Conditional β1-integrin gene deletion in neural crest cells causes severe developmental alterations of the peripheral nervous system

Thomas Pietri1,*, Olivier Eder1, Marie Anne Breau1, Piotr Topilko2, Martine Blanche1, Cord Brakebusch3, Reinhard Fässler3, Jean-Paul Thiery1 and Sylvie Dufour1,†

1UMR144, CNRS – Institut Curie, 26, rue d’Ulm, 75248 Paris Cedex 05, France
2U 368, INSERM – Ecole Normale Supérieure, 46, rue d’Ulm 75230 Paris Cedex 05, France
3Max Planck Institute of Biochemistry, Department of Molecular Medicine, Martinsried, 82152, Germany

*Present address: Department of Neuroscience, The Johns Hopkins School of Medicine, 725 North Wolfe Street, 1001 PCTB, Baltimore, MD 21205, USA
†Author for correspondence (e-mail: sylvie.dufour@curie.fr)

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Summary
Integrins are transmembrane receptors that are known to interact with the extracellular matrix and to be required for migration, proliferation, differentiation and apoptosis. We have generated mice with a neural crest cell-specific deletion of the β1-integrin gene to analyse the role of β1-integrins in neural crest cell migration and differentiation. This targeted mutation caused death within a month of birth. The loss of β1-integrins from the embryo delayed the migration of Schwann cells along axons and induced multiple defects in spinal nerve arborisation and morphology. There was an almost complete absence of Schwann cells and sensory axon segregation and defective maturation in neuromuscular synaptogenesis. Thus, β1-integrins are important for the control of embryonic and postnatal peripheral nervous system development.

Key words: Integrin, Peripheral nervous system, Neural crest cells, Conditional knockout

Introduction
The ontogeny of neural crest cells (NCC) is a major morphogenetic process involving various biological steps, such as epithelium-mesenchyme transition, cell migration and cell aggregation. NCC differentiate to generate a diversity of cell types, including craniofacial structures, the peripheral nervous system (PNS) and melanocytes (Le Douarin, 1982; Le Douarin and Kalcheim, 1999). NCC interact with the extracellular matrix (ECM) during their differentiation. Integrins are αβ heterodimers that bind ECM components and cell-surface receptors, and control cell adhesion, migration, differentiation and survival (Hynes, 2002). Previous studies have shown that integrins play a major role in the control of NCC adhesion and migration. In vitro, these cells express a large repertoire of integrins, including α1β1, α3β1, α4β1, α5β1, α6β1, αθβ1, αVβ1, αVβ3 and αVβ8 (Kim et al., 1996; Desban and Duband, 1997; Testaz et al., 1999). In vivo, functional analyses with integrin antibodies, antisense oligonucleotides and peptide competitors have shown that mouse and avian NCC use multiple β1-integrins (Boucaut et al., 1984; Bronner-Fraser, 1986; Poole and Thiery, 1986; Kil et al., 1998).

Constitutive knockout of the β1-integrin gene leads to the loss of twelve members of the integrin family and leads to death of the embryo during the peri-implantation period due to inner cell mass failure (Fässler and Meyer, 1995; Stephens et al., 1995). This constitutive β1-knockout model demonstrates the essential role of β1-integrins during early embryogenesis, but is of no use for investigating integrin functions during later stages of development or in differentiated cells, such as NCC derivatives. By contrast, neither the invalidation of individual α-integrin subunits (for reviews, see Bouvard et al., 2001; Hynes, 2002) nor the production of knockout chimaeric mice (Fässler and Meyer, 1995) has made it possible to delineate precisely the roles of specific integrins during NCC development, with the exception of α5β1-integrins, which have been implicated in cranial NCC survival (Goh et al., 1997), and α5β1- and α4β1-integrins, which have been implicated in the proliferation or survival of glial cell precursors (Haack and Hynes, 2001).

The Cre-LoxP conditional gene disruption system has been used to investigate the effect of integrins on NCC ontogeny in more detail. P0-Cre mice crossed with mice carrying a floxed β1-integrin allele generated animals with the conditional mutation targeted specifically to Schwann cells (SC), which are derived from NCC (Feltri et al., 2002). This conditional knockout revealed the role played by β1-integrin receptors in postnatal PNS development. The loss of β1-integrins impedes interactions between SC and axons, causing dysmyelinating neuropathy (Feltri et al., 2002).

We recently developed the Ht-PA-Cre transgenic mouse line, in which Cre recombinase activity is targeted specifically to NCC at the start of migration (Pietri et al., 2003). We report here the effects of specific inactivation of the β1-integrin gene in migratory neural crest cells. The crossing of Ht-PA-Cre mice with β1 floxed mice generated animals in which the NCC produce no β1-integrin but do produce β-galactosidase under the control of the endogenous β1-integrin promoter (Potocnik et al., 2000). The mutation targets the glial and sensory neuronal compartments of the PNS as well as the other NCC derivatives.
We show here that $\beta_1$-integrin receptors control PNS development at both early and late stages. The absence of $\beta_1$-integrins delayed the migration of SC along nerves and resulted in abnormal axo-glial segregation, especially for sensory axons. Substantial changes were observed in the pattern of subcutaneous and muscular innervation. In addition, the arborisation and fasciculation of the innervating fibres and their targeting to the distal tissues were abnormal, and neuromuscular synaptogenesis appeared to be blocked in a state of immaturity. Finally, the mutation caused the death of the animals by the age of three weeks, owing to multiple defects affecting various NCC derivatives.

Materials and methods

Mouse maintenance and genotyping

Homozygous Ht-PA-Cre mice were crossed with mice heterozygous for the $\beta_1$-integrin gene ($\beta_1^+/\beta_1^+$) to generate Ht-PA-Cre;$\beta_1^+\beta_1^+$ mice, referred to as mutants, and Ht-PA-Cre;$\beta_1^+/\beta_1^+$ mice, referred to as controls in the text. Wild-type and $\beta_1^+/\beta_1^+$ heterozygous embryos were used as controls for integrin levels in homozygous and heterozygous NCC-derived tissues. Mice were reared and experiments carried out according to the guidelines of the CNRS ethics committee. Ht-PA-Cre and $\beta_1$-integrin mouse genotyping was carried out as described by Pietri et al. and Potocnik et al. (Pietri et al., 2003; Potocnik et al., 2000), respectively.

Reagents and antibodies

The partially purified mouse monoclonal antibody (clone 2H3) against the 160 kDa neurofilament (NF-160) protein was obtained from Developmental Studies Hybridoma Bank. Mouse monoclonal antibodies against the nuclear factor Hu-D (clone 16A11) and against the major myelin protein P0 were gifts from J. A. Weston (Marusich et al., 1994) and J. J. Archelos (Archelos et al., 1993), respectively. The rabbit polyclonal antibody against laminins was obtained from Sigma. The rabbit polyclonal antibodies against tenascin and S100, and the mouse monoclonal antibody against fibronectin were obtained from Chemicon. In some cases, we used a rabbit polyclonal antibody against S100 produced by Dako. The mouse monoclonal antibody (clone SY38) against synaptophysin was obtained from Progen. The rabbit polyclonal antibody against cleaved caspase 3 (Asp175) was obtained from Cell Signaling. The rabbit polyclonal antibody against Kif67 was obtained from NovoCastra and the goat polyclonal antibody against parvalbumin was purchased from Swant. The $\alpha$-bungarotoxin ($\alpha$-BTX) biotin-conjugated compound extracted from Buranus multicinctus venom was purchased from Molecular Probes. Secondary antibodies conjugated with Alexa 488, cyanin 3 or horseradish peroxidase (HRP) were purchased from Molecular Probes, Jackson Laboratories and Amersham Pharmacia Biotech, respectively.

Histology, immunohistochemistry and microscopy

For the detection of $\beta_1$-galactosidase activity, embryos or organs were dissected in cold phosphate-buffered saline (PBS), pH 7.6, supplemented with 5% foetal calf serum (FCS), were fixed in toto in freshly prepared ice-cold 1% formaldehyde/0.2% glutaraldehyde/0.02% Nonidet-P40 for 2-4 hours and processed as described elsewhere (Dufour et al., 1994).

Whole-mount immunostaining was performed on embryos fixed in methanol Carnoy fixative or 4% PFA. Samples were incubated overnight in blocking solution composed of 0.3% Triton X100, 0.5× Blocking Reagent (Roche Applied Science), and 10% foetal calf serum in PBS and then for 2 days each with primary and secondary Alexa 488- or HRP-conjugated antibodies. Samples were thoroughly washed between incubations. HRP activity was detected with the VECTOR® peroxidase substrate kit, according to the manufacturer’s instructions (Vector Laboratories), followed by benzyl-benzoate solution (BABB) treatment for the detection of deep stained structures.

For histological and immunohistochemical analysis of sections, fixed samples were dehydrated and embedded in paraplast Plus. They were then cut into serial sections of 7-10 µm and the wax removed. Some whole-mount stained embryos were serially sectioned at a thickness of 200 µm on a vibratome. Sections were incubated for 2 hours in blocking solution consisting of 10% FCS/0.1% Triton X100/0.5× Blocking Reagent (Roche Applied Science) in PBS. The sections were then incubated overnight at 4°C with primary antibodies in blocking solution, rinsed several times in PBS and incubated with secondary antibodies for 2 hours at room temperature in the dark.

Cell proliferation was assessed by counting more than 2500 DRG cells in every second section of three different embryos of each genotype and analysing staining for the Ki67 marker. Results are expressed as the percentage of cells that were Ki67-positive. Statistical significance was determined with the ANOVA unpaired t-test.

Acetylcholine receptors (AChR) were detected with $\alpha$-BTX. The abdominal, diaphragm, soleus and gastrocnemius muscles of animals at times P1 and P21 were dissected in cold PBS, pH 7.6, and were then incubated for 2-3 hours at 37°C in $\alpha$-BTX in minimum essential culture medium. The samples were rinsed three times, for 1 hour each, in cold PBS, fixed in 4% PFA for thirty minutes and processed as for whole-mount samples used for immunostaining.

Whole-mount stained samples and histological sections were photographed under a Leica M28 stereomicroscope (Leica Microsystems SA) equipped with a JVC 3CCD colour camera. Confocal images were obtained for muscle preparations on a Leica TCS4D confocal microscope based on a DM microscope interfaced with an Ar/Kr laser. Stacks of images were mounted with Metamorph 5.0, and normalised to the same width on the z-axis.

Semi-thin sections and electron microscopy

Postnatal sciatic nerves were isolated from control and mutant animals perfused with 0.5% glutaraldehyde in phosphate buffer (PB, pH 7.4), fixed in 0.5% glutaraldehyde for 1 hour at 4°C and washed in PB. $\beta_1$-Galactosidase activity was detected histochemically by incubating samples in PB supplemented with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride and 1 mg/ml Bluo-gal (5-bromo-3-indolyl-$\beta$-galactosidase, Sigma) as a substrate for 12 hours at 30°C. The following day, samples were post-fixed by incubation in 1.6% glutaraldehyde in PB for 1 hour at 4°C followed by 1 hour in 1% OsO4 (Sigma) in PB. They were then washed in PB, dehydrated in ethanol (30%, 50%, 70%), stained by incubation with 1% uranyl acetate in 70% ethanol for 1 hour, dehydrated in ethanol (80%, 90%, 100%), infiltrated and embedded in Durcupan (Fluka). Ultrathin sections were cut and stained with uranyl acetate and lead citrate. The sections were observed with a Tecnai 12 (FEI, Phillips) electron microscope.

Results

Generation of conditional knockout mice lacking $\beta_1$-integrins in NCC

The aim of this study was to investigate the role of $\beta_1$-integrins in the relationships between the various component-derived NCC of the PNS (Fig. 1A, lower panel). $\beta_1$-integrin gene knockout has been reported to be lethal at E5.5 because of inner cell mass failure, whereas heterozygous animals are normal and fertile (Stephens et al., 1995). We restricted inactivation of the gene encoding the $\beta_1$-integrin subunit to the
NCC by crossing mice homozygous for the floxed \( \beta_1 \)-integrin allele (\( \beta_1^{fl/\beta_1^{fl}} \)) with mice homozygous for Ht-PA-Cre transgene and heterozygous for the \( \beta_1 \)-integrin gene (Ht-PA-Cre/\( \beta_1^{fl/\beta_1^{fl/\beta_1^{+/-}} \)). Cre recombinase-mediated deletion of the floxed \( \beta_1 \) gene resulted in recombination to give an intact \( \lambda c Z \) reporter gene under the control of the endogenous \( \beta_1 \)-integrin gene promoter, which was able to produce \( \beta \)-galactosidase (Fig. 1A, upper panel). This made it possible to follow the spatiotemporal pattern of floxed allele recombination in the affected cells visually (Potocnik et al., 2000). Crosses typically generated Mendelian segregation of the two types of embryonic genotype: Ht-PA-Cre;\( \beta_1^{fl/\beta_1^{-/+}} \) animals, which were heterozygous for the \( \beta_1 \)-integrin gene in NCC cells and their derivatives, and wild-type for this gene in other tissues. The Ht-PA-Cre;\( \beta_1^{fl/\beta_1^{+}} \) embryos were used as controls because \( \beta_1 \)-integrin gene heterozygosity has no effect on mice phenotype (Fassler and Meyer, 1995; Stephens et al., 1995). As with the Ht-PA-Cre;\( \beta_1^{fl/\beta_1^{-}} \) mutants, \( \beta \)-galactosidase activity was detected in the developing enteric nervous system (Fig. 1B, white arrowheads).

In control (Ht-PA-Cre;\( \beta_1^{fl/\beta_1^{+}} \)) and mutant (Ht-PA-Cre;\( \beta_1^{fl/\beta_1^{-}} \)) embryos, \( \beta \)-galactosidase activity was detected at around E8. Labelled cells were found in mesenchyme of the pharyngeal arches, in the frontonasal region and around the optic pit (not shown). At E13, the developing muscular and cutaneous innervations were labelled for \( \beta \)-galactosidase, as observed on 200 \( \mu m \) transverse sections of the trunk of a control embryo (Fig. 1B). Staining was detected on the peripheral nerves, the dorsal root ganglia (DRG) and sympathetic ganglia (SG). At higher magnification (Fig. 2I,J), \( \beta \)-galactosidase activity was found to be homogeneous in the control and mutant ganglia, indicating that both glial and neuronal precursors were targeted, as previously observed (Pietri et al., 2003). \( \beta \)-Galactosidase activity was also detected in the developing enteric nervous system (Fig. 1B, white arrowheads).
Disappearance of β1-integrins in NCC derivatives of mutant embryos

The β1-integrin subunit is ubiquitously produced during embryogenesis. In the mutant, we observed a progressive loss of the β1-integrins in targeted structures. Two days after deletion of the gene in migrating NCC, faint expression of β1-integrins was still detected in NC-derived cells such as the mesenchyme of branchial arches at E10 (Fig. 1C, arrowhead) and DRG cells at E12.5 (Fig. 1E, arrowhead), compared with the controls (Fig. 1D,F, arrowhead, respectively). A complete loss of β1-integrins from the surface of the NC-derived cells was achieved at E13 in the DRG of the mutant embryos (Fig. 1G, arrowhead) whereas in the heterozygous (Ht-PA-Cre; β1+/−) and control embryos, NC-derived cells produced detectable amounts of β1-integrins (Fig. 1H,I, respectively, arrowhead). Transverse sections of E13 mutant and control embryos were labelled for Hu-D (a specific marker of early differentiating neurons), DAPI and β1-integrins. In the DRG of the mutant embryos, the neurons (stained for the Hu-D marker; Fig. 1J,K, respectively, arrowhead), did not express β1-integrins compared to the control embryos (Fig. 2E,F, arrowheads, respectively). Cells not producing the Cre recombinase, such as vessels in the DRG and surrounding tissues (Fig. 1G, arrows), displayed β1-integrin levels similar to those observed in heterozygous embryos (Fig. 1H, arrows).

PNS development is altered in mutant embryos

At E10.5, the condensing DRG displayed no phenotypic alteration in mutants. These structures, which displayed β-galactosidase activity, were similar in size and structure to those of control embryos (Fig. 3A,B, respectively). Hu-D was distributed similarly in the mutant and control mice (Fig. 3C,D, respectively). By contrast, 70% of the E10.5 mutant embryos (n=17, from four different litters) displayed changes in the patterning of the cranial nerves (Fig. 3F) with respect to control embryos (n=15; Fig. 3E). Fifty-nine percent of the mutants exhibited a decrease in the number of vagus nerve (X) roots (Fig. 3F, white arrowhead), 24% had a fusion of nerves IX and X (Fig. 3F, black arrowhead) and 12% had an absence of nerve IX, whereas the placode-derived ganglion IX was present (not shown). The phenotypically altered mutants often displayed two of these defects (see Fig. 3F).

From E11.5, in contrast to what was observed on E10.5, nerves IX and X were present and not fused in mutants (Fig. 3H; n=18). Most of the mutant embryos displayed a cranial nerve pattern very similar to that of the controls (Fig. 3G; n=12). The more severely affected E11.5 mutant embryo exhibited some alterations of the fasciculation of vagus nerve roots (Fig. 3H, white arrowhead) compared with the control embryos (Fig. 3G).

At E12.5-E13.5, the trunk nervous system displayed abnormalities, with the partial disappearance of β-galactosidase staining at the sites of muscular and subcutaneous innervation of the thoracic-abdominal body wall and limbs in the mutant embryos (Fig. 4B,D). Four different litters were analyzed and all the mutants exhibited this phenotype. On 200 μm transverse sections, β-galactosidase-positive structures were not observed in the distal part of the innervation network in mutant embryos (white arrows, Fig. 4D), in contrast to what was observed in controls (Fig. 4A,C).

β-Galactosidase is difficult to detect in axons, on whole-mount embryonic preparations, because it is mainly localized in the cytoplasm of the cell body. Therefore, to determine whether the defect in the distal part of the innervation network in the mutants was due to the absence of axons or glial cells, we carried out NF-160 immunolocalisation on 200 μm transverse sections of E13.5 embryos stained for β-galactosidase activity. Axons were present and reached their


Fig. 3. Morphology of the PNS in control and mutant embryos on E10.5 and E11.5. (A,B) Detection of β-galactosidase activity after targeted deletion of the floxed β1-integrin locus in whole-mount control (A) and mutant (B) embryos, in the DRG and spinal nerves in the trunk on E10.5. (C,D) Hu-D immunolocalisation in transverse sections of control (C) and mutant (D) thoracic DRG of E10.5 embryos. (E-H) Whole-mount NF-160 immunostaining labels cranial sensory nerves in control (E,G) and mutant (F,H) embryos on E10.5 (E,F) and E11.5 (G,H), respectively. E11.5 embryos were treated with BABB. The position of nerves is indicated in E. The black arrowhead in F illustrates the fusion of nerves IX and X in the mutant on E10.5. The white arrowhead indicates a decrease in the number of nerve X roots at the same stage. On E11.5, structures are similar in the control (G) and mutant (H) embryos, except for certain changes to the fasciculation of nerve X roots in the mutant, which exhibits the strongest alteration (white arrowhead in H).

Fig. 4. Morphology of the spinal nerves in control and mutant embryos, on E12.5 and E13.5. (A-H) β-Galactosidase activity was detected in whole-mount preparations. Lateral view of control embryo (A) showing intense labelling of craniofacial structures and PNS. The mutant (B) displays an absence of labelling in the trunk PNS. The 200 μm transverse sections of whole-mount E13.5 control (C) and mutant (D) embryos reveal the loss of reporter gene expression in the distal part of the mutant innervation network (white arrows in D). (E-H) Detail of cutaneous lateral ramus and lateral muscular innervation. The immunolocalisation of NF-160 on similar 200 μm transverse sections from control (E,F) and mutant (G,H) embryos shows the presence of nerves in both cases. However, SC have been specifically lost from the distal part of the mutant nerves (black arrowhead in G,H).

distal targets in both mutants (Fig. 4G,H) and controls (Fig. 4E,F). However, SC precursors (SCP; with β-galactosidase activity) covering the axons were very few in number or absent in the distal part of the developing subcutaneous or muscular innervations in the mutant embryos (Fig. 4G,H, arrowhead), in contrast to control embryos (Fig. 4E,F). Moreover, if we observed few SCP in the distal part of the spinal nerves, they were always associated with the axons, suggesting that the interactions of the SCP to their axons are not completely abolished by the removal of the β1-integrin.

By contrast, on E16.5, SC were found at the apex of the nerves in the mutant embryos, as in controls (not shown). The
transitory absence of SCP along axons may be due to a decrease in SCP proliferation or to an increase in the apoptosis of these cells. Because it is difficult to quantify the glial cells number along the spinal nerves and because SCP are mainly originating from the DRG, we analyzed the proliferation and apoptosis rate within that structure. No significant difference was observed in the percentage of proliferative DRG cells on E12.5 and E16.5 for control (30.69±0.88% and 14.07±3.34%) and mutant (33.17±4.04% and 19.78±1.89%) embryos, as shown by detection of the Ki67 marker. In addition, very few DRG cells expressed the activated cleaved caspase 3 apoptosis marker and the numbers of such cells were similar in both mutant and control embryos at these stages (not shown). These results could not account for the lack of SCP on E13.5 in the distal part of the developing PNS. We therefore conclude that this phenotype is probably related to a delayed migration of these cells along the axons.

The subcutaneous nerve network at various sites, such as the area around the eyes, the lateral body wall and in the limbs, displayed similar morphological defects in all of the mutant embryos analyzed (n=13). At E13.5 and E14.5, the nerves in the mutants were always thinner and displayed a different pattern of arborisation from controls, as shown by whole-mount NF-160 immunostaining viewed from the ventral (Fig. 5A,C) and lateral (Fig. 5B-D and 5G-H) sides of the embryos and at the hindlimb level (Fig. 5E,F). In addition, the subcutaneous emergence sites of the sensory nerves always differed between the mutants and the controls (not shown). By contrast, at these stages, the extent of axonal growth was not significantly affected, even at the most distal part of the limbs in the mutants.

β1-Integrin gene deletion causes a postnatal lethality

Conditional disruption of the β1-integrin gene in migratory NCC and their derivatives did not result in the death of the embryo. All embryos reached term, as demonstrated by the Mendelian segregation of the two genotypes. Initially, no clear difference was detected between newborn control and mutant animals in terms of response to external stimuli (light or noise). All responded to tactile stimulation, but with the mutant animals displaying a weaker response (not shown). A few days after birth, the mutant animals began to display progressive defects in motor function, with the appearance of muscular weakness and muscular atrophy resulting in an abnormal posture (presented for P21 in Fig. 6A). The mutant animals were unable to climb on inclined surfaces and generally slipped on such surfaces. It was not possible to perform the RotaRod assay because the mutant animals were unable to hold on to the round bar and fell immediately. Mutant animals also put on body weight more slowly than did control animals (Fig. 6B). At birth, mutant and control animals (from five different litters) weighed a mean of 1.15±0.04 and 1.24±0.08 g, respectively, whereas after 3 weeks (P21) they weighed 6.07±1.01 and 11.45±0.52 g, respectively. This change in phenotype was observed consistently in the mutant animals of a given litters and in the various litters, with no difference in the response to the genetic change observed between male and female animals. Finally, the Ht-PA-Cre-dependent ablation of the β1-integrin gene led to the death of 90% of the mutant animals during the month after birth (Fig. 6C). The remaining 10% of mutant animals displayed no major defects, with only a mild motor coordination defect evident, and had a normal life expectancy.

During embryogenesis, we found that all of the mutant embryos analyzed displayed defects in the trunk PNS development. Our observations indicated that the expressivity of the phenotype is stable because all the embryos exhibited similar abnormalities. In addition, the penetrance of the phenotype is high and the phenotype is severe because 90% of the mutant animals displayed a progressive impairment of posture and mobility after birth, and died during the first month.
axons are present within these clusters and remain owing to the significant importance of the clusters of unsorted nerves (red arrows in Fig. 7D and 7B, respectively). However, only a few myelinated sensory axons revealed by their light blue staining, mutant nerves were composed of myelinated motor axons (Fig. 7A). This difference was already apparent at stage P1, but was less marked (not shown). The sciatic nerves of P21 control animals were composed of myelinated sensory axons (in contrast to whole-mount embryonic staining) and unsorted axons were not surrounded by a basal lamina as shown) were similar in the sciatic nerves of mutants and controls at both stages. Interestingly, at P21, some ECM-free spaces were detected in the mutant, reinforcing the idea that unsorted axons were not surrounded by a basal lamina as observed in Fig. 7E. In the mutants, the general organisation of the ECM was strongly disrupted at P1 and P21. This disruption was correlated with the delayed maturation of SC and changes in axo-glial segregation.

**β1-Integrin gene deletion leads to major changes in radial sorting in the sciatic nerves**

Morphological, ultrastructural and immunohistochemical analyses of sciatic nerves revealed a defect in SC differentiation, modified axon segregation (Fig. 7), and changes in ECM composition in the mutant animals (Fig. 8). Extensive β-galactosidase labelling of the mutant and control sciatic nerves revealed the activity of the β-galactosidase in the axons (in contrast to whole-mount embryonic staining) and therefore the identification of the sensory axons (β-galactosidase +) and the motor axons (β-galactosidase –) within the nerve. At P21, the sciatic nerve appeared to have a smaller diameter in the mutants (Fig. 7C) than in the controls (Fig. 7A). This difference was already apparent at stage P1, but was less marked (not shown). The sciatic nerves of P21 control animals were composed of myelinated sensory axons (Fig. 7B, red arrow) and motor axons (Fig. 7B, black arrow) of medium to large diameter, and small clusters of small-diameter unmyelinated sensory axons (Fig. 7B, red arrowhead). The mutant nerves were composed of myelinated motor axons (Fig. 7D, black arrow) and large clusters of unsorted axons of small to large diameter (Fig. 7E) mostly of sensory origin (as revealed by their light blue staining, β-galactosidase +; Fig. 7D, black arrowheads). Only a few myelinated sensory axons were observed in the mutant nerves compared with the control nerves (red arrows in Fig. 7D and 7B, respectively). However, owing to the significant importance of the clusters of unsorted axons, we could not exclude that a small number of motor axons are present within these clusters and remain consequently unsorted.

**β1-Integrin loss in SC leads to neuromuscular innervation defects**

The muscular activity is regulated by neuromuscular junction (NMJ) activity, which in turn requires coordinated interaction between SC, axons and muscle fibres (Burden, 1998; Sanes and Lichtman, 1999). We analysed the development of NMJs for various muscles obtained from P1 and P21 control animals and from mutant animals at P1 and from those at P21 that have developed motor and posture defects. Synaptophysin (synapo) and α-bungarotoxin (α-BTX) were used to detect the pre- (axonal terminal end) and post- (clusters of acetylcholine receptor, AChR) synaptic compartments of the synapse. The mutants differed from the controls in having a lower level of intramuscular innervation and in the formation of NMJs in abdominal muscles (Fig. 9) and the diaphragm, soleus and gastrocnemius muscles (not shown). At P1, the control abdominal muscle displayed a high density of AChR clusters co-localised with synaptophysin (Fig. 9A, inset), indicating the
apposition of the pre- and post-synaptic compartments, as previously described (Lin et al., 2001). At P21, fewer AChR clusters (Fig. 9C, insert) were detected, demonstrating that the known postnatal regulation of receptor number had occurred. By contrast, at P1, the muscles of mutants displayed high densities of AChR clusters, but most of these receptors were not connected to the post-synaptic apparatus (Fig. 9B, inset). At P21, the density of AChR clusters remained high in the mutant muscles, indicating that no postnatal regulation of receptor number had occurred. Some of these receptors were connected to nerve terminals (Fig. 9D, inset). The synapses in the P21 mutants appeared as ring-like structures (Fig. 9D, inset), resembling immature NMJs (Sanes and Lichtman, 1999).

NF-160 immunostaining labelled a dense mat of nerve fibres, mostly restricted to the surface of the muscle in the mutants (Fig. 9F), whereas many nerve fibres aligned with the muscle fibres were labelled in the controls (Fig. 9E). NF-160 immunostaining also suggested the existence of aberrant connections at the point of arrival of the axon at the NMJ in mutant muscles compared to the controls (Fig. 9E,F, insets). However, SC, which is identified by S100 immunolabelling, were located along the nerves and, in particular, at the NMJs in the muscles of both the controls and the mutants (Fig. 9E,F, insets).

Discussion
The Ht-PA-Cre mouse strain is a useful tool for investigating the contribution of β1-integrins to PNS development. From E8, all the expected targeted cells, such as the glial and neuronal NCC-derivatives, lost the floxed β1-integrin gene in our conditional mutants, as shown by the recombination-dependent expression of the lacZ reporter gene. We found that the loss of β1-integrins in the NC-derived cells leads to severe defects in the PNS, with delayed migration of SCP along the axons, embryonic alterations of the peripheral nerve network, a delay in the start of myelination in sciatic nerves, a severe alteration in the radial sorting of sensory nerves and defective NMJ maturation. The diversity and severity of the mutant phenotype demonstrates the crucial direct and indirect roles of β1-integrins in establishing both the glial and neuronal components of the peripheral nerves. The early death of the mutant animals in the postnatal period is probably caused by respiratory failure resulting from extensive alterations of the PNS, together with multiple defects in enteric and neuroendocrine derivatives (M.A.B. and T.P., unpublished), and reveals the importance of β1-integrins in NCC ontogeny.

β1-Integrins transiently control embryonic development of the cranial nerves
The conditional deletion of the β1-integrin gene began in the cephalic region of the embryos at stage E8 and followed a rostral to caudal progression in line with the generation of NCC. On E10, in most mutants, significant changes were observed in the epibranchial ganglia and nerves, of NCC origin, with altered vagus nerve roots and also fusions of the IXth and Xth nerves. Interestingly, defects of this type are not observed in the trigeminal ganglia, despite the NCC origin of the glial and neuronal compartments (Pietri et al., 2003). These defects, observed during a period in which the loss of β1-integrins from the cell surface is incomplete, illustrate differences between the epibranchial ganglia and nerves and the trigeminal ganglia to integrin levels during development.

Indeed β1-integrins were not lost immediately after recombination of the locus and ceased to be detectable at the
Integrins in PNS development

- Integrins are expressed at the cell surface at low levels, but the complete loss of β1-integrins strongly inhibits these processes.
- An increase in the apoptosis of cephalic NCC has been described in α5-integrin-null mice (Goh et al., 1997).
- The phenotypic effects on the cranial nerves of conditional mutants seem to be transient because on the following day, the cranial nerves and ganglia appeared similar to those of the controls.

**Fig. 8.** Immunohistochemical analysis of sciatic nerves on P1 and P21. Transverse sections of sciatic nerves of control (A,B,E,F,I-M) and mutant (C,D,G,H,N-R) embryos on P1 (A-D,I,K,N,P) and P21 (E-H,J,L,M,O,Q,R). Immunolocalisation of NF-160 (A,C,E,G), the major myelin protein P0 (B,D,F,H), laminin (I,J,N,O), fibronectin (K,L,OQ) and tenascin (M,R). The levels of labelling for different markers cannot be compared. Scale bars: in H, 25 μm for A-D and 50 μm for E-H; in R, 20 μm for I-R.

Cell surface only after 3 days of detectable β-galactosidase activity. This time lag to the loss of β1-integrins in the targeted structures limits the functional analysis of β1-integrin requirements during the early stages of NCC migration. In particular, no defects are observed in the branchial arches or in the colonisation of the heart in our mutants. A previous study showed that α4-integrin-null embryos die during early development and display abnormalities in heart development (Yang et al., 1995). In mouse and avian embryos, perturbation experiments with competitors have shown that α4-integrin controls cranial NCC migration (Kil et al., 1996; Kil et al., 1998). This suggests that, in our mutants, sufficient amounts of β1-integrins remain at the cell surface of the cranial NCC at these stages to support the correct development of these morphological processes. Consistent with this hypothesis, the elimination of β1-integrins earlier in development, when the NCC are still resident in the neural tube, results in the early death of the embryo (E12.5) (T.P., unpublished). In this case, the embryos die from haemorrhaging, accompanied by defects in NCC cardiac derivatives and in neural tube closure. Thus, various early morphological processes may occur if β1-integrins are expressed at the cell surface at low levels, but the complete loss of β1-integrins strongly inhibits these processes. An increase in the apoptosis of cephalic NCC has been described in α5-integrin-null mice (Goh et al., 1997). This phenomenon has not been observed in other knockout mouse models (reviewed by De Arcangelis and Georges-Labouesse, 2000; Sheppard, 2000). In our conditional mutants, the rate of apoptosis in the branchial arches was similar in mutants and controls (T.P., unpublished). This suggests that the increase in the rate of apoptosis observed in α5-null cranial NCC may be triggered by the combined effect of the loss of α5β1-integrin and a possibly defective ECM microenvironment in constitutive α5-integrin knockout embryos.

The phenotypic effects on the cranial nerves of conditional mutants seem to be transient because on the following day, the cranial nerves and ganglia appeared similar to those of the controls. At birth, mutant and control embryos displayed no obvious differences in sensorial capacities, with both groups responding to external noise and light stimuli. This suggests the existence of a compensatory mechanism, operating after E10.5, establishing a new path to guide the altered nerve to its...
normal destination, leading to normal sensorial behaviour later on. Our results indicate that β1-integrins are not required for the further development and maintenance of craniofacial structures.

β1-Integrins control embryonic development of the PNS

In vitro studies have clearly implicated β1-integrins in the control of neurite outgrowth for peripheral neurons (Tomaselli et al., 1993; Ivins et al., 2000; Vogelezang et al., 2001). In our conditional β1-integrin knockout embryos, the loss of β1-integrins in sensory neurons does not affect their axonal growth. The general trajectory of the proximal portion of these nerves appeared to be minimally affected in the mutant animals, with the nerve endings reaching distal targets. However, the organisation of the network is visibly affected because, from E12, the distal region of the nerves of the body wall appeared less branched and fasciculated. In addition, the pathfinding of the distal region of the spinal nerves was perturbed, particularly when they reach the subcutaneous level.

The spinal nerves contain a mixture of motor and sensory axons along most of their length, and the specific axon bundles segregate from one another only once they are in close proximity to their targets. Therefore, the motor axons, which are not targeted for the conditional deletion of the β1-integrin gene in our mutant animals, in the mixed nerves may help to compensate for or to mask the potential β1-integrin-dependent guidance defect of the neighbouring sensory axons.

In addition to defects in nerve branching and fasciculation, we observed that the distal region of the nerves was not covered by migrating SCP, as shown by the lack of β-galactosidase-expressing cells along the nerve fibres at E12-13.5, in the mutants. This lack of SCP could give account into the developmental defect of the innervation network in mutants. Consistent with this hypothesis, similar embryonic PNS alterations in nerve branching and pathfinding have been described in the ErbB2, ErbB3, neuregulin 1 and Sox10 mutants (Erickson et al., 1997; Woldeyesus et al., 1999; Morris et al., 1999; Britsch et al., 2001; Riethmacher et al., 1997), which display alterations in the development of NCC derivatives and lack SC. For example, in ErbB2 mutants in which the heart development defect has been rescued by the myocardial-dependent expression of ErbB2, the cutaneous sensory nerves display normal overall trajectories but are free of SC precursors, poorly fasciculated and disorganised (Woldeyesus et al., 1999; Morris et al., 1999). Taken together, these and our results strongly suggest that migratory SCP are required for correct organisation of the innervation network and nerve morphology.

The α4β1- and α5β1-integrins have been implicated in control of the survival and proliferation rates of SC obtained from explants of embryos lacking α4- and α5-integrins (Haack and Hynes, 2001). The SCP migrating along nerves are originated from the DRG. However, we detected no difference in the proliferation or apoptosis rates of the β1-null NCC derivatives in the DRG of our mutants at E12.5 or E16.5. In addition, the lack of SCP along nerves was transient because, by E16.5, as for the controls, SCs were detected in the distal region of the spinal nerves in mutants. Although we cannot exclude the possibility that a modification of proliferative or apoptosis rate may occur on SCP located along the nerves, the loss of β1-integrins (which is effectively complete by this stage) seems to reduce the rate of migration of the SC along
nerves. This is consistent with previous observations showing that SC lacking β1-integrins survive and proliferate normally in vivo (Feltri et al., 2002). As spatiotemporal regulation of the integrin repertoire occurs during SC ontogeny (Bronner-Fraser et al., 1992; Stewart et al., 1997; Milner et al., 1997; Previtali et al., 2001) other integrins, such as α6β4, αVβ3 and αVβ8, may have compensated for the possible migratory defect of SC induced by the loss of β1-integrins, by E16.5 in the mutants.

**β1-Integrins are required for radial sorting of the sensory axons in sciatic nerves**

In the course of development of peripheral nerves, cytoplasmic processes of remyelinating SC penetrate into the axon bundles to progressively achieve a one to one ratio between SC and axons. This phenomenon, starting just before birth, is named radial sorting and precedes the myelination process (Mirsy et al., 2002). In the mutant, the radial sorting of motor axons (not targeted by the mutation) occurs and the subsequent myelination of motor axon-associated SC is not affected. By contrast, there was an almost complete absence of myelinated sensory axons (distinguishable by β-galactosidase activity). Sensory axons remain in large dense clusters of unsorted axons, indicating that a defect in axo-glial segregation occurs in the mutant nerves.

Alterations in the radial sorting and myelination process have been described in P0-Cre;β1-/-β1Δ mutants (Feltri et al., 2002) where postnatal nerves were composed of numerous clusters of unsorted axons and of few myelinated fibres. In the P0-Cre;β1+/β1Δ mutants, the conditional disruption of the β1-integrin gene is restricted to the glial lineage; the SCP are targeted between E13.5 and E14.5 and the loss of the integrin subunit on these cells is achieved by E17.5, whereas the neuronal components of the sciatic nerve are not targeted. Therefore, in this study the phenotype appears to be SC autonomous. The authors attributed the partial loss of myelinated axons in the adult sciatic nerve as the result of a random escape process because of compensation by others receptors. In the Ht-PA-Cre;β1-/-β1Δ mutants, the conditional mutation is made on SC and in sensory neurons early during development, and we can visualize the targeted cells by their β3-galactosidase activity. The defects obtained on radial sorting of the Ht-PA-Cre;β1-/-β1Δ mutant sciatic nerves are in agreement with a crucial role of β1-integrins in SC to achieve the axo-glial segregation. We are able to discriminate the sensory axons from the motor axons within the sciatic nerves. This has allowed us to show a much more prominent defect of radial sorting on the sensory compartment than on the motor compartment. This observation suggests that the β1-integrins in neurons could be also required for this process. In addition, the absence of myelinated sensory axons indicates that the myelination process requires the β1-integrin functions both in SC and neurons. However, in the P0-Cre;β1+/β1Δ mutants (Feltri et al., 2002) the discrimination between the sensory and motor axons could not be made and it was not possible to determine whether the radial sorting defects affected only one or both types of neuronal fibres within the sciatic nerve. Therefore, it is possible that in the P0-Cre;β1+/β1Δ mutants, as for the Ht-PA-Cre;β1-/-β1Δ mutants, the radial sorting defect is restricted to the sensory compartment. In this case, additional hypotheses can explain the differential response of sensory and motor compartment of the sciatic nerves to the inactivation of the β1-integrin gene: (1) owing to their distinct embryonic origin, sensory and motor neurons may express different sets of cell adhesion molecules involved in the radial sorting process such that the sensory axon sorting mechanism required β1-integrins on glia; and (2) SC could be specialized in two distinct types, each ensheathing only motor axons or only sensory axons, and with distinct requirement for β1-integrin function. To definitively conclude on the specific role of β1-integrins in neurons and SC during the development of the PNS, it would be interesting to compare the radial sorting process of sensory and motor axons in animals in which the invalidation of β1-integrin gene is restricted to SC, to motor or sensory neurons, or to both SC and neuronal compartments.

The delay in the myelination of SC in the Ht-PA-Cre;β1-/-β1Δ sciatic nerves is consistent with results of two other studies that have implicated β1-integrins in the control of the myelination process. One of these studies shows that β1-integrin antibodies prevent SC co-cultured with DRG neurons to myelinate the sensory axons (Fernandez-Valle et al., 1994). Another study shows that glial cells that have been genetically modified to express dominant negative forms of the β1-integrins, were unable to remyelinate after their transplantation in the dorsal funiculus (Relvas et al., 2001). In addition, several lines of evidence demonstrate that laminins and their receptors are involved in PNS development and that SC require contacts both with the basal lamina and with axons for their survival and maturation during development (reviewed by Jessen and Mirsky, 1999; Previtali et al., 2001; Previtali et al., 2003; Mirsky et al., 2002). These interactions facilitate the activation of the signalling pathways mediated by various molecules including the focal adhesion kinase and paxillin (Chen et al., 2000) (reviewed by Previtali et al., 2001), further implicating β1-integrins in the initiation of myelination. These observations are consistent with our observation that myelination was delayed in mutant sciatic nerves. Together with changes to the ECM in the sciatic nerves, the general delay in myelination in mutants strongly suggests that the loss of β1-integrins alters the interaction of the SC with the basal lamina, as previously suggested (Feltri et al., 2002), and with axons. In addition, the loss of β1-integrins in targeted cells appears also to affect the ECM and basal lamina organisation. The presence of a basal lamina surrounding unsorted axons in the Ht-PA-Cre;β1-/-β1Δ sciatic nerves is consistent with a retraction of SC cytoplasmatic processes and previous reports for several types of peripheral demyelinating neuropathies (reviewed by Dyck et al., 1992). This similarity is corroborated by quantitative modifications of the ECM, in particular tenasin deposition in the sciatic nerves on P21, and indicates that demyelination may occur in the mutants. The absence of basal lamina surrounding the unsorted axons within the clusters in the mutant sciatic nerves at P21, strongly suggests that the SC did not individually ensheathe those axons in earlier stages of the maturation of the nerves. Therefore, our data strongly indicate that both processes – complete absence and retraction of SC cytoplasmatic process – may occur concomitantly in the mutant sciatic nerve.

**β1-Integrins are required in the terminal SC for the targeting of nerves and maturation of the neuromuscular junctions**

The defects in intramuscular innervation observed in several muscles at birth and following postnatal stages in the mutant animals suggest that SC with β1-integrins are required for
axonal target selection. It was recently suggested that glial cells may be involved in this process in Drosophila (Poec et al., 2001), but no similar mechanism has yet been described in mammals. By contrast to control animals, in which axons penetrated the muscle to innervate specific fascicles, most of the nerve network in the mutants remained along the surface of each muscle layer and failed to penetrate. On stages P1 and P21 in the mutant animals, SC covered the entire length of the motor nerves from base to tip and were found at the nerve endings. This suggests that β1-integrins are required for the interactions of SC with axons or basal lamina and to facilitate the entry of axons into the muscle layers and the targeting of AChR clusters. The perturbation of intramuscular innervation was more apparent on P21 than on P1 in the mutant animals, indicating the lack of a compensatory mechanism. At the postsynaptic level, on P1, no difference was observed in the number and structure of AChR clusters in the mutants, indicating that this failure for the axons to meet the targets was not due to the absence of targets. The mismatch between nerve endings and AChR clusters (pre- and post-synaptic compartments, respectively) was striking on both P1 and P21, confirming the independence of the formation of AChR clusters from the presynaptic apparatus.

The mutants responded to tactile stimulation at P1, which implies that there were some functioning NMJs at that stage. However this ability to respond deteriorated thereafter, suggesting that SC and sensory innervation were involved in this process.

Even in the presence of the three components of the synapse (axons, terminal SC and postsynaptic apparatus), NMJ observed at P21 in the mutant resemble to an immature NMJ as they have a ring-like structure. This type of structures is normally observed in the normal course of NMJ development from P7 to P10 (Burden, 1998; Sanes and Lichtman, 1999). In addition, a lack of regulation of the number of AChR clusters in the mutant occurred, at least until P21. In wild-type rodents, this mechanism occurred within the first 2 weeks after birth. Therefore, NMJ maturation in the mutants appears to be blocked at an early stage of their postnatal development. Several studies examining muscle re-innervations have suggested that terminal SC are involved in the maintenance and remodelling of the synapse (Son and Thompson, 1995). Our results point out the β1-integrins as major receptors for the proper activity of the terminal SC in the course of the NMJ development.

In normal NMJ development (reviewed by Sanes and Lichtman, 1999) or after nerve regeneration (Rich and Lichtman, 1989), a significant number of synapses are eliminated during organisation of the nerve-muscle connection. Synapse elimination involves the withdrawal of the axons and SC processes. This process was observed in the control animals, in which the number of AChR clusters and nerve terminals in the abdominal muscles decreased from P1 to P21. By contrast, in the mutants, the number of AChR clusters increased from P1 to P21. This phenomenon resembles the responses of muscles to denervation, by increasing the number of AChR clusters (Blondet et al., 1989). The increase in the number of AChR clusters in mutants coincides with progressive muscle fibre atrophy, reflecting the absence of stimulatory innervation. This suggests that the failure of many axons to establish functional NMJs leads to muscle to produce more AChR clusters and that the process of synapse elimination is probably not initiated. The specific alteration of the elaboration and maturation of the NMJs in our mutant animals shows a SC autonomic effect for the muscular innervation and reveals the major involvement of the β1-integrin specifically in the terminal SC functions.

We conclude from our results that β1-integrins are important for correct interactions between glial cells and axons, SC migration, axon morphology and the organisation of spinal nerves into networks en route to their peripheral targets. This study also highlights the functions of β1-integrins for both the neuronal and glial lineages of the PNS and at several steps in the differentiation of peripheral nerves, such as radial sorting and myelination, as well as the muscular synaptogenesis.

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