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Laminin deficits induce alterations in the development of dopaminergic neurons in the mouse retina

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Abstract

Genetically modified mice lacking the $\beta 2$ laminin chain ($\beta 2$ null), the $\gamma 3$ laminin chain ($\gamma 3$ null), or both $\beta 2/\gamma 3$ chains (compound null) were produced. The development of tyrosine hydroxylase (TH) immunoreactive neurons in these mouse lines was studied between birth and postnatal day (P) 20. Compared to wild type mice, no alterations were seen in $\gamma 3$ null mice. In $\beta 2$ null mice, however, the large, type I TH neurons appeared later in development, were at a lower density and had reduced TH immunoreactivity, although TH process number and size were not altered. In the compound null mouse, the same changes were observed together with reduced TH process outgrowth. Surprisingly, in the smaller, type II TH neurons, TH immunoreactivity was increased in laminin-deficient compared to wild type mice. Other retinal defects we observed were a patchy disruption of the inner limiting retinal basement membrane and a disoriented growth of Müller glial cells. Starburst and AII type amacrine cells were not apparently altered in laminin-deficient relative to wild type mice. We postulate that laminin-dependent developmental signals are conveyed to TH amacrine neurons through intermediate cell types, perhaps the Müller glial cell and/or the retinal ganglion cell.

Keywords

Extracellular matrix; Inner limiting; Membrane; Amacrine cell; Muller cell

Introduction

Laminins are heterotrimeric glycoproteins found in basement membranes and the extracellular matrix of nervous tissue (Colognato & Yurchenco, 2000). The component molecules (chains) fall into three families, each of which has multiple members: 5α , 3β , and 3γ chains have been identified. Of the approximately 45 possible combinations of these chains, only 16 have been identified (Yurchenco & Wadsworth, 2004; Yan & Cheng, 2006). Cell surface receptor molecules such as integrins (DeCurtis & Reichardt, 1993), dystroglycans (Barresi & Campbell, 2006), and syndecans (Suzuki et al., 2005) bind to laminins, and use this interaction to activate a variety of intracellular pathways involved in cell survival, growth and differentiation (Ivins

et al., 2000; Clegg et al., 2000). Laminin defects are implicated in several developmental disorders in humans and mice, including muscular dystrophy in both the peripheral, and central nervous systems (Colognato & Yurchenco, 2000; Miner & Yurchenco, 2004; Olson & Walsh, 2002). Mutations in the human $\beta 2$ gene result in Pierson's syndrome, and other ocular disorders (Zenker et al., 2004, 2005).

In the mammalian retina, the focus of the present study, several laminin isoforms have been identified (Hunter et al., 1992; Libby et al., 2000; Aisenbrey et al., 2006). Laminins are distributed widely within the retina, either in basement membranes (Bruch's membrane, inner limiting membrane, vascular basement membrane) or in the extracellular matrix, e.g., the outer plexiform layer (OPL) in which photoreceptors synapse with second-order retinal neurons and in the interphotoreceptor matrix. These studies demonstrated that the $\beta 2$ chain is located in the OPL and in basement membranes of the retina, whereas the $\gamma 3$ chain is deposited in retinal basement membranes. A prior study (Libby et al., 1999) found that disruption of the laminin $\beta 2$ chain altered photoreceptor development in several ways, including synaptic disruption and alterations of photoreceptor outer segment morphology. On the other hand, the inner plexiform layer (IPL), in which bipolar cells and inner retinal neurons make synaptic contacts, appeared unaltered. This prior study, however, did not exhaustively report the possible effects of laminin deletion on the inner retina. Because inner retinal neurons are found in multiple subtypes (MacNeil et al., 1999; Rockhill et al., 2002), here we explore the possibility that only certain of them might be affected by laminin deficits, leaving the overall appearance of the inner retina unchanged. Specifically, we examine possible effects of particular laminin chain deletions on the development of identifiable subtypes of inner retinal neuron: cholinergic, AII and tyrosine hydroxylase (TH)-containing amacrine cells.

Emphasis was placed on the TH amacrine cells because in preliminary screens it was the cell class in which clear developmental deficits related to laminin deletions were noted. Retinal TH-containing neurons are of two subtypes, each having a characteristic size, shape and domain of process arborization in the IPL. The larger, type I TH immunoreactive (IR) amacrine (Mariani & Hokoc, 1988) is known to be dopaminergic (Ehinger & Floren, 1978), whereas the neurotransmitter released by the smaller, type II TH neuron is not yet definitely established.

We compared neuronal development in wild type mouse retinas and in mice of the same strain in which the genes encoding either laminin $\beta 2$ or laminin $\gamma 3$ were deleted by homologous recombination techniques; we produced a mouse line with deletion of both $\beta 2$ and $\gamma 3$ chains by crossing these knockout animals. Our results indicate that both the $\beta 2$ laminin chain deletion and the double $\beta 2/\gamma 3$ laminin deletions were associated with substantial alterations in TH neuronal development, including increased apoptosis, delayed process outgrowth and altered TH levels; in contrast, the $\gamma 3$ laminin deletion was not linked to evident modifications of TH neuronal development. In none of the mutant mice we examined were changes noted in the development either of cholinergic or AII amacrine cells.

The same laminin chain deletions also resulted in disruption of the inner limiting membrane, a basement membrane that lines the vitreal surface of the retina. Moreover, the radial glial cells of the retina, the Müller cells, showed a marked disarray of vitreal end feet and inner retinal expansions, consistent with the finding that Müller cells express laminin receptor complexes and interact with laminins (Claudepierre et al., 2005, 2000; Noël et al., 2005; Méhes et al., 2002; Moukhles et al., 2000). Our data provide additional support for the idea that radial glial cells play crucial, if still poorly defined, roles in retinal development (Willbold & Layer, 1998; Blackshaw et al., 2004).

Methods and materials

Animals

The lines of mice we used were bred and maintained in the animal facility of Tufts University. All procedures involving animals were approved by the Tufts University Animal Care Committee and were in accordance with the standards established by the National Institutes of Health and the Association for Research in Vision and Ophthalmology.

The standard recombinant procedures used for generating the null mutations in $\beta 2$ have been reported elsewhere (Noakes et al., 1995). $\text{Lamc3}^{-/-}$ mice were generated using standard recombination methods; a complete description is given elsewhere (Li, Y., French, M., Burgeson, R.E., Koch, M., & Brunken, W.J., unpublished observation). In brief, a targeting vector for homologous recombination was created in which a 2.2 kb spanning exon 1 and part of intron 1 of the Lamc3 gene was deleted and replaced with promoterless IRES β -Geo cassette. The targeting construct was linearized and electroporated into 129/SvJ ES cells. Neomycin-resistant ES cell clones were selected, expanded, and injected into C57Bl/6 blastocysts for the production of chimeric mice. Mice from both lines ($\beta 2^{+/-}$ and $\gamma 3^{+/-}$) were backcrossed to wild type C57Bl/6J mice. $\beta 2$ null mice have renal and neuromuscular deficits that are lethal typically during the fourth postnatal week; therefore they were maintained as heterozygote lines. $\gamma 3$ null mice show only very subtle differences from wild type and are fertile; we maintained them as heterozygote lines. To generate the compound null ($\beta 2^{-/-}$, $\gamma 3^{-/-}$) mice, we mated $\beta 2^{+/-}$ mice with $\gamma 3^{-/-}$ mice and using breeding pairs of $\beta 2^{+/-}$, $\gamma 3^{-/-}$ to generate all the progeny examined in this study. While these mice are on a mixed genetic background of C57Bl/6J and 129Sv/J, the founder mice of this line had been backcrossed at least five times to C57Bl/6J. In all cases, littermates were used as controls. $\beta 2^{+/+}$, $\gamma 3^{+/+}$ animals were littermates of the $\beta 2$ null mice and the genotypes of the progeny were determined by PCR from genomic tail DNA.

Dissection and tissue processing

Mice were euthanized by a brief exposure to CO_2 , and then decapitated. The eyes were enucleated, the cornea and the lens removed and the eyecups fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 for 1 h at room temperature. Thereafter the eyecups were washed 3 $\times$ 20 min in phosphate-buffered saline (PBS, 7.4 pH). After cryoprotection in sucrose solutions of increasing concentration (10, 20, 30% in PBS), frozen sections (10 μm) were cut in a cryostat. The sections were mounted on Superfrost slides (Fisher Scientific), air-dried and processed for immunocytochemistry as described below. For whole mount preparations, eyecups were fixed, washed, and the retinas freed and processed for immunocytochemistry, using either 3,3 diaminobenzidine tetrahydrochloride (DAB) or fluorescent secondary antibodies to visualize the immunostained cells.

Immunocytochemistry

1. DAB staining—Whole mounts were pretreated with DMSO for 30 min followed by 3 $\times$ 10 min washes with 0.3% Triton-X 100 in PBS to increase the penetration of antibody molecules. After pre-incubation in blocking solution (5% donkey serum, 1% bovine serum albumin, 0.1% Triton-X 100 in PBS) whole mounts were incubated in anti-TH antibody (Table 1) overnight at 4°C, washed in PBS (6 $\times$ 10 min) and placed into solution containing biotin-conjugated anti-rabbit IgG antibody (1:250) (Table 2) for 6 h at room temperature. After 6 $\times$ 10 min washes in PBS, preparations were incubated in extravidin-peroxidase complex (1:100) (Sigma) for 4 h. The retinas were rinsed in PBS prior to their incubation in 0.05% DAB in PBS for 15 min, followed by 0.05% DAB and 0.01% hydrogen peroxide dissolved in PBS. The reaction was stopped by a rinse in PBS.

2. Double label immunofluorescent staining—Retinal sections were treated with 0.1% Triton-X 100 in PBS and incubated in blocking solution as described above. Mixtures of the primary antibodies (Table 1) were left on the sections for 2–4 h then the sections were washed 6×5 min in PBS. Immunoreactivity was detected by incubation with immunofluorescent secondary antibodies conjugated to appropriate fluorochromes (Table 2) for 2–3 h. After rinsing in PBS (6×5 min), sections were mounted with Vectashield (Vector Laboratories). Two controls were performed for immunocytochemical labeling. Omission of the primary antibodies in both the single- and double-labeling experiments resulted in no staining. Cross-reactivity of the non-corresponding primary and secondary antibodies was not detected. Reactions were carried out on tissues derived at least from three different animals.

Indolyl phosphate staining

For demonstration of human placental alkaline phosphatase (PLAP) activity in 2 and 3 days old transgenic mouse retinas, eyecups were fixed in 4% paraformaldehyde for 2 h at room temperature. Whole mount preparations were warmed to 65°C for 30 min to inactivate other phosphatases. They were incubated in the dark for 3 h in 0.1% 5-bromo-4-chloro-3-indolyl phosphate and 1% nitroblue tetrazolium solved in 0.1 M Tris-HCL buffer (pH 9.5) followed by rinsing in 0.02 EDTA solution. As a result, purple-blue reaction product appeared at the surface of the neurons.

Western blotting

To obtain sufficient protein, four P20 retinas were processed for Western blotting from each genotype (wild type (wt); $\beta 2^{-/-}:\gamma 3^{+/+}$; $\beta 2^{+/+}:\gamma 3^{-/-}$; $\beta 2^{-/-}:\gamma 3^{-/-}$). The tissue was homogenized by sonication and the protein extracted in lysis buffer containing 50 mM TRIS-HCL, 150 mM sodium chloride, 1% Nonidet P-40, 1% SDS, 0.5% sodium deoxycholate, 1% PMSF, 1% $\alpha 2$ -macroglobulin. After establishing the protein concentration by BCA Protein Assay Kit (Pierce), the samples were boiled for 5 min in sample buffer (62.5 mM Tris-HCL, 10% glycerol, 2% SDS, 5% mercaptoethanol, 0.05% bromophenol blue, pH 6.8). Ten μ g proteins from each sample was loaded and separated by electrophoresis through a 10 % polyacrylamide gel. The proteins were transferred to PVDF membrane (BioRad) by semi-dry immunoblotting. Membranes were pre-incubated in a blocking buffer (5% milk protein, 0.1% Tween-20 in TBS) for 1 h at room temperature prior to the incubation with anti-TH antibody (1:5000) overnight at 4°C. The membranes then were rinsed in Tris-buffered saline, 0.1% Tween-20 (TBS-Tween) 3×10 min, and incubated with horse-radish peroxidase-conjugated anti-rabbit IgG (1:10,000) (Molecular Probes) for 2 h at room temperature. Finally, the membranes were thoroughly washed in TBS-Tween solution, soaked in chemiluminescent substrate (PerkinElmer LAS Inc) for 1 min then exposed to film for signal detection.

Statistics

Data populations are given as means $\pm$ standard errors. Data groups were evaluated for significant differences ($p < 0.05$) by a Student *t*-test.

Reverse transcription and real time PCR

Total RNA was extracted from wt, $\beta 2$ null, $\gamma 3$ null and compound null retinas aged P20 and P4 using RNeasy Kit (Qiagen) according to the manufacturer's instructions; cDNAs were synthesized from 60 to 120 ng of total RNA. Primers designed for real time PCR were as follows: for mouse TH, the forward primer was GCCGTCT CAGAGCAGGATAC, the reverse primer was AGCATTTCCATC CCTCTCCT; for the mouse β actin, which served as an internal control, the forward primer was GGCTATGCTCTCCCTCACG, the reverse primer was CTTCTCTTTGATGTCACGCACG. For the mouse RPL13A control, the forward primer was GAGGT CGGGTGAAGTACCA and the reverse primer was TGCATCT

TGGCCTTTTCCTT. Reactions were carried out in a 25 μ l volume using Quantitect SYBR Green PCR Kit (Qiagen). Each experiment was repeated twice in triplicate. The results were analyzed by the Δ Ct method that reflects the difference in cycle threshold for the target gene relative to that for β actin in each sample.

Data processing

Micrographs were taken with an OrcaER digital camera (Hamamatsu Ltd, Tokyo, Japan) and digital images recorded with the help of an image analyzer program (Openlab Imposition Ltd., Lexington, MA or Auto Quant Imaging, Watervliet, NY). The digital images were adjusted for intensity and contrast in Adobe Photoshop 7.0. Cell density, cell lengths and nearest neighbor analyses were performed on retinal whole mounts, using a semiautomatic computer-assisted image analysis system (Openlab). The microscopic field was examined with a 20x objective on a video screen. The distance of TH-IR neurons to their nearest neighbor was measured and the data were grouped and plotted in 10 μ m increments. A sample size of approximately 2000 neurons was used to determine cell dimensions. These data were obtained from four retinas derived from four different animals.

Results

Comparison of wild-type and laminin-deficient retinas at P20

As a first step in identifying laminin-dependent alterations in neuronal development, we studied mouse retinas after 20 days of post-natal development (P20). Mice open their eyes around P14 and by P20 retinal dopaminergic neurons have achieved their adult dimensions (Wulle & Schnitzer, 1989). We examined dopaminergic, cholinergic, and AII amacrine cells, using antibodies specific for each of these subtypes of amacrine cell. In addition we used an anti-calretinin antibody, which labels several types of inner retinal neuron.

Subtypes of TH-immunoreactive neuron in the mouse retina

Two subtypes of TH neuron are found in mammalian retinas (Mariani & Hokoc, 1988). The type I TH cells are relatively large, with perikaryal diameter or long axis between 11–20 μ m. They immunostain robustly with an anti-TH antibody (Figs. 1a, 1b). A particular advantage of staining for TH is that this enzyme is found throughout the dopamine cell, permeating its finest processes, which in the rodent retina can be 0.2 μ m in diameter and hundreds of microns long (Witkovsky et al., 2005). Most type I TH cell processes are distributed within the most distal sublamina (sublamina 1) of the inner plexiform layer (IPL). Type II TH neurons are much smaller, with perikaryal diameter or long axis typically less than 10 μ m. Their TH immunoreactivity is modest and their processes arborize primarily in the middle (sublamina 3) of the IPL (Fig. 1c).

In each of the four studied genotypes (wild type, $\beta 2^{-/-}$; $\gamma 3^{+/+}$; $\beta 2^{+/+}; \gamma 3^{-/-}$, $\beta 2^{-/-}; \gamma 3^{-/-}$), type I TH neurons were characterized by cell density, perikaryal size, and nearest neighbor analysis. All these data were derived from examination of retinal whole mounts in which the entire retina was sampled. Hereafter, we refer to these genotypes as wt, $\beta 2$ null, $\gamma 3$ null and compound null, respectively. Type I TH neurons were at a significantly higher density in wt retina compared to either the $\beta 2$ null retina or the compound null retina, whereas their density in the $\gamma 3$ null retina did not differ significantly from wt (Table 3; *p* values given are calculated by reference to wild type).

The dimensions of type I TH cell bodies were estimated from their longest axis and the mean $\pm$ S.E. values are as follows: wt, 14.60 ± 0.04 ; $n = 2278$; $\gamma 3$ null, 15.68 ± 0.05 ; $n = 3450$; $\beta 2$ null, 14.87 ± 0.06 ; $n = 2605$; compound null, 13.66 ± 0.09 , $n = 1191$. Given the large sample sizes, the differences in the means, although small, are all significant ($p < 0.01$; Student *t*-test),

indicating that in the compound null retinas, the perikarya of type I TH neurons are somewhat smaller than the comparable cells in wt, $\gamma 3$ null or $\beta 2$ null mice.

Fig. 2 illustrates the nearest neighbor distribution of type I TH neurons in wt and each type of laminin-deficient retina we examined. Wild-type and $\gamma 3$ null nearest neighbor distributions were essentially identical, whereas the distributions of type I cells in the $\beta 2$ null and compound null retinas were skewed, with an evident shift of the nearest neighbor distributions to greater distances, as would be expected from the lower densities of type I TH neurons in these laminin-deficient retinas compared to wt (cf., Table 3).

Type 2 TH neurons

Although type II DA neurons are present in wt and $\gamma 3$ null retinas, their TH-IR in these two groups was very weak, preventing a reliable survey of their numbers in whole mount preparations. We were unable, therefore, to calculate a nearest-neighbor distribution for these cells. They were detectable, however, in vertical sections of the retina (Fig. 1a). In contrast, the immunoreactivity of type II TH neuronal perikarya in $\beta 2$ null and compound null retinas was, surprisingly, stronger (Fig. 1b) than in littermate $\gamma 3$ null littermates or in wt retinas of the same age (Fig. 1a), suggesting that the TH content of type II TH neurons is increased when the $\beta 2$ laminin chain is genetically ablated.

Thus, to summarize the findings for TH neurons at P20, the $\gamma 3$ null retina was not different in any measure from the wt. The $\beta 2$ null and compound null retinas showed similar alterations from the wt in the following ways: (1) type I DA cells are at a lower density; (2) type II DA cells show a greater TH immunoreactivity (3) in the compound null retina, type I TH perikaryal dimensions are slightly smaller than in the wt retina.

Given that (1) and (2) tend to produce opposite changes in total retinal TH, the possible differences in retinal TH content between wt and $\beta 2$ null retinas could not be predicted. To obtain further information on this point, we performed immunoblots on extracts from P20 pooled retinas from each genetic group, using β -tubulin as a standard. Wild type retinas were compared either to $\gamma 3$ null ($n = 1$), $\beta 2$ null ($n = 3$), or compound null retinas ($n = 1$); an example, comparing wt and $\beta 2$ null retinas, is illustrated in Fig. 3. The bands were scanned and their optical densities measured. After correction for differences in β -tubulin content, the ratios of optical densities are: $\gamma 3$ null/wt = 1.16; $\beta 2$ null/wt, 0.32 $\pm$.06; compound null/wt = 0.17.

Because the sample sizes were small, and we did not linearize our immunoblot detection methods, these data provide only a semi-quantitative indication of differences in TH content. Nevertheless they are consistent with the anatomical measures, in that in both $\beta 2$ null and compound null retinas, the type I TH neurons are at a lower density compared to wt. The ratios of TH content, however, indicate a greater difference between wt and $\beta 2$ null or compound null retinas than would be predicted solely by the measured differences in type I TH cell densities. Thus they may indicate additional differences in TH dendritic/axonal dimensions between wt and laminin-deficient mice. In fact, given the very long dendritic and axonal processes of TH neurons, and the observation that all such processes are filled with TH enzyme, most of the TH is estimated to be within them rather than in the perikaryon. A working calculation, based on the geometry of type I TH neurons in primate (Dacey, 1990) and rat retinas (Witkovsky et al., 2005) supposes that the perikaryon is a sphere, 15 μ m in diameter containing a nucleus 7 μ m in diameter, that each type I TH cell has four dendrites 300 μ m long and 1 μ m in diameter and four axons, 500 μ m long and 0.2 μ m in diameter. Assuming further that TH is uniformly distributed; these dimensions suggest that less than 20% total TH enzyme is in the perikaryon, the balance being spread among the dendritic and axonal processes. Thus, even small differences in TH process dimensions between wt and laminin-deficient mice would strongly influence the ratios of total TH. This possibility is considered in greater detail below in relation

to the developmental component of our study of TH neurons in wt versus laminin-deficient retinas. Type II TH neurons also contribute to total retinal TH, but based on dimensional considerations and their relatively low TH-IR compared to that of type I TH neurons, we estimate that their contribution to total retinal TH content is small (<5%).

Effect of laminin deficiency on basement membrane and glial Müller cell structure in the P20 mouse

Prior studies of the distribution of $\beta 2$ containing-laminins in vertebrate retinas (Hunter et al., 1992; Libby et al., 1996, 1999, 2000) showed that they are concentrated in the basement membranes and in the photoreceptor layer. They are sparse or absent, however, in the inner nuclear and plexiform layers, where the amacrine cell bodies and dendrites are located. This suggests an indirect mechanism whereby laminin deficits might affect amacrine cell development. In this regard, the Müller glial cell of the retina is an obvious point of focus. Müller cells attach to the retinal basement membrane (inner limiting membrane) and span the entire width of the retina. Recent studies suggest that Müller cells have multiple roles in retinal neuronal development (Reh & Levine, 1998; Blackshaw et al., 2004), including as a scaffold for neurite extension.

We began by examining the basement membrane of the retina, using antibodies directed against perlecan, an intrinsic molecule (heparin sulfate proteoglycan) of the basement membrane. Fig. 4 illustrates that in P20 wt retinas the basement membrane is a continuous sheet, but in both $\beta 2$ null and compound null retinas, the basement membrane showed discontinuous staining, indicating local disruptions of its structure. A more detailed and complete study of the effect of laminin deletion on basement membrane integrity is the subject of another study (Daly et al., 2006).

We similarly compared Müller cell structure in wt versus laminin-deficient retinas using either an antibody against glutamine synthetase (GS), a Müller cell specific marker (Vardimon et al., 1986), or an antibody directed against vimentin (VIM), an abundant intermediate filament protein found in Müller cells (McGillem et al., 1998), particularly in the proximal portion of the cell, i.e., that part which lies close to the retinal inner surface. A striking difference was revealed in Müller cell organization. In wt retinas, each Müller cell has a basal terminal expansion that attaches to the basement membrane; the alignment of these end feet creates an orderly array of glial cell terminal expansions (Fig. 5a). In either $\beta 2$ null or compound null retinas, however, the basal membrane was discontinuous, showing numerous small breaks at many points over the retinal surface. At these breaks, Müller cells did not attach to the basement membrane; instead their basal terminals formed a disorganized array (Fig. 5b) from the inner plexiform layer (IPL) through the ganglion cell layer. At other points, the Müller cell terminals aggregated at blood vessels (Fig. 5c). These observations suggest that attachment of the Müller cell end foot to the inner limiting membrane is critical in maintaining the polarity and organization of the Müller cell. Removal of all $\beta 2$ -containing laminins appears to disrupt significantly this attachment mechanism. In the compound null retina, in these regions of basement membrane disruption, the Müller cell had a reduced overall length and a disarrayed disposition of its fine lateral branches within IPL, relative to wt retina, as described and illustrated below in the section on development. No differences in basement membranes or Müller cell organization were noted in between WT and $\gamma 3$ null mice.

Effect of laminin deficiency on cholinergic, AII amacrine and calretinin-IR in the P20 mouse

Cholinergic amacrine cells were revealed by immunostaining with an antibody against choline acetyltransferase (ChAT; Zhang et al., 2005), whereas AII amacrines were labeled with an antibody against disabled-1 (Dab-1), a developmental marker (Rice & Curran, 2000). The anti-calretinin antibody immunostains numerous cells among the ganglion and amacrine cell layers

of the mouse retina, and also reveals three prominent bands of staining in the IPL. In relation to their cell density, process distribution and robustness of immunostaining, cholinergic and AII amacrine cells did not differ between wt and laminin-deficient retinas (not illustrated). With regard to calretinin immunostaining, in most regions of the retina, we noted no disruptions of the lamination pattern in the IPL in $\beta 2$ null and compound null mice relative to wt, although the IPL dimensions were somewhat reduced in the compound null mouse retina relative to wt. A comparison of calretinin-IR in wt, $\beta 2$ null and compound null retinas is illustrated in Fig. 6. It is important to note that the middle lamina of calretinin reactive processes arise from processes of type II TH neurons (Zhang, 2004); the normal appearance of this layer demonstrates that these cells are not disrupted by the mutations.

Laminin deficit-induced alterations in TH neuron development

The constellation of differences described above between P20 wt and laminin-deficient retinas suggests that the combined absence of $\beta 2$ - and $\gamma 3$ -containing laminins induces alterations in TH neuron, basement membrane and Müller cell development. These alterations are similar but less severe in the $\beta 2$ null retina. In order to study the onset of these defects, we extended our observations to the first two postnatal weeks when Müller cells and TH neurons become post-mitotic and undergo differentiation (Rapoport et al., 2004). In younger animals, there is less overlap of the dendritic or axonal arbors of neighboring TH cells making it possible to examine the arbors of individual TH neurons.

Developmental dependence of type I TH neuron cell density in wt and laminin-deficient retinas

We characterized the laminin-induced deficits in TH neuronal development by reference to the sequence of TH cell post-mitotic maturation in wt retinas. Because of the difficulty of seeing the smaller type II TH neurons in wt retinas our observations are confined to type I TH neurons. The development of type I TH neurons in mouse retina was studied in detail by Wulle and Schnitzer (1989); nevertheless we found substantial differences between our respective data bases in relation to the age at which TH-IR first appears and the distribution of TH neurons within the retina (see Discussion).

The density of type I TH neurons as a function of post-natal age is compared in Fig. 7 for wt, $\beta 2$ null, and compound null retinas. We made maps of the retinal distribution of TH cells, and computed cell densities from them. At postnatal day 3 (P3), type I TH cell density in wt retinas was low and variable (10–32 cells/mm²; $n = 3$ retinas from three different animals). Between P3–5, however, cell density increased rapidly to about 50 type I TH cells/mm². In the $\beta 2$ null retinas, the same growth pattern emerged but delayed by about 1 day with respect to wt retinas. The compound null retinas revealed an even greater delay; between P3–5, type I TH neurons were either completely absent, or present at a very low density. In the insert to Fig. 7, regression lines are fitted to the temporal sequence of Type I TH cell generation in wt and laminin-deficient retinas. Data for wt and $\beta 2$ null retinas are fitted by hyperbolas, whereas the data for compound null retinas are better matched by a linear function.

Fig. 7 thus illustrates two points about $\beta 2$ null and compound null retinas relative to their wt counterpart: a delayed appearance and a lower density. Cell density differences between wt and either $\beta 2$ null or compound null retinas are statistically significant ($p \ll 0.01$; cf. Table 3). Additionally Fig. 7 documents that for both wt and laminin-deficient retinas, there is a rather sharp reduction of approximately one-third in type I TH cell density between ages P10 and P12, with cell density remaining constant thereafter to P20. This loss is not attributable to an increase in retinal area, because the dimensions of the eye do not change appreciably between P10 and P12.

Growth pattern of type I TH neurons in wt and laminin-deficient retinas

In wt retinas, between P3-P6, type I TH cells have an inhomogeneous rate of development, in that in some local areas, cells have long processes with robust TH-IR, whereas in adjacent areas the development of the type I TH cell processes is retarded, and even the TH-IR of the perikarya is reduced. This patchy growth pattern gradually disappears between P6 and P15.

A comparison of type I TH neurons in wt, $\beta 2$ null and compound null retinas at different postnatal ages is depicted in Fig. 8. Panels a, b, c, illustrate the appearance of type I TH cells in wt, $\beta 2$ null and compound null retinas, respectively, at P7. The morphology, dimensions and TH-IR of type I TH neurons is similar between wt and $\beta 2$ null retinas, although the cell density is lower in the $\beta 2$ null than in the wt retina. In the compound null retina, however, the type I TH neurons at P7 show a markedly reduced TH-IR and restricted process outgrowth relative to their wt counterparts. It is apparent that at P10 (Figs. 8d–8f) and P12 (Figs. 8g–8i) the wt retinas develop an increasingly overlapping pattern of dendrites and axons (Figs. 8d, 8g), which is densest in the most distal (sublamina 1) portion of the IPL (the focal plane of all the images of Fig. 8). This network is reduced in the $\beta 2$ null retina (Figs. 8e, 8h) and is still sparser in the compound null retina (Figs. 8f, 8i). Parenthetically, at any age examined we found no distinctions between wt and the $\gamma 3$ null retinas.

During the third postnatal week the most prominent developmental change in type I TH neurons is the elaboration of axon terminal rings containing the presynaptic apparatus for the release of dopamine (Witkovsky et al., 2004). Between P12 and P20, the TH-IR network of axon terminals in the $\beta 2$ null retina approaches the density seen in the wt (Figs. 8j, 8k). Since, at P20, type I TH cells remain at a lower density than in wt retina (Table 1), the implication is that in the $\beta 2$ null retina, each type I TH cell generates extra axon terminals over what is seen in the wt retina. With regard to the compound null retina, its process development is poorer than that of wt retina at any developmental stage, including P20 (Fig. 8l). The density of axon terminals is reduced and the dendrites of the type I TH cell are thinner than those seen in wt retinas.

Thus, the data of Fig. 8 indicate that in compound null retinas, the growth of dendrites in type I TH retinas is reduced compared to wt. That is, even when the type I TH perikarya of wt and compound null have comparable TH-IR, the dendrites of those cells in wt retinas are longer, of greater diameter and more abundant. An alternative possibility, however, is that type I TH neurons in compound null retinas elaborate a full complement of dendritic and axonal processes, but that TH enzyme is somehow distributed only into a fraction of the existing dendrites, leaving others devoid of enzyme. Because in both wt and $\beta 2$ null retinas TH is distributed uniformly to all processes, including those of the finest caliber, we think this possibility is unlikely.

One feature of type I TH neuron development in laminin-deficient retinas is not revealed in Fig. 8. It is that the patchy development, i.e., adjacent retinal regions showing quite disparate degrees of TH-IR and process outgrowth, is still observed at P12 in compound null retinas (Fig. 9), although at this age it is no longer seen in wt retinas (not illustrated).

Activation of the TH gene in wt and laminin-deficient retinas

To gain some insight into the onset of TH protein production, we looked at the activation of the TH gene. To examine this point in wt mice we took advantage of a genetically modified mouse, produced in the Raviola laboratory (Gustincich et al., 1997), in which the TH promoter is linked to alkaline phosphatase. The background strain of these mice is the same, C57BL/6J, as the mutant mice employed in the present study. Positive staining for alkaline phosphatase is an indicator that the TH gene has been activated. We found that in P2 mice, perikarya at the same retinal position and the same low density as type I TH cells stained for alkaline

phosphatase (not illustrated). This datum shows that in wt mice by P2 the TH gene is activated. The first appearance of TH protein at P3 (cf. Fig. 3) thus indicates that at least 24 h are required to begin to synthesize and transport TH protein to its target sites. In the rat retina, essentially all amacrine cells are post-mitotic by P0 (Rapoport et al., 2004). In this regard, the finding that in wt mice the apparent density of type I TH neurons at P3 is about half that at P5, suggests that the appearance of TH protein is spread out over 2–3 days, progressively making more type I TH neurons visible through TH-IR.

Unfortunately, we could not apply the alkaline phosphatase method to our laminin-deficient mice since they lacked the transgene reporter. Thus, we approached the same question by assaying the retinas for TH mRNA using quantitative real time PCR. All the mice of a single litter were harvested for this experiment to ensure that the samples were from mice of the same age (P3). Genotyping was performed on tail DNA; $\gamma 3$ null mice were compared to compound null mice. Q-PCR tests were run with two internal controls: β -actin and RPL13a. Changes in the cycle threshold (Ct) were used to quantify our results. The Ct for the control littermates ($\gamma 3$ null) was 29.4 and 28.6 for the compound null using β -actin as reference; the Ct's were 29.4 versus 29.1, respectively, using RPL13a as reference. These values are essentially identical, indicating that the TH mRNA message levels are about the same in the compound null as in the wt mouse. We conclude that the delay in appearance of TH protein in the compound null mouse relative to the wt (cf. Fig. 7) is attributable to post-transcriptional regulation of TH protein synthesis. A second experiment of this type carried out at P4, which included wt and each of the three laminin-deficient genotypes gave identical results, i.e., mRNA message was present to the same degree in each.

Cholinergic and All amacrine cells

Beginning at P6 a comparison was made between wt and compound null retinas of cholinergic and AII amacrine cells, using the specific antibodies, anti-ChAT and anti-Dab-1, respectively. Compound null retinas were examined because the most severe developmental deficits were seen in that phenotype, relative to either the $\beta 2$ null or the wt or $\gamma 3$ null phenotypes. The criteria used were: (1) age at which specific immunoreactivity appeared, (2) neuronal geometry and retinal position, (3) cell density. Although TH neurons could be assayed in whole mounts, for cholinergic and AII amacrine cells vertical retinal sections were used. We examined sections on a daily basis between P4–P12, and based on the criteria stated above no differences were found between wt and compound null retinas. Representative sections illustrating ChAT-IR at P8 and Dab-1-IR at P10 in wt and compound null retinas illustrate this point (Fig. 10).

Basement membrane and glial Müller cells

Sections from the same P6–P12 compound null retinas used to examine cholinergic and AII amacrine cells also were assayed for dysmorphic signs in basement membrane and the Müller glial cell. Both anti-VIM and anti-GS were effective for revealing Müller cell morphology, although no GS staining was apparent earlier than P8.

An examination of Müller cell morphology in wt retinas shows that at all developmental stages examined, from P3–P20, at their proximal ends Müller cells have a terminal shaped like a vertically oriented conic section which is in contact with the basement membrane. The main stalk of the Müller cell arises from this terminal and extends vertically throughout the retina, giving off numerous side branches into the IPL. Anti-VIM-IR was confined to the most proximal portion of the Müller cell, whereas anti-GS-IR revealed the full extent of the Müller fiber. Fig. 11 compares at P12, the appearance of Müller cells in wt and compound null retinas. Relative to the wt (Fig. 11a), in the compound null retina, regional disruptions of the basement membrane occur; in these regions the Müller cells show dysmorphic features (Figs. 11b, 11c) including an overall reduction in length of the Müller cell. At higher magnification (Fig. 11c),

it can be seen that the vertical shafts of the Müller cells were thinner in the region of the break and had reduced side branches in the IPL, compared to glial cells on either side of the break.

Our data show that, in the compound null retinas, Müller cell and basal membrane abnormalities were evident throughout the period in which post-mitotic TH neurons began to extend their dendritic and axonal arbors and to acquire TH-IR. On the other hand, at early developmental stages (P3–P10), the Müller fibers in $\beta 2$ null retinas were not dysmorphic. They were of the same length as their counterparts in wt retinas, maintained the same strict vertical orientation and showed the same age-dependent sequence of process arborization within the IPL as noted in wt retinas. By P20, however, Müller glial cells in $\beta 2$ null retinas also were dysmorphic in some respects (cf. Fig. 5) as reported earlier (Libby et al., 1999).

Discussion

Principal findings

Laminin mutations result in a variety of development disorders in humans and mouse, of which the best studied are those in the periphery, including skin and muscular dystrophy (Colognato & Yurchenco, 2000). Moreover, laminin mutations do disrupt CNS development in human and mouse (Miner & Yurchenco, 2004; Olson & Walsh, 2002). Indeed, mutations in the human $\beta 2$ gene result in Pierson's syndrome, and other ocular disorders (Zenker et al., 2004, 2005). In addition, mutations in laminin receptor genes and in the cytoskeletal elements linked to them produce disruptions of brain and eye (Beggs et al., 2003; Lunardi et al., 2006). As three novel laminins have been isolated from CNS, laminin $\alpha 3\beta 2\gamma 3$, $\alpha 4\beta 2\gamma 3$, and $\alpha 5\beta 2\gamma 3$, all of which contain the $\beta 2\gamma 3$ pair, we set out to understand the function of these molecules as well as other $\gamma 3$ and $\beta 2$ containing laminins by using a reverse genetic approach. To this end, we produced a compound laminin null animal lacking all production of both $\beta 2$ and $\gamma 3$ laminin chains. These mice have profound disruptions of CNS development with cortical, cerebellar, and retinal dysplasia. These aspects of the phenotypes of this mouse are discussed in other studies (brain; Radner et al., 2004; retina, Daly et al., 2006; Pinzón-Duarte et al., 2006; systemic, Li & Brunken, unpublished data). The present study is focused on a subtle defect in the laminin mutant mice—the apparent selective disruption of the dopaminergic phenotypes in the retina. The retinal defect may have an analog in the cerebellum in which we find that Purkinje cells in the compound null animal have elevated levels of TH immunoreactivity (Radner et al., 2004). We have not determined if dopamine levels in basal forebrain structures are elevated.

In comparing the development of type I TH neurons in wt vs. $\beta 2$ null or compound null laminin-deficient retinas we noted four main differences. In both $\beta 2$ -laminin-deficient genotypes (1) the first appearance of type I TH neurons was delayed; (2) the density of type I TH neurons was reduced; (3) the immuno-reactivity of TH in type I TH neurons was reduced, but that of type II TH neurons was increased. Additionally, in compound null, but not $\beta 2$ null, the process growth of type I TH neuronal dendrites, was restricted. In the compound null retinas, the Müller glial cells showed multiple dysmorphisms, including shorter overall length, misaligned end feet and reduced process outgrowth in the IPL; more extensive characterization of the Müller cell and basement membrane phenotypes are presented elsewhere (Pinzón-Duarte et al., unpublished data). These altered arrangements were associated with breaks in the basement membrane lining the inner retinal surface. It is important to note that while the mice used in these studies, were on mixed backgrounds (see Materials and methods); all mice were backcrossed to C57BL/6J mice at least five times, thus achieving over 90% homozygosity. To further exclude strain differences, we used littermates as controls (see Materials and methods for details). As all animals were dated from birth (P0), increases or decreases in gestational time were not assayed. However, in other studies involving embryonic ages in which we used a post-coital timing scheme, we did not find changes in the pace of developmental so that the relative ages of mutant and littermate controls were identical. Moreover, the apparent selective

effects on the TH system noted here appear to rule out a simple developmental delay as the mechanism for the laminin-dependent alterations in development.

Possible mechanisms linking laminin deficiency to altered neuronal development

We found that the development of TH neurons in retinas of $\gamma 3$ null mice was indistinguishable from those of wt mice. On the other hand, the compound null mouse, in which both $\beta 2$ and $\gamma 3$ laminin components were deleted, showed more severe deficits in TH development than in the $\beta 2$ deletion alone. The spatial and temporal patterning of laminin component chains is regulated developmentally and laminin assembly and component regulation is complex. During the course of normal development, numerous “substitutions” of laminin chains are observed (Miner & Yurchenco, 2004). Moreover, the apparent promiscuity of laminin chain partnering and the homology of laminin domain structure of some of laminin chains support two substitutions—laminin $\beta 1$ is likely to substitute for laminin $\beta 2$ and laminin $\gamma 1$ for laminin $\gamma 3$. Both $\beta 1$ and $\gamma 1$ are expressed early in development, before either $\beta 2$ or $\gamma 3$, and have broader distribution, indeed they account for most of the known isoforms (16 ca.) of laminin. However, the observation that the phenotype of the $\beta 2^{-/-}:\gamma 3^{-/-}$ mouse has a more profound disruption than either the $\beta 2^{-/-}:\gamma 3^{+/+}$ or the $\beta 2^{+/+}:\gamma 3^{-/-}$ mouse suggests that these substitutions are not perfect. Specifically, our observations suggest that while $\beta 2\gamma 1$ laminins may be very good substitutes for $\beta 2\gamma 3$ (based on the apparent lack of a retinal phenotype), $\beta 1\gamma 3$ laminins are a poorer substitute for the $\beta 2\gamma 3$ (based on the disruptions seen), and finally, $\beta 1\gamma 1$ is a very poor substitute for $\beta 2\gamma 3$. This argument has focused on those molecules containing both $\beta 2$ and $\gamma 3$, but we recognize that laminins contain either one or the other chains are also found, further complicating the genetic analysis. Thus, we cannot ascribe the defects we observe in these mutant animals, to any specific laminin molecule.

Effects of laminin deficiency on dopaminergic neurons

The focus of this study has been the specific disruption of the dopaminergic system. Two other amacrine cell types we examined, AII and cholinergic amacrine cells, were not affected by laminin deficits as judged by the following criteria: (1) post-natal age at which these cell types appeared; (2) level of immunoreactivity; (3) laminin pattern in the IPL; (4) perikaryal size; and (5) density of the cell populations. In addition it appears that Type II TH amacrine cells were not adversely affected in the laminin mutants we examined. Not only was their TH IR increased relative to Wt mice (Fig. 1) but their processes appeared to be robust. That is, in Fig. 6, the tri-laminar appearance of calretinin-IR process in the IPL was strong in all genotypes. The middle lamina of calretinin immuno-reactivity contains the processes of Type II TH neurons (Zhang, 2004).

Three aspects of TH-expression are altered: the number of cell adopting the TH-fate (as measured by density and nearest-neighbor analysis), the time course of TH expression, and TH cell dendritogenesis. Each of these aspects is likely to result from different developmental mechanisms. Indeed, for retinal ganglion cells, cell fate, cell body positioning (spacing) and dendritic arborization are regulated independently of each other (Lin et al., 2004). A similar separation of developmental processes is likely to occur in amacrine cells.

The disruption of the TH system must be indirect because, although $\beta 2$ and $\gamma 3$ chains immunoreactivity is found in the outer plexiform layer and retinal basement membranes (ILM and Bruch's membrane) in mammalian retina (Libby et al., 2000; Aisenbrey et al., 2006), it is absent at the border of inner nuclear and inner plexiform layers where the TH neurons reside. What cells might couple the TH system to laminins? Two major retinal cell classes make contact with these laminin-containing compartments in the neural retina, the retinal ganglion cells and the Müller glial cells. Primary disruptions of either or both cell classes may account for some or all of the phenotypes we have observed within the TH system.

Possible involvement of Müller cells in laminin-dependent developmental changes

One possibility is that the laminin deficiencies we report here lead to altered TH neuron development by a pathway involving the following sequence: (1) loss of β 2- and/or γ 3-containing laminins results in a defective basement membrane characterized by many local tears and disruptions in structure (cf. Fig. 4); (2) disrupted basement membrane prevents Müller glial cells from attaching to it (cf. Fig. 5); (3) failure of Müller cell attachment results in dysmorphic growth and defective Müller cell process layering in the IPL (cf. Fig. 11c); (4) during the period of maximum TH neuronal process growth and synapse formation—P3 to P12—Müller cells promote the dendrite expansion of TH cells.

Several lines of indirect evidence support the Müller cell hypothesis outlined above. Müller cells have the appropriate molecules. They are at least one source of β 2 laminin (Libby et al., 1997) and they express laminin receptor molecules including, integrins (Sherry & Proske, 2001), dystroglycan-associated complex (Moukhles et al., 2000; Claudepierre et al., 2000), and a transmembrane collagen (Claudepierre et al., 2005). Functional data indicating laminin-dependent interactions come from Méhes et al. (2002), who report that laminin-1 increases Müller cell motility and process outgrowth and from Halfter (1998), who found that disruption of the basement membrane led to Müller cell end foot retraction. It is worth noting in this context that retinal detachments produce many of the same disruptions of retinal architecture that we have observed in laminin mutants including sprouting of Müller cells (Fisher et al., 2005) and remodeling of inner retinal neurons. Finally, on a more speculative note, glial cells, perhaps including Müller cells, are important regulators of synapse formation in the CNS; thus the Müller cell may play a role in promoting synapse formation and concomitantly dendrite elongation (Steinmetz et al., 2006). Thus, it may be the defects in TH dendritogenesis are the result of a failure of Müller cell-aided synapse formation and dendrite elongation.

It is unlikely, however, that defects in the Müller cells can account for all of the developmental changes we noted in TH cells—specifically with respect to cell fate adoption. We found a lowered density of type I TH neurons in the laminin-deficient mice compared to wt animals at every age studied from P6 to P20. Studies of cell birthdays in mouse (Young, 1985) and in rat retinas (Rapoport et al., 2004) demonstrate, on average, that retinal amacrine cells are born before Müller glial cells and complementary studies, in mouse, show that programmed cell death of retinal amacrine cell occurs before cell death of Müller cells (Young, 1984). However, the range of times over which these two processes (birth and death) occur in the two classes of retinal cell (amacrine and Müller) overlap considerably. The decrease in TH cell density may indicate more apoptotic death of TH cells in laminin-deficient mice prior to their differentiation. We lack any experimental evidence for the notion that laminin-deficiencies somehow induce increased apoptosis; indeed the original report on at least one of these mutants, the β 2 null, ruled out an increase in cell death (Libby et al., 1999). In non-neural systems, integrins have been described as contributing to cell survival (Meredith et al., 1993; Bates et al., 1995); in neural systems laminins promote neural and glial survival (Chen & Strickland, 1997; Yu et al., 2005). Recent reviews of apoptosis in the retina implicate a wide variety of molecules that influence cell growth and death (Linden et al., 1999, 2005); it is possible that laminins are among these but further experiments are needed to define a role for them in retinal apoptosis.

Potential molecular components of laminin-induced alterations

Another component of altered TH cell development we noted in the laminin-deficient mice is changed levels of TH enzyme production: a decrease in type I TH cells and an increase in type II TH cells. The TH gene is well known to be affected by activity-dependent gene promoter factors such as AP-1 and CREB (Ghee et al., 1998; Lewis-Tuffin et al., 2004). Borba et al. (2005) showed that blockage of cAMP production by pituitary adenylyl cyclase-activating

polypeptide (PACAP) slowed the rate at which TH-IR appeared in cultured chick retina. Their data suggests the possibility that laminin-deficiency somehow results in lowered cAMP levels, resulting in a slowed activation of the TH gene. It has been demonstrated that Müller cell potassium channel scaffolding is laminin-dependent ($\alpha\beta1\gamma1$) (Noël et al., 2005); thus, it is possible that alterations in Müller cell ionic fluxes ultimately affect TH expression.

It is well known that laminins bind to target molecules located on both glial cells and neurons. The well-studied laminin binding molecules are the integrin family (DeCurtis & Reichardt, 1993; Hynes, 2002) and the dystroglycans (Barresi & Campbell, 2006). Integrins are found in multiple subtypes, and specific laminin-integrin binding partners have been identified (Hynes, 2002). We have shown that $\alpha3$ integrins bind to retinal $\beta2$ -containing laminins (Manglapus, M.K., Claudepierre, T, Hunter, D.D., & Brunken, W.J., unpublished observation). Moreover, it has been shown that integrin components are widely distributed in the retina (Sherry & Proske, 2001) and that multiple integrin components are found within the Müller glial cells. DeCurtis and Reichardt (1993) showed that in the chick retina, integrins were required during early development for ganglion cells to attach to laminin and to show process outgrowth; moreover, disruption of $\beta1$ integrin signaling causes a collapse of retinal ganglion cell dendritic arbors (Marrs et al., 2006). Thus, it is plausible that deletion of laminins result in a disruption of integrin signaling in the IPL.

Indeed, in the compound mutant, there is a reduction of ganglion cell density (Pinzón-Duarte et al., 2006; Pinzón-Duarte et al., 2007); this no doubt translates into a reduction of ganglion cell dendritic arborizations in the IPL. Others have shown that ganglion cell dendritic development is a pivotal event in early IPL lamination (Kay et al., 2004); the loss of ganglion cells in these animals' results in delayed amacrine cell dendritogenesis, which progressively recovers late in development in most regions of the retina as bipolar cell axons invade the IPL. However, Wong and colleagues (Kay et al., 2004) show that there are focal regions in the retina in which the stratification of amacrine cell processes in the IPL remains disrupted. Those effects are similar to those we see in the TH population—thus, it is possible that the loss of ganglion cells from these retinas contributes to the focal disruptions of TH-cell stratification we have observed. One possible connection between retinal ganglion cells and TH-cells is BDNF. BDNF is produced by retinal ganglion cells (Vecino et al., 2002); BDNF receptors (TrkB) are expressed on TH-cells (Cellerino & Kohler, 1997); and finally BDNF expression has profound effects on TH-cell development (Cellerino et al., 1998). In the BDNF $-/-$ mouse TH-cell dendritogenesis is inhibited and delayed; in contrast, intravitreal injection of BDNF in postnatal rat results in increased TH-cell density and dendritogenesis (Cellerino et al., 1998). Thus, it is possible that laminin disruption produces an reduction in ganglion cell derived BDNF, which in turn retards TH-cell development. The apparent selective effect of the laminin deletions on TH cells might be explained by an effect mediated via BDNF, rather than Müller cells, as one might expect Müller cells to produce a more generalized effect. Indeed, Müller cell disruption in these mice is profound and appears to lead to profound disruption of late born cell types (photoreceptors and bipolar cells; Pinzón-Duarte et al., 2006; Daly et al., 2006). Thus, it is likely that multiple indirect mechanisms underlay the observed phenotype.

Developmental sequence of TH neuron postnatal development

We found several differences in the development of TH neurons relative to what was reported by Wulle and Schnitzer (1989). These differences are not species-dependent since both investigations utilized C57BL/6J mice. Specifically, Wulle and Schnitzer (1989) reported that in wt mice type I TH neurons first appeared at P6, and that these neurons first appeared only in central retina. We find, in contrast, that type I TH neurons first appear at P3, similar to what we reported earlier for rat retina (Witkovsky et al., 2005) and that the early populations of type I TH neurons are found in all retinal locations, including the extreme periphery. Finally, Wulle

and Schnitzer (1989) indicate a steady increase in the density of type I TH neurons from P5 to P25, whereas we noted a rise in density from P3 to P10, followed by a fall to P15, remaining constant thereafter.

The reasons for these discrepancies are not entirely clear. In our hands, the rabbit anti-TH antibody from Chemicon is more sensitive than its counterpart from Eugene Tech. The fluorescent secondary we used for the developmental sequence reveals more fine TH processes than does the DAB method. Possibly a more sensitive primary antibody reveals TH-IR at P3-P5, when TH levels are far from peak. Still, at age P6 and older, TH perikarya are readily detected with either set of antibodies, so this methodological difference does not explain why we obtained different cell densities and retinal distributions of type I TH neurons.

In summary, we provide evidence that genetic deletion of particular laminin chains leads to dysmorphic changes in TH neurons. This effect is likely mediated indirectly by an alteration of the Müller cell or the ganglion cell population or both. Disruptions of these latter cells (Müller and ganglion cells) are likely to be secondary effects of the disruption of the basement membrane in these animals. Earlier it was shown that the same laminin chain deletions adversely affect photoreceptor development (Libby et al., 1999). Our study thus contributes to a growing body of data showing that components of the extracellular matrix surrounding nerve cells exert a profound influence on the maturation and function of neural circuits.

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Fig. 1.

Type I and type II TH cells in the mouse retina. All panels illustrate vertical sections from P20 mouse retinas immunostained with an anti-TH antibody. Wt and b2ko refer to wild type and $\beta 2$ null genotypes, respectively. Retinal layers for this and subsequent figures: phot, photoreceptor layer; opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, ganglion cell layer. **(a)** A large type I TH neuron at left and a small type II TH neuron (asterisk) at right. Note three bands of TH-IR in the ipl. **(b)** The same two cell types in a $\beta 2$ null retina. Note that the type II neuron (asterisk) shows stronger TH-IR than its counterpart in a wt retina. **(c)** A type II neuron (asterisk) sends a process (arrows) into the intermediate band of TH-IR in the ipl, whereas the processes of the type I TH neuron are concentrated in the upper band of TH-IR in the ipl. Marker bar = 20 μm for all panels.

Fig. 2.

Nearest neighbor distribution for type I TH neurons. The box at right indicates the mouse genotype: wt, wild type; $\beta 2$ ko, $\beta 2$ null; $\gamma 3$ ko, $\gamma 3$ null; double ko, compound null. wt and $\gamma 3$ ko retinas have similar distributions; $\beta 2$ ko and double ko retinas are skewed to the right, reflecting a lower density of type I TH cells (cf. Table 3).

Fig. 3.

Immunoblots of TH and β -tubulin in the mouse retina. The genotypes of the tested retinas (β 2ko, wt) are indicated above. Note that TH-IR is higher in wt compared to β 2 null retinas, whereas the β -tubulin standards are identical. M_r standards in kD are indicated at left.

Fig. 4.

Basement membranes in mouse retinas of different genotypes. Inner limiting membranes at the retina-vitreous border were stained with an anti-perlecan antibody. **(a)** In the wt mouse, the basement membrane is a continuous line. Other stained profiles in this panel are blood vessels. **(b)** In the $\beta 2$ null retina the basement membrane is discontinuous. **(c)** In the compound null (dko) retina, the breakup of the basement membrane is still more apparent. Marker bar in a 5 μm for all panels.

Fig. 5. Müller glial cells in P20 retinas. In panels a, b, anti-glutamine synthetase-IR in green, anti-perlecan-IR in red. In panel c, anti-vimentin-IR in green, anti-perlecan-IR in red. **(a)** wild type retina. Anti-perlecan immunostaining is confined to the basement membrane (arrows) and blood vessels. Terminal expansions of Müller glial cells about the basement membrane; the vertical stalk of one Müller glial cell is positioned to the right of the asterisk. In the ipl Müller cells give off numerous side projections from their vertical stalks. **(b)** $\beta 2$ null retina. The basement membrane is largely absent. Müller cells show hypertrophy and misalignment of their end feet, which are associated with blood vessels (arrows). In the ipl there is disorganization of Müller cell side branches relative to wt control. **(c)** Flat mount view. In a compound null retina, Müller cell end feet, here stained with an anti-vimentin antibody, are oriented towards a blood vessel (asterisk). Marker bar in (a) is 10 μm and applies to all panels.

Fig. 6.

Anti-calretinin immunoreactivity in P20 mice. Labels at lower right indicate the genotype of the mouse. Panels a–c illustrate that in either the $\beta 2$ null (b) or the compound null retina (c), calretinin-IR did not differ from that seen in the wt retina (a). Three bands of anti-calretinin-IR are seen in the ipl. Marker bar in (a) is 20 μ m for all panels.

Fig. 7.

Density of type I TH neurons in mouse retina as a function of age. Box at lower right indicates the genotype. Each point is the data from a single retina, except for postnatal day (P) 20, where $n = 4$. Note that in wild type retinas, type I TH neurons appear at P3. Their density increases rapidly to a plateau at P8–10, falling thereafter by about 1/3. $\beta 2$ null and compound null retinas show similar growth profiles, except that their onset is delayed and the maximum densities achieved are lower (cf. Table 1), relative to wild type retinas. Inset shows initial portions of cell density increase for wt, $\beta 2$ and compound null retinas fitted by regression lines (see text for further Discussion).

Fig. 8.

A comparison of TH-IR in type I TH neurons in mouse retinas of different genotypes. All panels illustrate views of flat mounted retinas. The left hand vertical column illustrates wild type retinas, the middle column $\beta 2$ null retinas, and the right hand column compound null retinas. Post-natal ages (P7, P10, P12, P20) for each horizontal row are indicated at left. Note that relative to wild type retinas, type I TH neurons in $\beta 2$ null retinas have about the same dendritic growth, but are at a lower density. In the compound null retinas, dendritic growth is reduced and TH-IR is less intense relative to wild type retinas. Marker bar in (c) is 20 μ m for all panels.

Fig. 9. Patchy distribution of TH-IR in compound null P12 retinas. Panels (a) and (b) illustrate TH-IR in flat mount of central retina for a wild type (a) and a compound null (b) retina. In the wild type retina, TH-IR is distributed homogeneously. In the compound null retina, TH-IR shows a sudden change from robust staining (to the right of the diagonal line) to poor staining (left of the diagonal line). Several such sudden alterations in TH-IR are seen in each compound null retina. Marker bar in (a) is 50 μm for both panels.

Fig. 10.

Comparison of ChAT and Dab-1 immunoreactivity in wild type and compound null retinas. Each panel illustrates a vertical section through the retina; a,c, wild type retinas, b,d compound null retinas. Anti-choline acetyl transferase (ChAT)-IR is illustrated for P8 retinas; anti-disabled 1 (Dab-1)-IR is shown for P10 retinas. There is no difference in immuno-reactivity between wild type and compound null retinas for these markers. Marker bar in (a) is 20 μ m for all panels.

Fig. 11.

Müller glial cells in developing mouse retinas of different genotypes. All panels illustrate vertical sections through the retina stained with an anti-glutamine synthetase antibody; (a) wild type P12 retina, (b, c) compound null P12 retina. In (a) the Müller glial cells show a strict vertical orientation with a layering of fine offshoot processes in the inner plexiform layer (ipl). In (b) the compound null retina shows a disruption of Müller glial cell orientation at a local break in the basement membrane (arrows). The portion marked by a box is shown at higher magnification in (c). In the region of the break, the Müller cell vertical stalks are thinner (asterisks) and the offshoot processes are reduced compared to the immediately adjacent region (arrows). Marker bar in (b) is 20 μm for a, b and 4 μm for c.

Table 1

Primary antibodies

Name	Host	Dilution	Supplier
anti-Tyrosine hydroxylase	rabbit	1:1000	Chemicon
anti-Choline-acetyl-transferase	rabbit	1:500	Chemicon
anti-Disabled-1	rabbit	1:1000	Chemicon
anti-Calretinin	mouse	1:1000	Chemicon
anti-Vimentin	rabbit	1:2000	Chemicon
anti-Glutamine synthetase	mouse	1:2000	Sigma
anti-Perlecan	rat	1:1000	Chemicon

Table 2

Secondary antibodies

Name	Host	Dilution	Supplier
anti-rabbit IgG-Alexa 488	donkey	1:250	Molecular Probes
anti-rabbit IgG-Alexa 594	donkey	1:250	Molecular Probes
anti-mouse IgG-Alexa 488	donkey	1:250	Molecular Probes
anti-mouse IgG-Alexa 594	donkey	1:250	Molecular Probes
anti-rat IgG-Alexa 594	donkey	1:250	Molecular Probes
anti-rabbit IgG-biotin conjugated	donkey	1:250	Molecular Probes

Table 3Density (cells/mm²) of type I TH neurons in P20 mouse retina

Wild type	$\gamma 3^{-/-}$	$\beta 2^{-/-}$	$\beta 2^{-/-}; \gamma 3^{-/-}$
42.7 $\pm$ 2.3	45.3 $\pm$ 3.8	29.3 $\pm$ 1.7	26.0 $\pm$ 2.4
	p = 0.29	p << 0.01	p << 0.01