Constant, 1994).

Cell proliferation assay in vitro
Freshly purified OP from either wild-type or TN-C-null cultures (prepared from the basal monolayer in the described brain cultures obtained by the mechanical dissociation method from cultures of newborn brains by immuno-affinity column chromatography (Faissner and Kruse, 1990) was placed on TN-C-null astroglial matrix at the concentration of 10 μg/ml in PBS and incubated overnight at 37°C before washing and cell plating. For the αβ3 function blocking experiments, rat OP cells were used instead of mouse cells, as discussed in the text. OP cells were plated on LabTek chamber slides (Nunc) either precoated with PDL alone (control) or PDL followed by an overnight incubation at 37°C with purified TN-C at 10 μg/ml in PBS. OP cells were grown for 18 hours in SA TO medium in different concentrations of PDGF, with or without the F11 mouse monoclonal anti-PDGFαR antibody (a kind gift from M. Horton, London) at 20 μg/ml, before incubation in BrdU and processing for the proliferation assay as previously described.

TUNEL labelling in vivo
Cryostat sections (10 μm) of cortex and corpus callosum were prepared as above and labelled using the terminal deoxyribonucleotide transferase-mediated dUTP nick end labelling method, using a commercially available kit (ApopTag, Intergen, NY) according to the instructions. Labelled cells were counted in two separate frontal anterior brain sections. For the corpus callosum, the whole region was counted and for the cortex, an area of seven fields of 500 μm × 500 μm at the external edge of both cerebral hemispheres was counted as for the studies on cell proliferation.

RESULTS
Oligodendrocyte precursor migration in TN-C-deficient mice
To investigate the effect of TN-C deficiency on migration of OP cells in vivo we localised OP cells at different developmental stages by in situ hybridisation using cRNA probes complementary to PDGFαR mRNA. This marker is restricted to the oligodendrocyte lineage (Pringle et al., 1992) and has been used in previous studies to determine the distribution of migrating OPs (Bartsch et al., 1994). We initially compared TN-C deficient mice (as described by Saga et al., 1992) with age-matched wild type mice from a F1 C57BL/6J × CBA backcross designed to match the genetic background of the original TN-C knockout animals.

To examine OP migration we chose to study the optic nerve. OP cells start to enter the optic nerve from the chiasm at birth and then migrate toward the retina (Bartsch et al., 1994). Cell migration is responsible for all the OP cells within the nerve, and both the single source of cells and the uniform direction of migration along a narrow tract makes the optic nerve ideal for studies on migration in vivo. To compare the distribution of OP cells in wild-type and TN-C-deficient nerves, we serially sectioned the nerve for 1.8 mm from the retinal end towards the chiasm and counted the number of PDGFαR mRNA-positive cells in sections at different distances from the retina as described in the Materials and Methods. In wild type newborn (P0) animals very few PDGFαR mRNA-positive cells were detectable in the optic nerves and were all restricted to regions greater than 1.2 to 1.8 mm from the retina (Fig. 1, P0). In contrast, in the knockout animals, PDGFαR mRNA-positive cells were seen between 0.9 and 1.2 mm from the retina, with significantly more cells found in the nerve than in wild type (Fig. 1B, C, P0). In postnatal day 2 (P2) animals, the number of PDGFαR mRNA-positive cells and the distance travelled from the chiasm increased in both wild-type and knockout mice, and cells positive for PDGFαR mRNA were first found 300 μm from the retina (Fig. 1, P2). However, the number of PDGFαR mRNA-positive cells was significantly greater in TN-C-null mice in each region of the optic nerve (Fig. 1, P2). By P5, the density of PDGFαR mRNA cells had increased still further in each region of the optic nerve (Fig. 1, P5) and, in agreement with previous studies, the OPs had now reached the retinal end of the optic nerve (Bartsch et al., 1994). We did not find any differences in cell numbers between TN-C-null mice and wild-type mice at this (Fig. 1D,E, P5) and later stages (Fig. 1, P12 and adult). However, from P12 to adult, the number of cells containing PDGFαR mRNA decreased and, in contrast to earlier developmental stages, the PDGFαR mRNA-positive cells in the adult optic nerve were now uniformly distributed along the different segments. At all stages in both wild-type and knockout mice, no PDGFαR mRNA-positive cells entered the part of the optic nerve immediately adjacent to the retina, although a few cells were occasionally seen between 250-300 μm from the retina (Fig. 1).

These results show that in TN-C knockout mice OP cells appear sooner in the optic nerve than in wild-type animals and are then found at points nearer the retina as OPs migrate along the nerve. To exclude the possibility that this result reflected differences in age and genetic background between litters, rather than differences in TN-C expression, we next compared heterozygous and homozygous null littermates generated by backcross experiments. The original TN-C-null mice were crossed into the F1 C57BL/6J × CBA background, as described in Materials and Methods, and the serial sectioning and in situ hybridisation analysis repeated. As in our first set of experiments, we found that PDGFαR mRNA-positive cells were found at points nearer the retina and were present in greater numbers at all distances from the retina in the homozygous mice at P0 and P2 (Fig. 2, P0 and P2). To further confirm this result on an inbred genetic background we used 129 mice in which the TN-C deficiency had been bred back onto a parental 129 line for 10 generations, so providing an almost completely pure 129 background. Once again, when
we compared heterozygous and homozygous null littermates we found that PDGFαR mRNA-positive cells were found at points nearer the retina at P2 (Fig. 2) in the null mice. This confirms that the different distribution of OPs associated with a lack of TN-C is not restricted to a single genetic background.

Migration of OP cells in vitro

The in vivo experiments above show that environments lacking TN-C promote faster OP migration. To determine whether this effect was a direct consequence of TN-C loss, we next performed in vitro migration assays using an agarose drop assay (Milner et al., 1996), using rat OP and astroglial matrix substrates prepared from either wild-type or knockout mice. As expected, OP migration at 1 and 2 days was significantly greater over the matrix that lacked TN-C (Fig. 3). However, the addition of purified TN-C at 10 or 40 μg/ml to the substrate for 18 hours before the assay (Fig. 3), or at 10 μg/ml in solution for the duration of the assay (not shown), did not reduce the migration rate of cells on TN-C deficient matrix to wild-type levels. This suggests that the enhanced migration on the null matrix is mediated by indirect effects of TN-C deficiency on the ECM, in addition to the loss of any direct effects of TN-C that we have described previously using purified TN-C substrates (Frost et al., 1996; Kiernan et al., 1996).

Proliferation of OP cells in TN-C-null mice in vivo

Although a role for TN-C in the proliferation of neural cells has not been shown previously, TN-C has been shown to stimulate proliferation of other cell types in vitro (Jones and
Jones, 2000). We therefore next asked whether TN-C was involved in cell proliferation within the developing CNS in vivo. The original TN-C-null mice (Saga et al., 1992) and matched same age wild-type mice (C57BL/6J-CBA F1) were used for in vivo BrdU labelling to analyse cell proliferation during postnatal development. For these studies in early postnatal mice, we used two injections of BrdU 1 hour apart, with the rationale that any increase in cell cycle time associated with a reduced rate of cell proliferation would be revealed by a reduction in the percentage of labelled cells. A potential shortcoming of this protocol is that it assumes the length of S phase remains constant as the OP cell cycle time lengthens. A recent study using cumulative labelling in embryonic development suggests that this may not be the case, and that lengthening of S phase is also seen (van Heyningen et al., 2001). However, any lengthening of S phase in the TN-C-null mice would increase the labelling index, and so diminish rather than exaggerate the sensitivity of our protocol in the detection of reduced rates of proliferation in these mice.

During early postnatal development, forebrain OP cells are generated from pre-progenitor cells in the SVZ surrounding the lateral ventricles (Levison et al., 1993; Levison and Goldman, 1993; Levison and Goldman, 1997; Luskin and McDermott, 1994). Consequently, our first experiment was an analysis of BrdU incorporation in this area between P0 to P17. We found a significant reduction at all ages in the number of BrdU-positive cells in the SVZ of TN-C-deficient mice when compared with heterozygous littermates (Student's t test: *P<0.05, **P<0.01, ***P<0.001).

In order to examine OP proliferation directly, we used the 129 mice and performed double immunostaining for BrdU and NG2 chondroitin sulfate proteoglycan, a marker for OP cells in vivo (Levine and Stallcup, 1987; Levine et al., 1993), which is colocalized with the PDGFrR (Nishiyama et al., 1996). As shown in Fig. 5, a significant number of the BrdU-positive cells in the cortex were also NG2 positive, while in the SVZ, very few of the BrdU-positive cells were NG2 positive. In all cases, however, there was a significant reduction in the number of NG2-positive cells that were BrdU positive at either or both P7 and P10 (Table 1, Fig. 5), showing directly that deficiency of TN-C reduces OP proliferation in the CNS.

To confirm these results using age and genetic background-matched littermates, we performed a second set of experiments using TN-C-deficient heterozygous and homozygous mice crossed into the F1 C57BL/6J × CBA background. We examined these mice at P2, P7 and P10, and once again we found a significant decrease in the number of BrdU-positive cells at all ages in the SVZ of the homozygous mice, in comparison with the heterozygous mice (Fig. 4). In addition to the SVZ, we also compared the number of BrdU-positive cells in the corpus callosum, cortex and striatum of these littermates. Differences in the numbers of BrdU-positive cells were found in the cortex and the striatum at P7 and P10, with fewer BrdU-labelled cells seen in homozygous mice when compared with heterozygous mice (Fig. 4A). We also found a reduced amount of BrdU-positive cells in the corpus callosum at P7 but not at P10 (Fig. 4).

We also compared the levels of cell proliferation between heterozygous and TN-C-deficient homozygous littermates in the 129 genetic background. Once again we found reduced levels of BrdU labelling in SVZ and cortex at both P7 and P10 and in corpus callosum at P7 (Table 1). This result confirms that the reduced cell proliferation associated with TN-C deficiency is present on at least two genetic backgrounds.
Proliferation of OP cells on astroglia extracellular matrix lacking TN-C

The experiments above confirm a role for TN-C in the regulation of OP proliferation in vivo. OP proliferation is also driven by the mitogen PDGF in vitro (Pringle et al., 1989) and in vivo (Calver et al., 1998; Fruttiger et al., 1999). We therefore next examined the interaction between PDGF and TN-C in OP proliferation. These experiments were performed using either wild-type or TN-C-deficient OP cells grown in culture on astrocyte-derived matrix from either wild-type or TN-C-deficient mice. The proliferation index of OP cells was determined after two days of plating by exposure to BrdU for 6 hours. As shown in Fig. 6A, in the absence of PDGF, the basal level of proliferation of OP cells was only 3-4%. In control experiments using wild-type OP cells on wild-type matrix, there was a significant proliferative response to both 1 and 10ng/ml PDGF, in agreement with previous studies.
Glial precursor behaviour in tenascin-C knockout mice (Pringle et al., 1989). However, in the absence of TN-C (i.e. knockout OP cells on knockout matrix) there was no response at either concentration (Fig. 6A). Experiments in which either the OP cells or the matrix were TN-C deficient revealed that the PDGF response was more dependent on the genotype of the cells than the substrate. The response of wild-type cells on knockout matrix was greater than that of knockout cells on wild-type matrix (Fig. 6A). However optimal responsiveness to PDGF required both sources of TN-C, with the response of the wild-type cells on knockout matrix significantly less than that of the same cells on wild-type matrix (Fig. 6A).

In order to confirm that a lack of TN-C is directly responsible for the loss of PDGF responsiveness in the OP cells, we repeated these experiments adding purified TN-C (see Materials and Methods) at a concentration of 10 mg/ml. This exogenous TN-C was able to restore the response to PDGF of both TN-C-deficient and wild-type OP cells grown on TN-C-deficient substrates. As before, however, wild-type OP cells were more responsive to the mitogenic effect of PDGF, with approximately twofold greater BrdU labelling after the addition of exogenous TN-C (Fig. 6B).

Involvement of \(\alpha\nu\beta3\) integrin in TN-C mitogenic functions in OP cells

The experiments above show that TN-C is required for the mitogenic response of mouse OP cells to PDGF in cell culture. In order to investigate the mechanism by which TN-C interacts with PDGF signalling pathways, we focussed on the \(\alpha\nu\beta3\) integrin. This integrin is an established TN-C receptor that has been shown to mediate TN-C growth control effects in other cell types and to mediate interactions between integrin and growth factor receptor signalling pathways (for a review, see Jones and Jones, 2000). Additionally, we have shown previously that \(\alpha\nu\beta3\) integrin is expressed during OP differentiation and regulates OP proliferation (Blaschuk et al., 2000; Milner and ffrench-Constant, 1994; Milner et al., 1997). We therefore performed proliferation assays in vitro in which BrdU incorporation into OP cells was determined in the presence or absence of a function-blocking \(\alpha\nu\beta3\) antibody, either on control substrates (PDL) or on substrates containing purified TN-C (PDL+TN-C at 10 \(\mu\)g/ml, see Materials and Methods). In the absence of any well-characterised blocking
antibodies against mouse αvβ3, we used rat OP cells and the monoclonal anti-rat αvβ3 antibody F11 (Helfrich et al., 1992). Initial experiments established that, as in the mouse cells, the mitogenic response of rat OP cells to PDGF was enhanced by exogenous TN-C (Fig. 7). The F11 antibody blocked this TN-C-mediated effect, but had no effect on control PDL substrates on which PDGF was still able to stimulate OP cell proliferation (Fig. 7). These data therefore demonstrate that TN-C acts via one of its receptors, αvβ3 integrin, to enhance the mitogenic effects of PDGF in OP cells.

**Cell death in the CNS of TN-C-deficient mice**

The reduction on OP proliferation in the corpus callosum and cortex of the TN-C deficient mice would be expected to result in a reduced number of oligodendrocytes available for myelination in these areas. However, as we have described previously, the architecture of the myelinated tracts is normal in these mice (Kiernan et al., 1999). Many newly formed oligodendrocytes undergo programmed cell death, unless they establish appropriate axonal contact (for a review, see Barres and Raff, 1999). One potential mechanism to explain the normal myelination in the TN-C-deficient mice would therefore be a compensatory reduction in the level of programmed cell death in these tracts during development. To examine this, we performed TUNEL labelling to determine the levels of cell death during myelination in corpus callosum and cortex. As shown in Fig. 8, we found significantly reduced levels of TUNEL labelling in the TN-C-deficient mice at P17 in corpus callosum and P5 in cortex. There was no significant difference at earlier developmental times in corpus callosum, while very few dying cells were seen in the normal or TN-C-deficient cortex after P5.

**DISCUSSION**

Our results show an important role for the extracellular matrix molecule TN-C in the regulation of neural precursor cell proliferation and migration, as revealed by reduced proliferation of SVZ cells and OP cells in transgenic mice that lack TN-C (Saga et al., 1992). These mice have previously been reported as normal (Saga et al., 1992). Although subsequent studies have revealed changes in behaviour (Fukamauchi et al., 1996; Kiernan et al., 1999) and neurotransmitter levels (Fukamauchi et al., 1996) in the CNS, as well as abnormalities of neuromuscular junction architecture (Cifuentes-Diaz et al., 1998) and repair in the kidney (Nakao et al., 1998), no developmental abnormalities have been described previously. Our results are therefore important in that they demonstrate for the first time a significant role for TN-C in the basic processes of cell growth control during normal development.

The reduction in cell proliferation in the TN-C-deficient animals was revealed by BrdU studies in vivo, with double labelling experiments showing directly that there was a reduction in OP proliferation. Cell culture studies using cells obtained from either wild-type or knockout animals show that the effect on cell proliferation reflects a change in the sensitivity of the OP cells to the mitogen PDGF. Cells that lack TN-C and are grown on an astrocyte-derived extracellular matrix also derived from TN-C-deficient animals show almost

**Table 1. Decreased rate of NG2-positive cells incorporating BrdU in the CNS of TN-C-null mice with a 129 genetic background**

<table>
<thead>
<tr>
<th>Region</th>
<th>Age</th>
<th>Genotype</th>
<th>Total number of BrdU-positive cells</th>
<th>% of BrdU-positive cells in the NG2-positive cell population</th>
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<tr>
<td>SVZ</td>
<td>P7</td>
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<td></td>
<td></td>
<td>+/-</td>
<td>546.67±66.71*</td>
<td>20.41±3.16</td>
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<tr>
<td></td>
<td>P10</td>
<td>+/-</td>
<td>266.67±16.12*</td>
<td>23.10±2.16</td>
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<tr>
<td></td>
<td></td>
<td>+/-</td>
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<tr>
<td>Cortex</td>
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<tr>
<td></td>
<td></td>
<td>+/-</td>
<td>102.00±12.53*</td>
<td>14.31±1.43</td>
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<tr>
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<td></td>
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<td>+/-</td>
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<td></td>
<td>P10</td>
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<td></td>
<td></td>
<td>+/-</td>
<td>67.33±11.29</td>
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</table>
no response to PDGF. Importantly, this response is restored by the addition of exogenous TN-C, confirming that this molecule is necessary for the mitogenic effect of PDGF on OP cells. As experiments using transgenic mice expressing different levels of PDGF have shown that this mitogen is limiting for OP proliferation in vivo (Calver et al., 1998; Fruttiger et al., 1999), any alteration in the sensitivity of the precursor cells to PDGF should alter cell proliferation. We propose, therefore, that the observed changes in the transgenic mice reflect a partial loss of response to PDGF in three areas of the CNS where TN-C is expressed during development: the SVZ, cortex and white matter tracts (Bartsch et al., 1992; Bartsch et al., 1994; Crossin, 1996; Crossin et al., 1989; Gates et al., 1995; Gotz et al., 1997; Jankovski and Sotelo, 1996; Miragall et al., 1990; Mitrovic et al., 1994).

TN-C has been shown to stimulate cell proliferation in a number of other non-neural cell types in vitro (Jones et al., 1997; Ohta et al., 1998; Seiffert et al., 1998), and one previous study has also described reduced cell proliferation in vivo in association with a model of renal glomerulonephritis (Nakao et al., 1998). Interactions such as we document here between TN-C and growth factor signalling have been described in several studies for PDGF, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF; for a review, see Jones et al., 1998) and we have previously suggested a model in which the sequential signalling of PDGF°v and the PDGF°v receptor will co-immunoprecipitate with alpha(v)beta3 and ligand binding to alpha(v)beta3 will enhance PDGF signalling responses (Schneller et al., 1997). However, the association between alpha(v)beta3 and the PDGF receptor suggested by our work has not been shown previously. alpha(v)beta3 contributes to the regulation of OP cell proliferation in cell culture, and we have previously suggested a model in which the sequential signalling of alpha(v)beta1, alpha(v)beta3 and alpha(v)beta5 regulates OP migration, proliferation and differentiation, respectively (Blaschuk et al., 2000; Milner et al., 1996; Milner and ffrench-Constant, 1994; Milner et al., 1997). Our demonstration here that TN-C acts through the alpha(v)beta3 integrin to potentiate PDGF mitogenic activity in OPs adds significant support to this hypothesis. It emphasises the role of alpha(v)beta3 in OP proliferation, demonstrating the association with PDGF signalling pathways known to regulate OP proliferation in vivo (Calver et al., 1998; Fruttiger et al., 1999) and identifying an essential role for an ECM ligand of alpha(v)beta3, which is known to be expressed in the pathways of OP migration during development (Bartsch et al., 1992; Bartsch et al., 1994).

We have also examined OP migration during postnatal development in the TN-C-deficient mice, as TN-C has been shown to inhibit OP migration and adhesion in cell culture (Frost et al., 1996; Kiernan et al., 1996). Our studies show that OPs, as identified by the expression of the PDGF receptor, have migrated further along the optic nerve at P0 and P2 in the

**Fig. 8.** Reduced cell death in the postnatal brain of TN-C-null transgenic mice. At P5, P10 and P17, TUNEL-positive cells were counted in the corpus callosum and the cortex of wild-type (WT) and TN-C-null mice (TN-C°/°) in two separate brain sections from three different animals of each genotype, and shown as mean±s.e.m. Note the decrease in cell death at P17 in the corpus callosum and at P5 in the cortex of TN-C-null mice (Student’s t test: **p<0.02; ***p<0.01).
abundance of TN-C, so confirming for the first time a role for TN-C in the regulation of inhibition of migration in vivo. Based on cell culture studies, the inhibition of OP migration by TN-C has been proposed as a mechanism to limit the movement of OPs in vivo and so prevent myelination in two areas of the CNS, the retina and the molecular layer of the cerebellum. High concentrations of TN-C produced by astrocytes at the optic nerve head and at the border of the molecular layer have been proposed to act as barriers during development (Bartsch et al., 1992; Bartsch et al., 1994). Although we showed previously that adult mice lacking TN-C showed no change in the pattern of myelination at the optic nerve head and cerebellum (Kiernan et al., 1999), we could not exclude the possibility that OPs enter these regions during development but are then lost by programmed cell death (Calver et al., 1998). The present developmental study argues that this is not the case; even in the optic nerves that lack TN-C, cells arriving prematurely at the optic nerve head were never seen to enter the retina. TN-C is not therefore required for the barrier function of this region, and our finding that OPs also never entered the molecular layer of the cerebellum (E. G., unpublished) shows that TN-C is also not required as a barrier to OP migration in this structure.

In contrast to the experiments examining proliferation in which the addition of purified TN-C to the in vitro assays restored OP proliferation to wild-type levels, the addition of exogenous TN-C to our migration assays, either to the substrate or in solution, did not reduce the level of migration. This was surprising given our previous finding that purified TN-C substrates inhibit OP migration (Kiernan et al., 1996), and points to additional indirect effects of TN-C loss mediated by changes either in the levels or in the organisation of other molecules in the ECM. As we have also described previously, the inhibition of OP migration by TN-C is substrate dependent (Frost et al., 1996) and we suggest that the changes in the TN-C-deficient ECM render the cells insensitive to the direct effects of purified TN-C. TN-C interacts with a number of other ECM molecules (Jones and Jones, 2000) and the reorganisation of the matrix associated with TN-C deficiency could therefore alter the 3D architecture and the interactions of cells with these other molecules. This is an important conclusion, as it emphasises that the effects of deficiency deduced from the phenotype of null mice reflect both direct and indirect effects of the loss of any one molecule, with in vitro experiments, such as those performed here, helpful in distinguishing these possibilities.

Given the roles for TN-C in proliferation and migration demonstrated by this present study why, as we have described previously (Kiernan et al., 1999), is the final pattern of myelination normal in TN-C-deficient animals? Our results showing a decrease in TUNEL labelling suggest that this reflects the presence of corrective mechanisms during normal development. Overproduction of OPs normally occurs, with as many as 50% of newly formed oligodendrocytes subsequently undergoing programmed cell death (Barres et al., 1992) regulated, at least in part, by the availability of axonal targets for myelination (for a review, see Barres and Raff, 1999). Variations in the numbers of OP cells in different regions of the CNS that reflect changes in proliferation and migration could therefore be corrected at a later stage of development. In the case of reduced proliferation, the reduction in the level of cell death we observe at the final stages of myelination (at which time axonal targets would normally be limiting) would correct the phenotype. These corrective cellular mechanisms are quite distinct from compensatory molecular mechanisms that may operate within individual cells as has been shown, for example, in the MyoD knockout where Myf5 can compensate for the loss (Rudnicki et al., 1993). They may represent an important cause of the apparent normality of many transgenic mice. Correction may, however, be inadequate in the presence of other genetic or environmental perturbations of development and this may provide the selection pressure required to explain the high degree of conservation seen for TN-C across a range of vertebrate species.

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