Neurological changes as a consequence of basement membrane defects – studies in the nidogen knockout mouse

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To

my Parents

ABSTRACT

Introduction: The basement membrane is a highly specialised extracellular matrix found underlying all endothelia and epithelia and surrounding many mesenchymal cells, in particular myocytes, peripheral nerves and adipocytes. Basement membranes have numerous physical and signalling functions that alter with the specific tissue type and stage of development. Nidogens are in mammals a family of two proteins nidogen -1 and 2. *In vitro* studies have indicated that the 150 kDa glycoprotein nidogen-1 may play a central role in the supramolecular organization of basement membranes through binding to a range of extracellular proteins. To elucidate their importance in the basement membrane, mice were generated with mutated alleles of the NID-1 gene. Ultrastructural analysis of kidney and skeletal muscle from mice lacking nidogen-1 failed to reveal differences in morphology and basement membrane structure. However, these mice show signs of neurological impairment with ataxia, especially of the hind limbs, and spontaneous seizure activity. Nidogen-2 staining in these animals is increased in certain basement membranes, particularly in cardiac and skeletal muscle, where it is normally found in scant amounts, suggesting that the loss of nidogen-1 may be compensated by nidogen-2. Mice have been generated lacking nidogen-1 and one or both alleles of nidogen-2. Mice lacking both nidogen-1 alleles and heterozygous of nidogen-2 show more severe neurological defects, than those lacking only nidogen -1.

Aim of the project: To study the behavioural, electro-physiological, neurological and cellular aspects of the nidogen knockout mice (NID1 - -/NID2 + +) and (NID1 - -/NID2 + -), to gain a further insight into the function of this protein family.

Results: The neurological defects were studied using rotarod tests, *in vivo* EEG recordings and in vitro hippocampal and neocortical field potential recordings analysing input/output relationships and short- and long term plasticity (paired-pulse behaviour and LTP). *In vivo*, the animals displayed massive functional deficits in the rotarod test and epileptiform discharges in EEG recordings. *In vitro*, in the hippocampus, 28% of the slices showed spontaneous, and another 33% evoked spontaneous epileptiform activity. Significant increases of the input/output ratio of synaptically evoked responses in CA1 and dentate gyrus, as well as of paired pulse accentuation, and loss of perforant path LTP was observed. By contrast, in the neocortex, the input/output ratio and paired-pulse accentuation were reduced. To augment the *in vivo* studies, mouse and human forms of nidogen-1 and-2 were cloned, recombinantly expressed and purified. Laminin was extracted from both control and nidogen knockout mice and the biochemical aspects of basement membrane deposition which seemed to be altered in the absence of nidogen was studied. A possible down-regulation of laminin and its receptors was investigated.

Discussion: The results reveal the epileptic nature of the mice in the absence of nidogen-1. Alterations in synaptic plasticity and network function in the nidogen-1 null animals are indicative of a novel role for a nidogen-1, a protein found only in basement membranes. Also, it suggests that nidogen-1 is important for maintaining the structural integrity of basement membranes.

ZUSAMMENFASSUNG

Hintergrund: Die Basalmembran stellt eine hochspezialisierte extrazelluläre Matrix dar, die unter allen Endo- und Epithelien zu finden ist, sowie viele mesenchymale Zellen, vor allem Myocyten, periphere Nerven und Adipocyten, umgibt. Basalmembranen erfüllen mannigfaltige Gerüst- und Signalfunktionen, die vom Gewebetyp und dem ontogenetischen Reifungsstadium desselben abhängen. In Säugetieren treten Nidogene als eine Familie zweier Proteine auf - Nidogen-1 und -2. *In vitro* Untersuchungen deuten darauf hin, daß das 150 kDa große Glycoprotein Nidogen-1 eine wesentliche Rolle bei der supramolekularen Organisation von Basalmembranen durch Bindungen an eine Reihe unterschiedlicher extrazellulärer Proteine spielt. Um die Bedeutung von Nidogenen in der Basalmembran näher zu beleuchten, wurden Mäuse mit Allelmutationen des Nidogen-1 Genes erzeugt. Ultrastrukturelle Untersuchungen der Nieren und des Skelettmuskels von Mäusen, denen Nidogen-1 fehlt, haben keine Unterschiede in der Morphologie der Basalmembran belegen können. Allerdings zeigen diese Tiere deutliche neurologische Defizite, die vor allem in tonischen Streckungen und Myoklonien der Hinterläufe und spontaner Krampfaktivität bestehen. Der immunhistochemische Nachweis für Nidogen-2 zeigt in einigen Basalmembranen ein stärkeres Signal, vor allem im Herz- und Skeletmuskel, in denen Nidogen-2 unter normalen Bedingungen in nur sehr geringen Mengen anzutreffen ist. Dies deutet darauf hin, daß der Verlust von Nidogen-1 ggf. durch Nidogen-2 kompensiert wird. Neben Nidogen-1 Knockout Tieren wurden auch Mäuse hergestellt, denen Nidogen-1 und eines bzw. beide Allele des Nidogen-2 Genes fehlten. Dabei konnte gezeigt werden, daß der Phänotyp in Mäusen, denen Nidogen-1 fehlte und die darüber hinaus heterozygot für Nidogen-2 waren, deutlich ausgeprägter war als in jenen Tieren, denen nur Nidogen-1 fehlte.

Ziel des Projektes: Um einen tieferen Einblick in die Funktion der Proteinfamilie zu erlangen, sollen die Nidogen Knockout Mäuse (NID1 - -/NID2 + + und NID1 - -/NID2 + -) elektrophysiologisch, neurologisch und auf zellulärer Ebene charakterisiert werden.

Ergebnisse: Funktionell ist sowohl bei - -/+ - als auch bei - -/+ + Tieren eine signifikant verminderte Leistung im Rotarod-Test nachzuweisen. Im epikorticalen 24h-EEG der betroffenen Mäuse zeigten sich praktisch ununterbrochen epileptische Potentiale, während dies in keinem der Kontrolltiere auftrat. *In vitro* waren in 2/7 Präparaten von - -/+ - Mäusen spontane epileptiforme Entladungen im Hippocampus zu beobachten; bei afferenter Stimulation der Moosfasern traten zudem in 2/6 Schnitten epileptiforme Potentiale auf. Dabei war das Verhältnis zwischen Reizintensität und Signalamplitude in - -/+ -, und weniger in - -/+ + Präpraten, signifikant zu größeren Potentialen verschoben (Schaffer-Kollateral- und Tractus perforans Stimulation). In beiden Eingängen war auch die Doppelpuls-Potenzierung in - -/+ - Schnitten vergrößert, ebenso wie die Langzeitpotenzierung bei Tractus-perforans-Aktivierung. Um die *in vivo* Untersuchungen noch zu erweitern, wurden sowohl Maus als auch humanes Nidogen-1 und –2 kloniert, rekombinant exprimiert und aufgereinigt. Ausserdem wurde aus Nidogen Knockout Mäusen und Kontrolltieren Laminin extrahiert und die Zusammensetzung der Basalmembran biochemisch charakterisiert, die bei Abwesenheit von Nidogen-1 verändert scheint.

Diskussion: Bei Abwesenheit von Nidogen-1 zeigen die Mäuse einen epileptischen Phänotyp. Veränderungen in der synaptischen Plastizität und der Vernetzung lassen auf eine neue Rolle des Nidogen-1, einem Protein, das nur in Basalmembranen gefunden wird, schliessen. Weiterhin deuten unsere Ergebnisse auf eine Beteiligung von Nidogen-1 an der Aufrechterhaltung der strukturellen Integrität von Basalmembaranen hin.

Lebenslauf

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1. Introduction

1.1. Basement membranes

The extracellular matrix (ECM) is a network secreted and shaped around the cells of multicellular animals (metazoa). The basement membrane is a specialized element of the extracellular matrix consisting of a thin (40-120 nm) sheet of proteins and proteoglycans. The sequencing of the *C. elegans* genome in comparison to higher organisms shows that the basement membrane components are highly conserved throughout metazoan evolution, much more so than the rest of the ECM (Liddington et al., 2000). In hydra, the simplest and oldest of metazoa, the body wall is organised as an epithelium bilayer (endoderm and ectoderm) with an intervening basement membrane containing laminin and interstitial matrix components like type 1 collagen (Shimizu et al., 2002). In more complex organisms, like mammals, basement membrane components appear prior to implantation and mediate the very first tissue interactions during initial stages of development. All basement membranes in higher organisms have four major constituents: type IV collagen, laminin, perlecan and nidogen which form an intricate web of interactions. Apart from providing a structural base for cells to adhere to, individual basement membrane components serve as regulators of many biological activities such as cell growth, repair, differentiation, migration, proliferation and morphogenesis. The effects of various basement membrane components on cell functions are mediated via cell surface receptors like integrins and dystroglycan (Erickson and Couchman, 2000). The transmembrane syndecans and the glycosylphosphoinositide-linked glypicans, both members of cell surface heparan sulfate proteoglycan families modulate many signalling activities (Bernfield et al., 1999). Basement membrane matrices can also be found in association with SPARC (osteonectin), fibronectin, proteins present in specific basement membranes, minor (poorly characterized) components and growth factors that can adhere to heparan sulfate (Yurchenco, 1990). Many signalling molecules such as FGFs, VEGF, TGF-β1 and β2, as well as several chemokines and cytokines bind to heparan sulfate present on cell-surface proteoglycans.

1.2. Basement membrane components

Diversity in the composition of the basement membrane in different tissues at different stages of development arises not only through expression of different matrix molecules, but

also from the existence of multiple forms of individual molecules. In addition, synergistic interactions between growth factors, cell surface receptors and matrix molecules regulate cell behaviour during development (Adams and Watt, 1993).

1.2.1. Collagen IV

Collagen IV is a non fibrillar collagen and is expressed exclusively in all basement membranes. This collagen consists of a central domain containing a triple helix with the typical Gly-X-Y amino acid repeats, an N-terminal domain (7S) and a C-terminal noncollagenous globular domain (NC1), (figure 1.1). The multiple interuptions in the regular Gly-X-Y amino acid sequence of the collagenous region contributes to the flexibility of the molecule. Six α(IV) chains are known (α1(IV), α2(IV), α3(IV), α4(IV), α5(IV), α6(IV)) to form collagen IV (Mariyama et al., 1992; Zhou et al., 1994; Hudson et al., 1993).

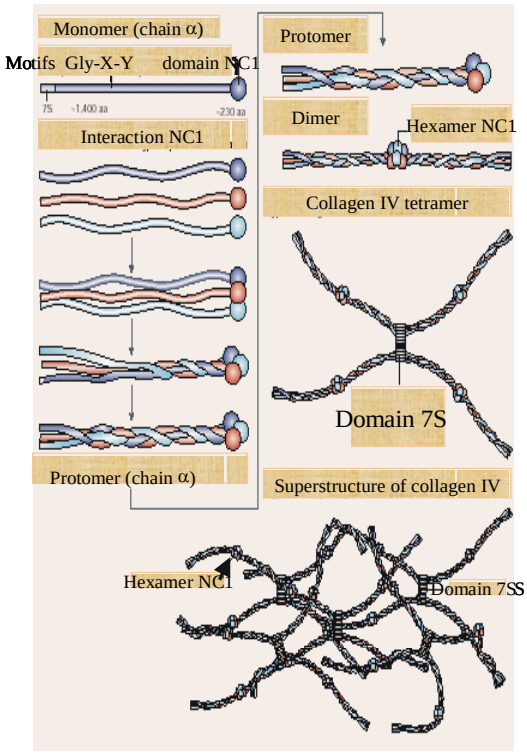


Figure 1.1: Collagen IV assembly and network formation. The triple helical formation is initiated by the NC1 domain. The NC1 domain associates with itself to form a dimer. 7S domains associate to form tetramers (modified from Kalluri, 2003).

The most abundantly found collagen IV is composed of the α1(IV) and α2(IV) chains. Some isoforms are tissue specific, for instance, α3(IV), α4(IV) and α5(IV) chains that associate to form the collagen IV isoform found in glomerular basement membranes. Mutations in any of the chains in this collagen IV isoform have been shown to result in the renal degenerative Alport's syndrome (Kashtan and Michael, 1993). The collagen IV network provides structural support and is important for basement membrane assembly through interactions with nidogen and perlecan (Mayer et al., 1995b; Timpl, 1996). Collagen IV is involved in several biological functions like migration, growth and cell differentiation (Yurchenco and Schittny, 1990).

α2β1 integrin is a receptor for collagen IV (Kapyla et al., 2000). Also, discoidin domain receptors 1 and 2 (DDR1 and DDR2) are a subfamily of the receptor tyrosine kinase class that are activated upon stimulation with various types of collagen. Collagen IV can activate DDR1 which can then modulate cell proliferation and metalloprotease expression (Vogel, 2002).

1.2.2. Perlecan

Perlecan is a member of the family of heparan sulfate proteoglycans (HSPG). First discovered in glomeruli (Kanwar and Farquhar, 1979), then isolated from the Engelbreth-Holm-Swarm mouse tumor (Hassell et al., 1980; 1985; Paulsson et al., 1987), it was described as 'pearl like' from rotary shadowing microscopy (figure 1.2). Perlecan is composed of a core protein of 80 nm which contains five globular domains (Paulsson et al., 1987a). The core protein has a mass of 390kD molecule which can be found as a 700kD molecule when it is glycosylated (Hassell et al., 1980). Perlecan can form homodimers and sometimes higher aggregates (Yurchenco et al., 1987) and it can also interact with several other extracellular matrix components such as laminin-1, collagen IV (Battaglia et al., 1992; 1993, Sasaki et al., 1998), nidogen-1 and 2 (Battaglia et al., 1992; Kohfeldt et al., 1998), fibulin-2 (Sasaki et al., 1995), fibronectin (Heremans et al., 1990) and the β-amyloid protein (Snow et al., 1995; Narindrasorasak et al., 1991). Perlecan is expressed in mice at the two cell stage prior to basement membrane formation, however, some perlecan knockout mice which survived until birth showed degradation of collagen fibrils in cartilage (which lacks a basement membrane), dwarfism, neuronal ectopy and exencephaly. Though basement membranes form, they

become disrupted with increasing mechanical stress indicating a role for perlecan in regulating basement membrane integrity (Costell et al., 1999; Groffen et al., 1999).

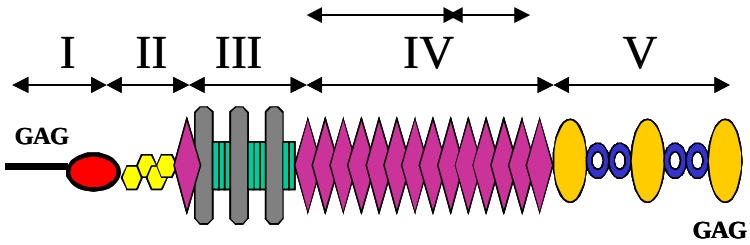


Figure 1.2: The first domain, domain I, contains heparan sulfate chains. Domain II is similar to low density lipoprotein (LDL). Domain III contains three regions similar to the L4 globular domain of laminin, each one separated by EGF domains which are also similar to the EGF laminin structure. Domain IV contains immunoglobulin like structures and domain V contains three domains similar to the neurexins, each one separated by two EGF structures. Domain III interacts with cell surfaces and domain IV binds to nidogen.

1.2.3. Laminins

Laminins are large (600-800kD), noncollagenous glycoproteins, composed of three genetically different chains α, β and γ. Laminins are predominant in basement membranes and are crucial for its formation (Tunggal et al., 2000). Laminin-1 was first isolated from embryonic carcinoma cells (Chung et al., 1979) and Engelbreth-Holm-Swarm (EHS) tumour cells (Timpl et al., 1979). The laminin family contains 15 isoforms (Burgeson et al., 1994; Timpl, 1996; Koch et al., 1999; Miner et al., 1997; Colognato and Yurchenco, 2000; Libby et al., 2000; Li et al., 2003). Until now five α chains (α1-α5), three β chains (β1-β3) and three γ chains (γ1-γ3) have been identified (figure 1.3).

The typical laminin molecule is composed of four arms; the three short arms of the prototype, laminin-1, molecule are contributed by the N-terminal regions of the α1, β1 and γ1 chains (figure 1.4) while the long arm is formed by an association of their C-terminals into a coiled-coil which is stabilized by inter-chain disulphide bridges. The α-chain has a C-terminal extension of five globular domains: LG1-LG5. There are two globular domains designated IV and VI, in the β and γ chains each, while the short arm of the α chain is composed of three globular domains, IVa, IVb and VI. The globules are separated by epidermal growth-factor-

like (EGF like) domains (Sasaki and Yamada, 1987; Engel et al., 1981; Sasaki et al., 1987,1988).

The overall domain structure of laminin-1 is maintained by some isoforms, while others like laminins 5, 7, 8 and 9 have N-terminal truncations occurring in the γ2, α3 and α4 chains (Rousselle et al., 1991; Champliaud et al., 1996).

The laminin subunits β1 and γ1 have the widest tissue distribution. α3, β3 and γ2 subunits are found in the skin and other epithelia with these β and γ isoforms being limited to hemidesmosomes. The α1 subunit is developmentally regulated and expressed highest in the early embryo, neuroretina, brain and developing kidney. Skeletal and cardiac muscle, peripheral nerve, capillaries, placenta and brain contain the α2 chain, while the α4 subunit is restricted to primary mesenchymal cells (adult muscle, lung, nerve, blood vessels and other tissues). The α5 subunit is expressed in diverse epithelia, kidney, developing muscle, nerve and synaptic basement membranes. The β2 subunit is restricted to neuromuscular junctions and the glomerulus and the γ3 subunit is found in non-basement membrane distributions in nerve epithelia and brain (Colognato and Yurchenco, 2000).

Laminin 1	α1 β1 γ1
Laminin 2	α2 β1 γ1
Laminin 3	α1 β2 γ1
Laminin 4	α2 β2 γ1
Laminin 5	α3 β3 γ2
Laminin 6	α3 β1 γ1
Laminin 7	α3 β2 γ1
Laminin 8	α4 β1 γ1
Laminin 9	α4 β2 γ1
Laminin 10	α5 β1 γ1
Laminin 11	α5 β2 γ1
Laminin 12	α2 β1 γ3
Laminin 13	α3 β2 γ3
Laminin 14	α4 β2 γ3
Laminin 15	α5 β2 γ3

Figure 1.3: Nomenclature of laminin isoforms (Burgeson et al., 1994; Tunggal et al., 2000).

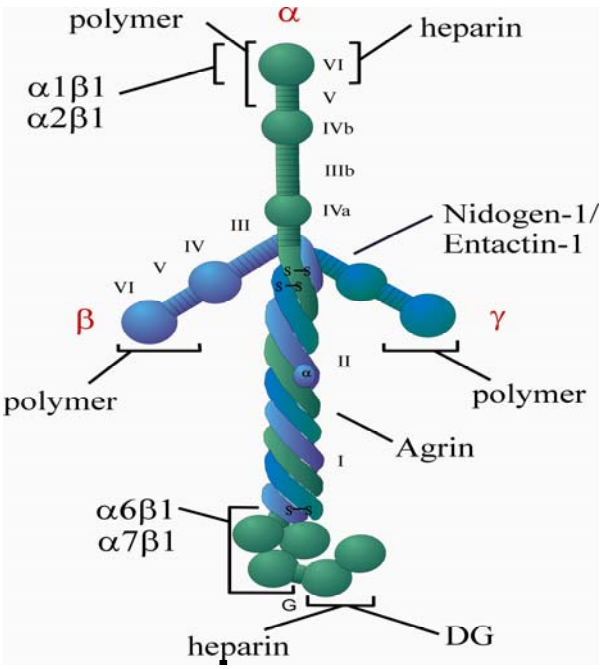


Figure 1.4: Schematic structure of the laminin molecule comprising an α,β and γ chain. The molecule has a cruciform shape (Engel et al., 1981) with three short amino-terminal arms which are involved in polymerisation. The long carboxy-terminal arm consists of domain I and II of all three chains and forms a coiled-coil α helix (Paulsson et al., 1985; Beck et al., 1993) which is terminated by a globular carboxy-terminal domain (G) contributed by the α chain only. The three-stranded coiled-coil domain is required for high-affinity binding to agrin (Kammerer et al., 1999). α1β1, α2β1, α6β1 and α7β1 indicate integrin binding sites. Other binding partners indicated are dystroglycan (DG), the polysachharide heparin and nidogen-1/entactin-1 (modified from Yurchenco et al., 1995).

The mechanism of laminin chain secretion *in vivo* is not well understood. However, cell culture studies demonstrate that the formation of the ternary disulphide-bonded αβγ complex is a pre-requisite for translocation from the endoplasmic reticulum to the golgi complex. Eventually after maturation of N-linked oligosaccharides, complete αβγ trimers are secreted (Hunter et al., 1992). The β and γ subunits cannot be secreted as single subunits, but the α chain can (Yurchenco et al., 1997). The laminin molecule after secretion can self-assemble as well to form a meshwork (figure 1.5), (Yurchenco et al., 1985). In the presence of Ca^{2+}, the N-terminal globular LN domains interact leading to the formation of large polymers (Yurchenco et al., 1992; Paulsson et al., 1988). As laminins-5 and 6 have N-terminal truncations and have only one or two LN domains respectively, they fail to polymerise (Cheng et al., 1997). The self-assembly of laminin-1 can be inhibited by chelating agents (Yurchenco et al., 1985) and proteolytic fragments that contain the LN domains also inhibit polymerisation (Yurchenco and Cheng, 1993). The long arm of laminin is available for cell

contacts and can bind to cell surface receptors like α-dystroglycan or integrins (Colognato et al., 1999; Cohen et al., 1997) which are then rearranged leading to a reorganisation of the cortical cytoskeleton (Colognato et al., 1999). Hence, apart from its role in basement membrane assembly, laminin polymerisation has also a regulatory effect on cells.

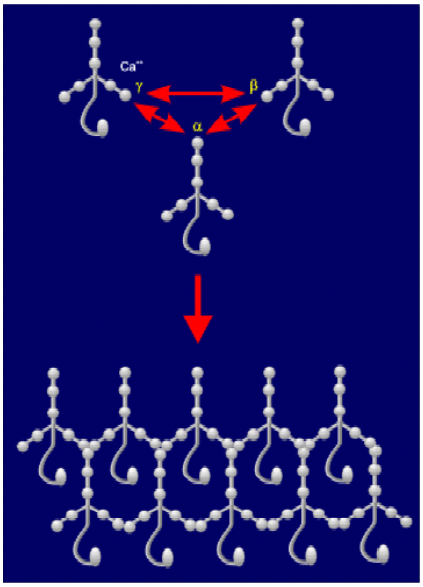


Figure 1.5: The three-arm interaction model of laminin polymerisation (Yurchenco and Cheng, 1994). The amino-terminal ends of the α, β and γ chains (red arrows) interact with each other in a Ca^{2+} dependent manner to produce an array. The long arms are free for interaction with cells.

The multiplicity of laminin isoforms provides for heterogeneity among basement membranes. The laminin family plays essential roles in differentiation, cell polarity, morphogenesis, structural integrity, migration, cell adhesion and signalling (Ekblom et al., 1998; Carter et al., 1991; Niessen et al., 1994; Rousselle and Aumailley, 1994; Lampe et al., 1998; Gonzales et al., 1999; Kim et al., 2000). The importance of the laminin family has been elucidated by the generation of knockout mice. The α2 -/- (Miyagoe et al., 1997; Kuang et al., 1998), α5 -/- (Miner et al., 1998) and γ1 -/- (Smyth et al., 1999) laminin-null mice result in fetal lethality. The α3 -/- (Ryan et al., 1999) mice had epidermolysis bullosa and the β2 -/- (Noakes et al., 1995) mice had defects in neuro-muscular and renal glomerulus; both knockouts resulted in neonatal lethality. The α4 -/- (Thyboll, 2002) mice were viable but had transient microvasculature bleeding.

1.2.3.1. Laminin Receptors

Dystroglycan

Dystroglycan is composed of two subunits α and β encoded by the gene DAG1 (Ibraghimov-Beskrovnaya et al., 1992). These result from a post-translational enzymatic cleavage. Dystroglycan is widely expressed in several tissues including heart, brain, muscle and epithelial cells. It has been described in skeletal muscle to be a component of the dystrophin associated protein complex. Dystroglycan links the extracellular matrix to the actin cytoskeleton. Indeed the α subunit is an extracellular subunit which interacts with extracellular matrix proteins through its N-terminal domain. The C-terminal domain of the α subunit interacts with the N-terminal domain of the β subunit, the C-terminal region of which interacts with dystrophin which is in turn linked to the actin cytoskeleton (Rentschler et al., 1999; Jung et al., 1995; Susuki et al., 1992; 1994; Moores et al., 2000). The α subunit interacts with neurexin (Sugita et al., 2001), agrin (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994), perlecan (Talts et al., 1999) and the laminin globular domains (Ervasti et al., 1993; Tisi et al., 2000; Hohenester et al.,1999). In humans, mutations in dystrophin cause Becker and Duchenne muscular dystrophies, while mutations in α2 laminin cause congenital muscular dystrophy. Even defects in glycosylation of these proteins induce severe diseases such as the Fukuyama congenital muscular dystrophy or the muscle-eye-brain dystrophy. Dystroglycan knockout mice die early during development (E5.5) with defects in the formation of the Reichert basement membrane (Williamson et al., 1997) and the question as to whether dystroglycan is important for basement membrane formation or basement membrane stability is still disputed. Some studies have demonstrated that dystroglycan is required for the formation of the subendodermal basement membrane in embryoid bodies (Henry and Campbell, 1998), while others report that such mediations may be indirect and that dystroglycan regulates the synthesis of basement membrane components rather than the assembly process itself (Li et al., 2002). Mice chimeric for dystroglycan expression in all tissues have been reported to develop severe muscular dystrophy (Cote et al., 1999). Mice with skeletal muscle-specific disruption of dystroglycan develop myonecrosis around 4-6 weeks of age, indicating that loss of dystroglycan alone is sufficient to cause muscular dystrophy (Cohn et al., 2002).

β1 Integrins

Integrins are the major receptors by which cells attach to the extracellular matrix. They are transmembrane type I glycoproteins and link the cytoskeleton to the ECM (Tamkun et al., 1986; Hynes et al., 1987). All integrins are αβ heterodimers. There are 8 known β subunits and 18 known α subunits that can associate to form 24 integrins. Both subunits of integrins are transmembrane glycoproteins, each with a single hydrophobic transmembrane segment. In most integrins, the cytoplasmic domains are short (50 amino acids or less). β4 is a notable exception: its cytoplasmic domain comprises over 1000 amino acids. The extracellular domains (>75kD for β subunits and >100kD for α subunits) associate to form αβ heterodimers (Hynes, 1992).

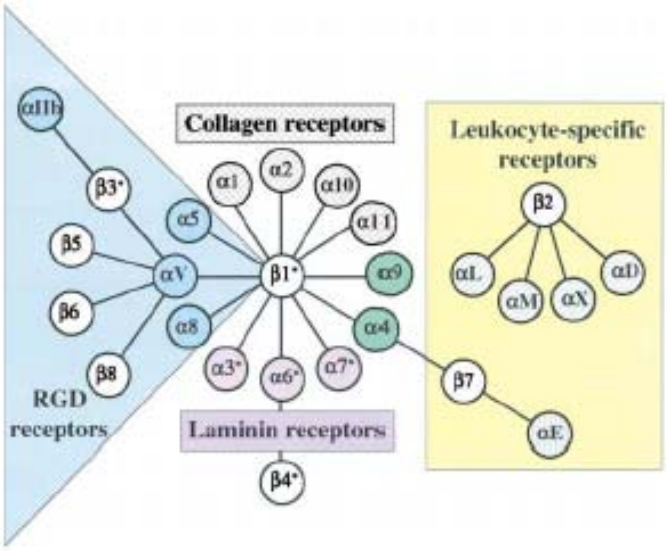


Figure 1.6: The integrin family. Integrins consist of two subunits α and β. 24 heterodimers are known till now. Each heterodimer interacts specifically with its ligand (modified from Hynes, 2002).

The β1 subunit associates with at least ten different α subunits and thus forms the largest subfamily of integrins (figure 1.6). The complexity is increased by the existence of several isoforms with alternatively spliced cytoplasmic domains (Altruda et al., 1990; Languino and Ruoslaht, 1992; Hogervorst et al., 1991). Inactivation of the β1 integrin gene in ES cells resulted in drastic effects on the morphology, adhesion and migration (Fassler et al., 1995) and failure in the formation of basement membranes in embryoid bodies (Fassler and Mayer, 1995; Aumailley et al., 2000). Conditional knockout of β1 integrin in the skin demonstrated its role in the processing and maintenance of basement membranes and formation of hemidesmosomes (Brakebusch et al., 2000). The presence of novel phenotypes

in α3 and α6 mice indicate their essential roles in multiple processes during embryogenesis (De Arcangelis et al., 1999).

1.2.4. Nidogen

Nidogen, an ubiquitous component of basement membranes was so named because of its ability to aggregate into nest-like structures (from Latin, 'nidus' means nest; 'genare' means to produce). However, this property later turned out to be unphysiological. Initially, called entactin, it was first isolated from F9 mouse teratocarcinoma cells (Hogan et al., 1980; Carlin et al., 1981) and then from mouse EHS tumour (Timpl et al., 1983) where it accounted for 2-3% of total protein. It was purified as 80kD and 45kD fragments that could be stained by the Schiff reaction and were bound partially by concanvalin A demonstrating that they were glycoproteins. It contains about 10% carbohydrate, with N-linked and O-linked oligosaccharide chains in similar proportions. By improving extraction and purification conditions, full length nidogen-1 was isolated as a 150kD protein (Paulsson et al., 1988). As nidogen is highly susceptible to proteolysis, specific semi-stable fragments had resulted from earlier purifications (Dziadek et al., 1985). A remarkable effect on extraction yields was observed from mouse EHS tumour with a physiological buffer containing EDTA, that resulted in a large quantity of intact laminin-nidogen complex. Analysis of the purified complex demonstrated that the two proteins were in a 1:1 ratio (Paulsson et al., 1988) indicating that the anchoring of these complexes to the ECM required divalent cations (Paulsson et al., 1987b). Rotary shadowing electron microscopy demonstrated the binding of nidogen to the centre of the cross shaped laminin molecule. Characterisation of the proteolytic fragments of the laminin-nidogen complex and their activity in ligand binding assays provided evidence that nidogen-1 bound to laminin with its C-terminal domain (Mann et al., 1988).

The whole amino acid sequence of nidogen-1 was deduced from cDNA clones isolated from expression libraries and demonstrated a large N-terminal globular domain (641 residues), five EGF-like repeats constituting the rod-like domain (248 residues) and a smaller C-terminal globular domain (328 residues), (Mann et al., 1989). Production of recombinant mouse nidogen-1 and its study established several facts about its structure and function. Nidogen-1 was found to have three globular domains G1-G3, with N-terminal domain G1 and G2 connected by a flexible link and G2 and G3, connected by a rod like domain. Binding to

collagen IV was mediated by the G2 domain and to laminin by the G3 domain with a K_D of 0.5 nM (Fox et al., 1991). The high affinity nidogen-binding site was localized to a single motif homologous to the epidermal growth factor like LE-4 present in the short arm domain III of laminin γ1 chain (figure 1.7), (Gerl et al., 1991, Mayer et al., 1993). This motif consisting of 56 amino acid residues which are folded into four loops (a-d) by disulphide bonds. This sequence is unique within the laminin γ1 chain (Engel, 1992) which explains the high specificity for nidogen binding. By use of synthetic peptides and recombinant mutants, the major binding site of loop a was localized to the heptapeptide NIDPNAV (position 798-804). A change of Asp 800 to Asn or Ala 803 to Val caused a strong reduction in binding activity, while only small changes were observed for changes Pro 801 to Gln and Ile 799 to Val (Poeschl et al., 1994).

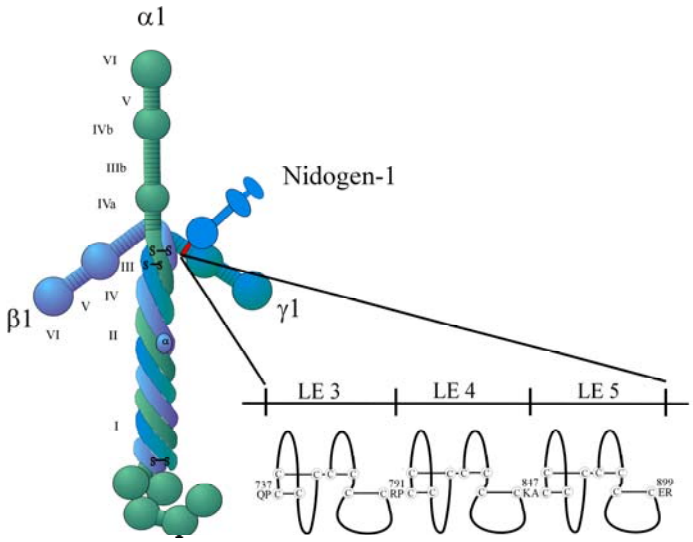


Figure 1.7: The interaction between laminin-1 and nidogen-1. Nidogen-1 binds with its globular domain G3 to the short arm of the γ1 chain. The nidogen-binding site, LE module 4 of the γ1 chain, is shown to be enlarged together with the neighbouring LE modules. LE modules contain cysteine residues which upon disulphide bonding form loops similar to those of EGF (Cooke et al., 1987).

A very low nidogen binding activity was demonstrated when the LE-4 domain present in domain III of the γ1 chain had been replaced by that of the γ2 chain. The central Asn and Val in the crucial heptapeptide binding sequence of γ1III4 is modified in γ2III4 by Ser. Hence, despite 77% sequence identity between motifs γ1III4 and γ2III4, both differed considerably in binding properties, in agreement with the different functions for laminin which vary in the γ chain composition (Mayer et al., 1995).

The crystal structure of the nidogen binding site on the laminin γ1 chain has been well analysed (Stetefeld et al., 1996, Baumgartner et al., 1996, Takagi et al., 2003). The mouse nidogen-1-G2 fragment which contains binding sites for collagen IV and perlecan is composed of an EGF-like domain and an 11-stranded beta-barrel with a central helix. Site-directed mutagenesis demonstrates that the conserved residues are involved in perlecan binding with a very high affinity to the core protein (Hopf et al., 2001).

The mouse nidogen-1 gene (NID-1) spans at least 65kb on chromosome 13 (Durkin et al., 1993) while human nidogen-1 gene is more than 90kb in length and maps to chromosome 1 (Olsen et al., 1989). Sequence comparison of mouse and human nidogen cDNAs reveal an 84% identity at the nucleotide level and 85% similarity at the amino acid level (Nagayoshi et al., 1989). Non-mammalian nidogen has been described in ascidian *Halocynthia roretzi* (Nakae et al., 1993), *Caenorhabditis elegans* (Lee and Chung, 1996), *Drosophila melanogaster* (Kumagai et al., 1999) and *Danio rerio* (Gong et al., 1997). Conservation of structural and functional domains of mouse and human nidogen-1 and comparison of the gene in lower animals indicate that the NID-1 gene has evolved from the shuffle of cysteine rich motifs (Nakae et al., 1993).

Studies comparing the expression of nidogen and various chains of laminin showed that laminin β1 and γ1 chains are expressed in the 2-4 cell stage, whereas nidogen-1 seems to appear with the α chain between the 8-16 cell stage morula (Cooper and Mac Queen, 1983; Dziadek and Timpl, 1985). Nidogen and laminin co-localised at the blastocyst stage and in post implantation basement membranes. During early embryogenesis nidogen-1 mRNA was found not only in cells of the ectoderm derived mesoderm but also in the cytoplasm of the endoderm and ectoderm, indicating that all three germ layers express it (Miosge et al., 2000). This result indicates that although nidogen-1 is predominantly a product of mesenchymal origin (Ekblom et al., 1994), it can also be generated by other cell types. The formation of the laminin-nidogen complex during embryogenesis is believed to occur by two different ways. In some cases, the biosynthesis of laminin and nidogen-1 occurs in the same cell and is followed by intracellular fomation and secretion of the laminin-nidogen complex (Wu et al., 1988). This may also enhance nidogen's stability against endogenous proteolysis (Dziadek, 1995). Typical examples of this form of secretion are the formation of the lens capsule (Dong and Chung, 1991) and the formation of Reichert's membrane by parietal endoderm (Thomas and Dziadek, 1993). The other pathway involves cellular co-operation, where epithelial derived

laminin and mesenchyme derived nidogen bind to form a complex in the extracellular space. This is indicated from antibody pertuberation experiments that block the laminin-nidogen interaction which interfered with morphogenesis (Ekblom et al., 1994; Kadoya et al., 1997).

Nidogen-2, a 200 kD protein was recently isolated from KUSA cells, a murine osteoblast like cell line, by the signal sequence trap method. Nidogen-2 shares 46% overall sequence identity at the amino acid level with nidogen-1, containing five EGF-like repeats and two thyroglobulin like motifs which are both cysteine rich (figure 1.8), (Kimura et al., 1998). Electron microscopy of recombinant nidogen-2 shows a similar G1-G3 domain arrangement to that of nidogen-1.

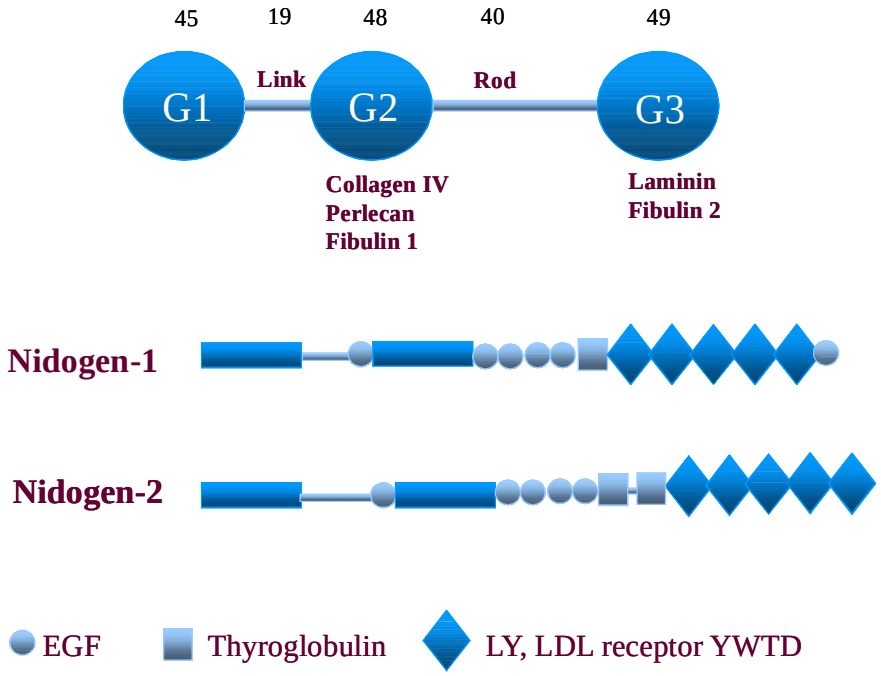


Figure 1.8: The domain organisation and modular structures of nidogen-1 and nidogen-2. Both nidogens comprise three globular domains. G1 and G2 are joined by a link region while G2 and G3 are connected by a rod-like structure. Different basement membrane proteins binding to the G2 and G3 domains of nidogen-1 are indicated (modified from Erickson and Couchman, 2000).

Immunofluorescence studies revealed colocalization of both nidogen-1 and -2 in various tissues but some differences in expression patterns were noticed particularly in heart and skeletal muscle (Kohfeldt et al., 1998). Nidogen-2 is typically enriched in endothelial basement membranes whereas nidogen-1 shows broader localisation in most basement membranes (Schymeinsky et al., 2002). In embryos, nidogen-2 mRNA was produced by

mesenchyme at sites of epithelial-mesenchymal interactions, but the protein was deposited on epithelial basement membranes as previously shown for nidogen-1 (Salmivirta et al., 2002).

The mouse nidogen-2 gene locus is approximately 60kb and is assigned to chromosome 14 (Schymeinsky et al., 2002) and human nidogen-2 spans approximately 50.5Mb on chromosome 14 (Kohfeldt et al., 1998). Except for the link region, a high sequence identity (77-92%) was found between mouse and human nidogen-2. However, mouse nidogen-2 binds to domain III of γ1 arm of laminin with a 10 fold lower affinity than mouse nidogen-1 whereas human nidogen-2 bound with 50-100 fold less affinity (Salmivirta et al., 2002). Nidogen-2 binds collagen IV, perlecan and fibulin-2 in a manner comparable to nidogen-1 (Salmivirta et al., 2002). The molar levels of nidogen-2 are however far lower (2-5%) than that of nidogen-1 (Miosge et al., 2002).

In order to characterize the biological significance of the laminin-nidogen interaction, two approaches were used. In the first genetic approach, the nidogen-binding module of the laminin γ1III4 chain was deleted. This resulted in mice that died immediately after birth with structural abnormalities in specific basement membranes of kidney and lung, resulting in renal agenesis and impaired lung development (Willem et al., 2002). In the second approach, the laminin-nidogen interaction was blocked by the addition of an excess of recombinantly expressed nidogen-binding sites during basement membrane formation in F9 embryoid bodies. This lead to defects in the basement membrane, increased permeability and abnormal differentiation suggesting that the laminin-nidogen interaction may be highly important for epithelial development (Tunggal et al., 2003).

Towards understanding the *in vivo* roles of the nidogens, mouse knockouts were made. Nidogen-1 deficient mice had ultrastructurally intact basement membranes in most tissues (Murshed et al., 2000). However, the animals showed signs of neurological impairment with ataxia, especially of the hind limbs and spontaneous seizure-like activity (Dong et al., 2002). Nidogen-2 deficient mice were normal and showed no ultrastructural alterations in basement membranes (Schymeinsky et al., 2002).

1.3. Basement membrane assembly

The assembly of basement membranes is of astonishing complexity and though several high affinity interactions in the form of networks and connecting elements determine the basic framework, many more, probably weaker, potential interactions exist and these may introduce cell or tissue specific features into the assembly. Several important conclusions have been drawn from studies of the roles laminin and collagen IV play in matrix structure and assembly (Sechler et al., 1998, Timpl et al., 1981). Firstly, the assembly of the basement membrane depends on self-interactions and interactions with other basement membrane proteins (figure 1.9).

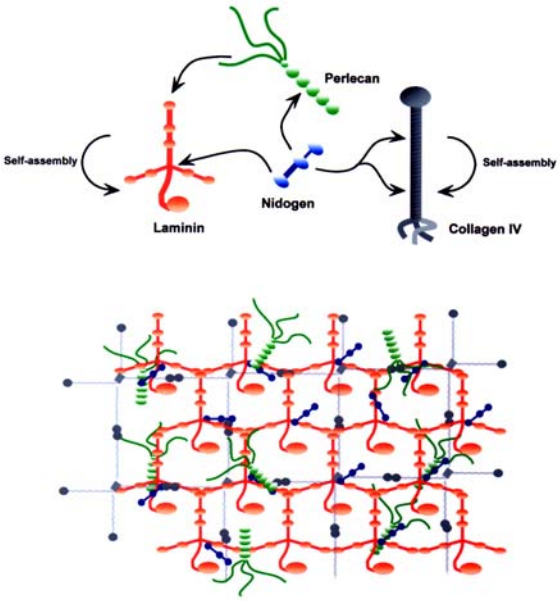


Figure 1.9: Schematic representation of the current model of basement membrane assembly (drawn after Yurchencho et al., 1995). The upper panel shows possible interactions among crucial basement membrane components and the lower panel depicts laminin and type IV collagen networks connected by nidogen-1.

Secondly, interactions with their receptors, for example, cell-surface integrins are required to localize laminin at appropriate sites for assembly (Schwarzbauer, 1999) and to increase the local protein concentrations at the cell-surface. This may also be enhanced by the negative charge at the lipid interface. Finally, integrins must be able to make productive connections with the actin cytoskeleton through their cytoplasmic tails, probably as a means of supporting the tension needed for binding and multimerization.

In vivo results suggest that there may be a receptor hierarchy between β1 integrin and dystroglycan for basement membrane assembly. Laminin assembly into oligomers might be initiated by dystroglycan and then integrins might be required to rearrange the oligomers into the appropriate configuration for formation of a polygonal network. Alternatively, multiple integrin binding sites at both ends of laminin and on type IV collagen might allow these receptors to organise the matrix over extended distances (Schwarzbauer, 1999). However, new reports provide evidence for a mechanism in which laminin polymerisation and interaction with the cells of the inner cell mass through a heparain binding sequence in LG4 initiates basement assembly and differentiation. In the presence of laminin-1, neither β1 integrin nor dystroglycan was needed for basement membrane assembly, instead they co-operated to sustain survival of the epiblast and regulate laminin α1 chain expression. (Li et al., 2002).

1.4. Basement membranes in embryonic development

Initial generation of cell diversity in the mouse embryo occurs at the morula stage when trophoblast and inner cell mass cells form presumably by response to their position. The inner cell mass gives rise to the embryo proper and the trophoblast cells form part of the extra-embryonic tissues. Endoderm differentiation from the inner cell mass is also in response to cell position, and divergence of parietal and visceral endoderm populations depends on further tissue interactions (Murray and Edgar, 2000). Deposited extracellular matrix is first observed by electron microscopy in preimplantation blastocysts on the fourth day of gestation (Leivo, 1983). A thin layer of material is seen on the blastocoelic surface of trophoblast cells, and a discontinuous layer is present between the primary endoderm layer and ectoderm cells of the inner cell mass. A structurally distinct basement membrane is first seen in the postimplantation egg cylinder between the visceral endoderm and ectoderm tissues initially and later also separating ectoderm, mesoderm and endoderm layers (Enders et al., 1978). It is believed that the appearance of laminin at the 2 cell stage and nidogen at the 8 cell stage are important for the development of the first basement membrane of the trophectoderm (Leivo et al., 1989).

Tissue morphogenesis during organogenesis is dependant on epithelial mesenchymal interactions and basement membranes play a crucial role in these events (Bernfield et al.,

1984). Information about the importance of basement membranes have been obtained from a number of molecular and functional studies. Epithelial branching morphogenesis is crucial for the development of several organs, such as lung, kidney, salivary and mammary gland (Werb et al., 1996; Shuger et al., 1991). Pertuberation experiments employing blocking antibodies, proteolytic fragments and synthetic peptides on basement membrane components resulted in drastic effects on organ cultures. Laminin, nidogen, heparan sulphate proteoglycans and tenascin-C were found to be involved in early lung morphogenesis (Schuger et al., 1998; Ekblom et al., 1994; Schuger et al., 1996; Chiquet M., 1992). The importance of the laminin and nidogen interactions were further elucidated in salivary gland branching morphogenesis (Kadoya et al., 1997). The distortions in the basement membrane induced by antibodies blocking the interaction could be counteracted by epidermal growth factor, which increased the production of nidogen-1 in the mesenchyme (Kadoya et al., 1997). Different isoforms of laminin, collagens, proteoglycans and certain cell surface receptors like $\alpha 6\beta 1$ integrin and dystroglycan have been shown to be important all through major stages of kidney development (Ekblom et al., 1996).

Developing nervous system involves the interactions of neurons and other supporting cells. Laminin, fibronectin, tenascin-C and glycosaminoglycans have been identified along axonal pathways, although evidence implicating them in axon-guidance is largely indirect, based for example, on the effects of these molecules in simple tissue culture experiments (Lander, 1989; Baron-Van Evercooren et al., 1982; Wehrle and Chiquet, 1990). When presented as a tissue culture substrate, laminin-1 is a potent stimulator of extension of a variety of classes of axons. As it is expressed in several regions of the developing central and peripheral nervous system in ‘corridors’ that mark the trajectory of several classes of axons, laminin-1 is believed to be a positive corridor cue that contributes to channeling of some axons (Rogers et al., 1986; Edgar, 1991) as well as neural crest cells (Erickson et al., 1980).

Results obtained by targeted deletion of basement membrane genes encoding the proteins found ubiquitously in the basement membrane, confirmed their importance in embryonic development as earlier observed in cell and organ culture experiments. Mice lacking the laminin $\gamma 1$ chain die at 5.5 day post coitum (dpc) through a failure of ectodermal and endodermal differentiation (Smyth et al., 1999; Murray and Edgar., 2000). Some of the mice deficient for perlecan die of cardiac rupture at 10.5 dpc because of basement membrane instability and others at birth with brain anomalies (Costell et al., 1999). Knockout of collagen

IV results in embryonic lethality at E10.5-E11.5, with thin and ruptured Reichert's membrane (Poeschl, unpublished data).

1.5. Neurological disturbances as a result of disruption in basement membrane genes and receptors

Several neurological disorders have been reported as a result of disruption of basement genes and their receptors, involving laminin γ1, perlecan, integrins, dystroglycan and merosin (laminin α2 chain). In mice with a targeted deletion of the nidogen binding site of laminin γ1, the pial basement membrane disintegrates at early stages of gestation. This results in disrupted neuronal migration indicating that an intact basement membrane is essential for proper cortical development (Halfter et al., 2002). In another study, perlecan null embryos that survived the first critical period between E10 and E12.5 developed brain anomalies and this was unexpected as no perlecan expression had been found in the central nervous system. However, defects in basement membranes separating the brain from the adjacent mesenchyme resulted in invasion of the brain tissue into the overlying ectoderm leading to abnormal expansion of neuroepithelium, neuronal ectopias and exencephaly (Costell et al., 1999). Targeted deletions of α3 and α6 integrins and of a brain-specific β1 integrin knockout shows extensive cortical ectopias (Georges-Labouesse et al., 1998; Anton et al., 1999; Graus-Porta et al., 2001). Dystroglycan-null brain loses its high-affinity binding to laminin, and shows discontinuities in the pial surface basal lamina (glia limitans) that probably underlie the neuronal migration errors (Moore et al., 2002). Several human heriditary diseases related to defects in basement membrane proteins such as the laminin α2 chain deficiency in congenital muscular dystrophy (Mercuri et al., 1996; DeStephano et al.,1996; Van der Knaap et al., 1997), Fukujama muscular dystrophy (Nakano et al., 1996) and Walker-Warburg syndrome (Williams et al., 1984) are accompanied by cortical dysplasias. Indeed, the only other report of seizures as a result of disturbances in a basement membrane gene is that of the laminin α2 chain. Patients with deficiency of laminin 2, 4 and 12 isoforms have classical congenital muscular dystrophy characterised by severe muscle weakness, markedly raised creatine kinase and characteristic white matter hypodensity on cerebral magnetic resonance imaging. In addition, 8-20% of these patients suffered from seizures. There was no consistent pattern of seizures, but they were observed in early childhood (Jones et al., 2001).

1.6. Seizures, memory and synaptic plasticity

A seizure (from the Latin *sacire* - to take possession of) is an abnormal, hypersynchronous discharge of a population of cortical neurons. Epilepsy is a disorder of the central nervous system characterised by recurrent seizures unprovoked by an acute systemic or neurologic insult. Next to strokes, epilepsy is the most common neurological disease. About 0.5 to 1% of the population suffer from epilepsy (Dichter and Ayala, 1987).

The basic anatomic and electrophysiologic properties of the cerebral cortex and the factors that determine the level of neural activity at the cellular and network level are important for understanding the concepts of epilepsy.

1.6.1. Anatomy of the cerebral cortex

The cerebral cortex develops from portions of the telencephalic vesicle. Cortical neurons begin to form a multilayered structure, which depending on the location, consists of three or six tiers (figure 1.10). Cells formed at the same time migrate to the same cortical layer; cells migrating later pass through deep layers to form more superficial laminae. A six-layer cellular arrangement is characteristic of the entire *neopallium*, which is referred to as the *neocortex* or *homogenetic* cortex. The structure is shown schematically in figure 1.10A. The *paleopalium* (olfactory cortex) and the *archipallium* (hippocampal formation) have three basic layers and collectively constitute the *allocortex* or *heterogenetic* cortex as shown in figure 1.10B (Carpenter, 1991).

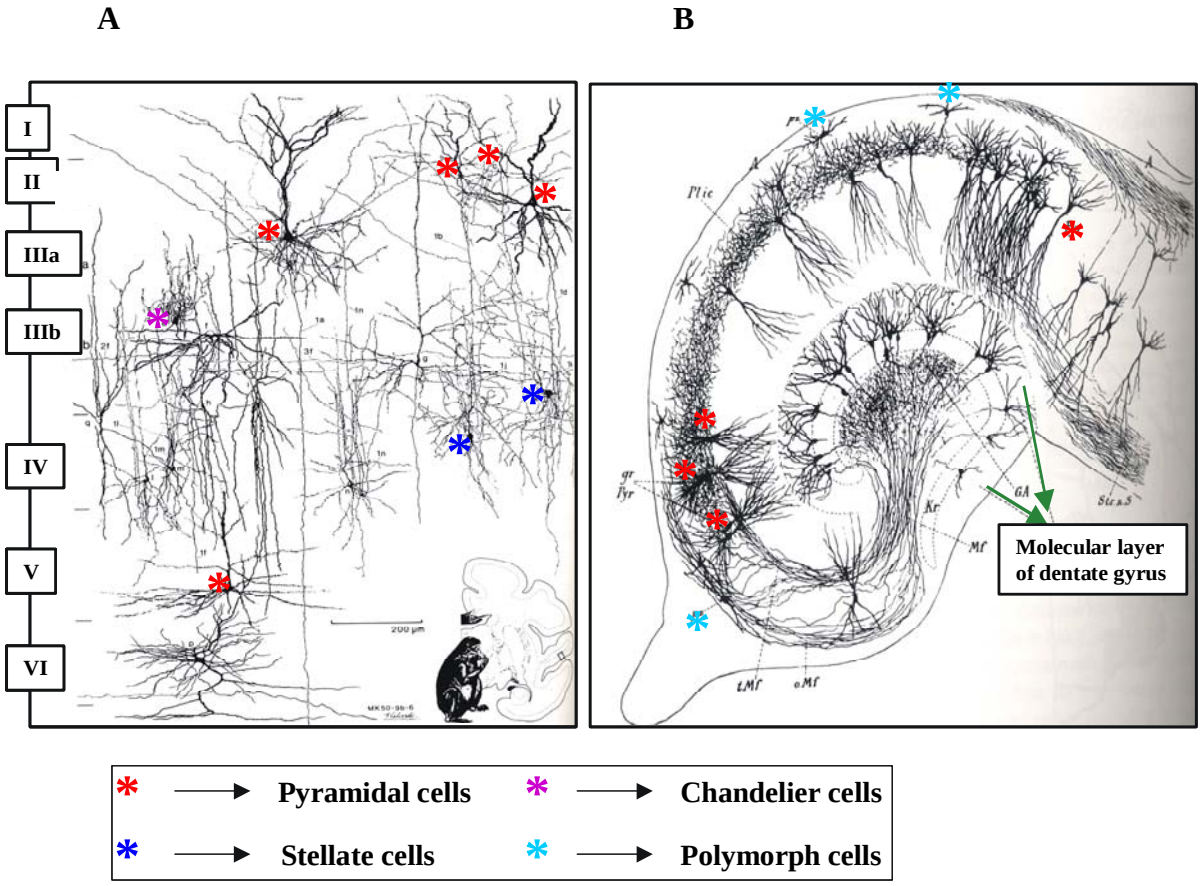


Figure 1.10: Anatomy of the cerebral cortex. (A) Structure of the temporal neocortex of the rhesus monkey as revealed by golgi impregnation. Small pyramidal neurons in layers II and III have restricted dendritic trees and form axonal collaterals with neighbouring cortical domains. Medium-to-large pyramidal cells in deep layer III and layer V have more extensive dendritic trees and furnish long cortico-cortical connections. Chandelier cells are common in the temporal neocortex. Stellate cells can be seen in layer IV and are probably taregts of thalamocortical fibres. Layer VI pyramidal cells are involved in certain cortico-cortical as well as cortico-thalamic projections (Valverde, 1986). (B) Transverse section through the hippocampus and dentate gyrus of a kitten. The cortex of the hippocampal formation has three fundamental layers. They are the polymorphic layer, the pyramidal layer and the molecular layer. Anatomy of the hippocampus is mentioned in detail in section 1.6.2.

The cortex including neo, archi and paleocortex consist of two general classes of neurons. The projection or principal neurons (e.g. pyramidal neurons) are cells that 'project' or send information to neurons located in distant areas of the brain. Interneurons (e.g. basket, chandelier and double bouquet cells) are generally considered to be local-circuit cells which influence the activity of nearby neurons (Hof et al., 1999). The pyramidal neurons are the largest and most numerous neurons of the cerebral cortex. Pyramidal neurons not only furnish the major excitatory output of the neocortex, but also act as a major intrinsic excitatory input through axonal collaterals. The other main excitatory input to pyramidal cells in the cortex is provided by the interneuron class referred to as spiny stellate cells, small multipolar neurons

with local dendritic axonal arborisations. Most interneurons form inhibitory synapses on principal cells or other inhibitory neurons. Recurrent inhibition can occur when a principal neuron synapses on an inhibitory neuron which in turn synapses on the principal cells to achieve a negative feedback loop. Some interneurons have rather extensive axonal projections in which case they can provide a strong synchronisation or pacer activity to large groups of neurons (Cobb et al., 1995; Somogyi et al., 1982).

1.6.2. Anatomy of the hippocampus

The hippocampus resembles in appearance a marine organism after which it was named (Latin: sea-horse). It is one of a group of structures forming the limbic system and is located deep within the temporal lobes just beneath the thalamus and posterior to the amygdala. A cross section through the hippocampal formation is shown in figure 1.11. The hippocampal formation is made up of the entorhinal cortex, subiculum, dentate gyrus and the ammon's horn (cornu ammonis) fields CA4, CA3, CA2 and CA1 in succession. Both CA2 and CA4 are transition zones and little is known about their function. CA1 and CA3 are the principal regions of the hippocampus (Laroche et al., 2000).

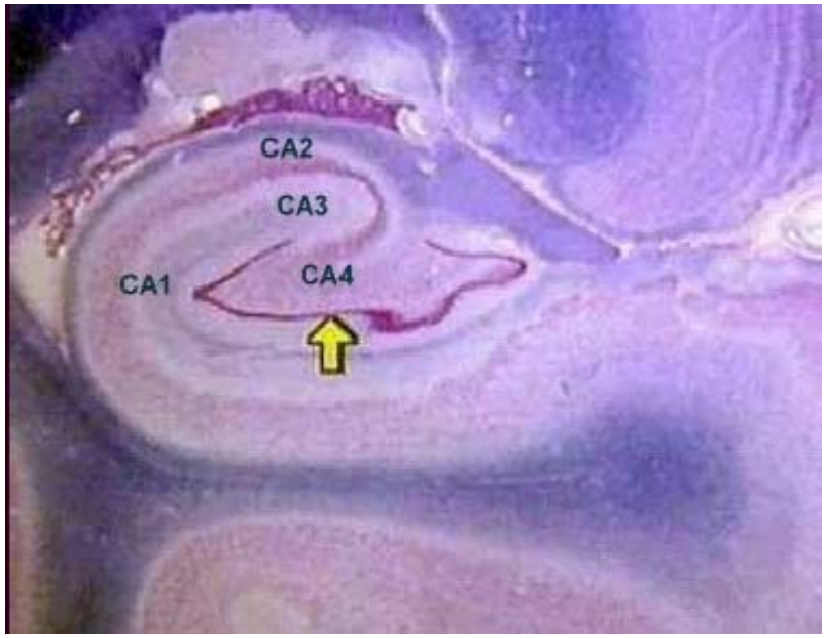


Figure 1.11: Nissl stained preparation of a cross sectional view of a primate hippocampal formation. Arrow indicates the dentate granule cell layer (created by the Electrophysiology laboratories at Trinity College, Hartfort, CT).

1.6.3. Electrophysiology and cytochemistry governing excitability

Investigations of epileptic mechanisms have attempted to determine whether the primary abnormality resided in altered intrinsic neuronal properties or in the aggregate properties of neuronal networks (Dichter and Ayala, 1987). Some of the properties of neurons and molecular factors that determine the level of neural activity are described below.

The nervous system makes use of two kinds of electrical signals. The first kind are graded, passive or localised potentials and the second kind are impulses, or action potentials. Local potentials that occurs at synapses are called postsynaptic potentials or more simply as synaptic potentials. Synaptic potentials can be excitatory or inhibitory. The size of the synaptic potential can be graded and is a reflection of the number and rate of activity of excitatory or inhibitory presynaptic nerve terminals giving rise to it. In contrast to the local potential, the action potential is a brief event that travels unattenuated along the axon. The movement of charge caused by a rapid influx of Na^+ ions generates a large and brief deviation in the membrane potential. The different types of ion channels in neuronal membranes allow complex patterns of action potentials to be generated and complex synaptic computations to occur within single neurons (McCormick, 1999).

Neurotransmitters are released subsequent to action potentials depolarising the pre-synaptic nerve terminal at a synapse. After liberation, neurotransmitters subsequently bind to post-synaptic receptors specific for that ligand. In so called ionotropic receptors, ligand binding results in channel activation and passage of ions into or out of the following postsynaptic cells. The major neurotransmitters in the brain are glutamate, gamma-amino-butyric acid (GABA), acetylcholine (ACh), norepinephrine, dopamine, serotonin and histamine (Deutch and Roth, 1999). Ca^{2+} acts as an intracellular messenger tying the electrical signal of pre-synaptic depolarization to the act of neurosecretion (Zucker et al., 1999). Other molecules, such as neuropeptides and hormones, play modulatory roles that modify neurotransmission over longer time periods.

The excitatory amino acid neurotransmitter, glutamate accounts for most of the fast synaptic transmission that occurs in the CNS (Fonnum, 1984). There are several subtypes of glutamate receptors. Glutamate receptors can be found postsynaptically on excitatory principal cells as well as on inhibitory neurons and have been demonstrated on certain types

of glial cells. The ionotropic subclasses are the N-methyl-D-aspartate (NMDA), alpha-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid (AMPA) and kainate receptors; these allow net cation influx upon activation by glutamate. They are differentiated from one another by cation permeability as well as differential sensitivity to pharmacological agonists/antagonists (Kandel et al., 1991; Karnup and Stelzer, 1999). All ionotropic glutamate receptors are permeable to Na^+ and K^+, and it is primarily the influx of Na^+ through these channels that contribute to membrane depolarization and generation of action potential. The NMDA receptor is also permeable to Ca^{++}. It is blocked by Mg^{++} in the resting state, but under conditions of local membrane depolarization, Mg^{++} is displaced and the channel becomes Ca^{++} permeable (Betz, 1990; Mayer and Westbrook, 1987). Excessive influx of Ca^{++} tends to depolarise the cell and is thought to contribute to Ca^{++} mediated neuronal activation, such as that seen in status epilepticus and ischemia (Nicoll et al., 1990). Under such conditions, glutamate levels are pathologically elevated for prolonged periods, NMDA receptors remain persistently activated and intracellular calcium reaches cytotoxic levels leading to cell death. Inhibitors of NMDA receptors can prevent such neuronal cell death (Choi et al., 1988). The other major type of glutamate receptor is the metabotropic receptor, which functions by means of receptor-activated signal transduction involving membrane-associated G-proteins. There are at least three subtypes of metabotropic receptors, based on differential agonist potency, mechanism of signal transduction and pre versus postsynaptic localization (Nakanishi, 1994; Lie et al., 2000; Baskys and Malenka, 1991; Xiao et al., 2001).

Experimental studies using animal epilepsy models have shown that NMDA, AMPA and kainate agonists induce seizure activity. Metabotropic agonists appear to have variable effects likely dependent upon their different location and mechanisms of signal transduction (Kullmann et al., 2000).

GABA is the main inhibitory neurotransmitter in the CNS and plays an important role in several physiological processes, including network synchronisation and generation of theta (4-7 Hz) and gamma (30-90 Hz) rhythms which are thought to be associated with cognitive functions (Cherubini and Conti, 2001). GABA interacts with two major subtypes of receptors: $GABA_A$ and $GABA_B$ receptors. $GABA_A$ receptors are found postsynaptically, while $GABA_B$ receptors are found post but also presynaptically, and can thereby modulate synaptic release. $GABA_A$ receptors are permeable to Cl^- ions; upon activation Cl^- flux, which in the adult brain is usually directed inwards, hyperpolarises the membrane and reduces action potential

likelihood. Therfore, substances which enhance $GABA_A$ receptor current, such as barbiturates and benzodiazepines, are well known to supress seizure activity (Bormann, 1988). $GABA_B$ receptors are associated with second messenger systems rather than Cl^- channels, in addition to their hyperpolarising action in postsynaptic neurons, they can also lead to attenuation of transmitter release due to their presynaptic location. The second messenger systems often result in opening of K^+ channels, leading to a hyperpolarising current (Kaupmann et al., 1997).

1.6.4. Effect of seizures on neural networks

Seizure activity is sometimes accompanied by cognitive dysfunction. Structural cerebral abnormalities which underlie the epileptic focus may directly cause cognitive dysfunction. On the other hand, seizure activity itself is thought to disturb the learning and memory function of an otherwise healthy brain outside the epileptic focus. In mammals, the hippocampus and prefrontal cortex are believed to be part of a unique memory system where the hippocampus plays the dual role in processing certain types of information and in the supervision of neocortical learning (Rolls, 1989). Important insights into the function of the hippocampus came from studies of patients when both hippocampi were surgically removed to alleviate the symptoms of temporal lobe epilepsy. Such patients suffered an important anterograde or new information amnesia (Laroche et al., 2000). They displayed a particularly noticeable and specific deficit in the capacity for consolidating short-term memory into long-term memory. That is, if told to remember a name or word, they could repeat it immediately, but all re-collection was lost if they were distracted, even briefly. Long term memories aquired prior to the surgery remained intact. Often memory impairment was found to be associated with bilateral lesions that were confined to the hippocampus. All these observations established the fact that long lasting changes in the efficacy of signalling between neurons in the hippocampus was important for memory consolidation (Bliss and Lomo, 1973; Reilly, 2001).

1.6.5. The hippocampal network

Signals of cortical origin are processed in the hippocampal system through a series of steps that include an entorhinal-hippocampal loop and a return projection to the neocortex. The hippocampus through this loop, called the trisynaptic circuit is reciprocally connected to

widespread areas of the remainder of the cerebral cortex. The three relays of the trisynaptic circuit being 1) Entorhinal cortex to the dentate granule cell layer, via the perforant path. 2) Dentate granule cell layer to the pyramidal cells of region CA3 by means of the mossy fiber system. 3) CA3 region to the CA1 region via the Schaffer collaterals which project to the entorhinal cortex, via the subiculum (figure 1.12).

One branch of CA3 leaves the hippocampus via the fornix and travels back to the neocortical areas of origin. The second branch, the Schaffer collaterals, forms synapses on the neurons of the CA1 subfield. CA1 axons either project to the entorhinal cortex (via the subiculum) or enter the fornix and project back to the neocortex (Laroche et al., 2000).

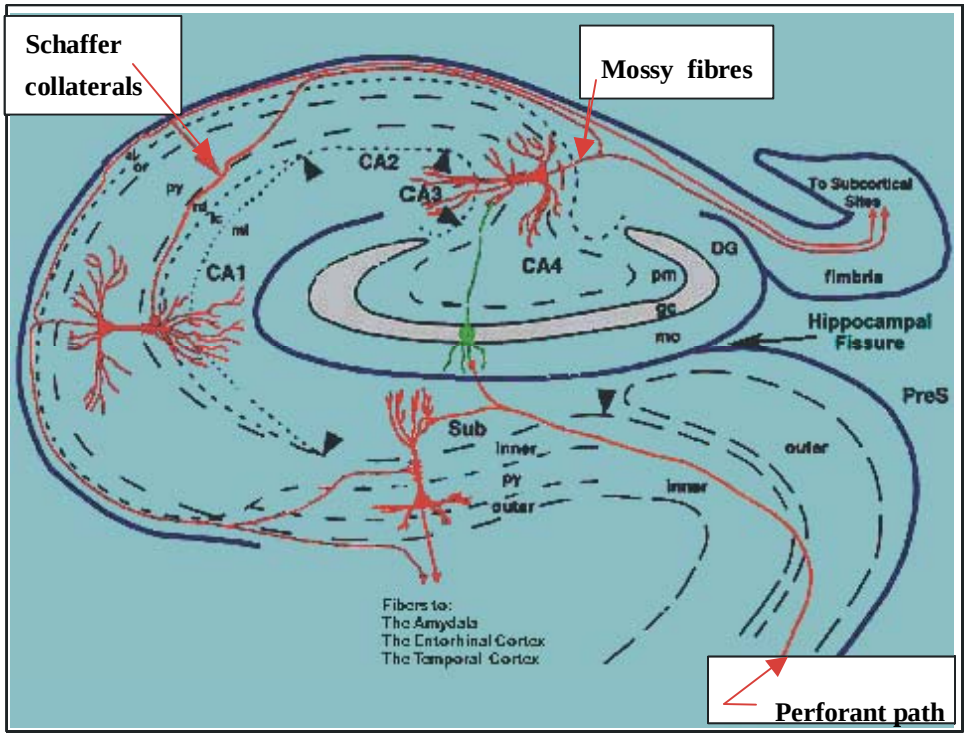


Figure 1.12: The Hippocampal Network: The trisynaptic pathway is an intricate internal circuit mechanism that is unique to the hippocampus. Considering the hippocampus on a coronal plane, the granule cell layer of the dentate gyrus is considered to be the first stage of the trisynaptic pathway. It is the target for the majority of entorhinal afferents carrying sensory information about the external world. These afferents reach the granule cells via the perforant pathway (so named because these axons *perforate* the hippocampal fissure). The CA3 subfield represents the second stage of the trisynaptic pathway. Pyramidal cells of this region are the principal targets of granule cell axons. The axons of CA3 pyramidal cells arise from the lower pole of the soma, or often from a primary basal dendrite. They give rise to extensive axonal arborisations and project to the CA1 subfield, as originally discribed by Schaffer (1892). Finally, the CA1 subfield represents the third and last stage of the intrahippocampal trisynaptic loop. It is a major target of CA3 pyramidal cell axons, the Schaffer collaterals.

These dendritic processes ultimately terminate in a tuft of thin branches in the stratum lacunosum moleculare usually reaching the hippocampal fissure (adapted from Benes et al., 1997).

1.6.6. Hippocampal synaptic plasticity

Mechanisms of neuronal development, learning, memory and circuit reorganisation include alterations of the strength of synaptic connections between neurons; in other words, neural networks are capable of plasticity. These changes in synaptic plasticity have been well studied in mouse and human hippocampi to understand the molecular mechanisms by which neural networks function.

Plasticity in epilepsy has been most commonly studied in the hippocampus and there are several reasons for this. First, the hippocampus is one of the areas of the brain that is quite susceptible to seizures and many types of epilepsy involve the hippocampus. In temporal lobe epilepsy, the hippocampus is a key player, although it is not the only one. Second, the laminar organisation of the hippocampus makes changes perhaps more noticeable than might be in another region with a less obvious lamellar structure.

The hippocampus exhibits both short and long term plasticty. As chemical synapses are not static, postsynaptic potentials (PSPs) wax and wane, depending on the recent history of presynaptic activity. At some synapses, PSPs increase during repetitive stimulation to many times the size of an isolated PSP (Zucker, 1989). Many changes in synaptic efficacy are due to changes in quantum content (the smallest unit in which the transmitter is secreted), (Fatt and Katz, 1952). At most synapses, a second impulse to a presynaptic fiber delivered shortly after the first gives rise to a large postsynaptic potential. This effect, called facilitation, has been shown to be due to an increase in the mean number of quanta of transmitter released by the presynaptic nerve terminal (Castillo and Katz, 1954; Dudel and Kuffler, 1961; Kumo, 1964). Repetitive activation of a synapse can, depending on the circumstances, induce several forms of short-or long-term postsynaptic alterations of responsiveness to subsequent stimuli.

Short-term plasticity, ie. short-term depression and facilitation, strongly influences neuronal activity in cerebral cortical circuits (Hempel et al., 2000). It can be tested using a stimulation paradigm delivered by paired pulse stimulation. In different regions of the brain, the response level of neurons to pulse stimulus (a pair of excitatory PSPs (EPSPs) or

inhibitory PSPs (IPSPs) separated by several milliseconds) varies from one type of neuron to the next. Short-term plasticity may be responsible for many cognitive abilities involving temporal processes (Buonomano et al., 1988). The nature of the plasticity may take on several forms. For instance, one kind of plasticity known as paired-pulse plasticity (PPP) of the EPSP involves an increase in the peak amplitude of the resulting EPSP, due to residual calcium increase after the first stimulus, known as paired-pulse facilitation (PPF). Another kind of plasticity involves decreased amplitude due to paired-pulse depression (PPD). Changes in the ratio of amplitudes of first and second potentials are generally accepted as modification in the presynaptic component of the synapse (Commins et al., 1998; Chen et al., 1996; Gottschalk et al., 1998), although alterations in postsynaptic AMPA receptors have also been reported during PPF experiments (Wang and Kelly, 2001).

'Long term potentiation' or 'LTP' is an enduring increase in the efficacy of signalling between neurons resulting from the application of brief high frequency electrical bursts. Currently, LTP is the dramatic electrophysiological neuroplasticity phenomenon with which to model memory processes (Bliss and Collingridge, 1993). Although LTP has been best studied in the hippocampus, it has been found throughout the limbic forebrain (Racine et al., 1983), in deep locations like deep cerebellar nuclei (Racine et al., 1986), the amygdala (Champman et al., 1990), the striatum (Boeijinga et al., 1993) and superior cervical ganglion of the spinal cord (Brown and McAfee, 1982). Since its discovery in the perforant path of the hippocampal formation, the great majority of the work related to LTP has been through electrophysiological investigations. During this time, evidence for a number of mechanisms for the induction and expression of this functionality have been been reported including pre and postsynaptic mechanisms, activation of previously silent synapses and involvement of both calcium/calmodulin dependent protein kinase C (figure 1.13), (Malenka et al., 1989; Malinow et al., 1989; Manabe, 1992; Malinow and Tsien, 1990; Bekkers and Stevens, 1990; Williams, 1989).

In the hippocampus, the two major forms of LTP are the NMDA-receptor-dependent (Collingridge et al., 1983) or opioid-receptor-dependent (Bramham et al.,1991). Although the latter is less well understood (Derrick et al., 1991; Breindl et al., 1994; terman et al., 2000; Carlo et al., 2000), this form of LTP is the predominant form of plasticity within extrinsic afferents to the hippocampal formation (mossy fiber CA3, lateral perforant path-dentate gyrus, lateral perforant path-CA3) than is NMDA-receptor-dependant LTP (medial perforant

path-dentate gyrus, medial perforant path-CA3). LTP may require a different stimulation pattern, may decay at a different rate, and may involve different mechanisms in different synaptic pathways (Madison et al., 1991; Brown et al., 1990). For example, synapses made by axons in the Schaffer collateral tract onto pyramidal cells in the CA1 region exhibit both NMDA and non-NMDA receptor dependent LTP. Glutamate is released as a neurotransmitter at these synapses, and the CA1 cells have both NMDA and non-NMDA receptors in their postsynaptic membranes. Both forms of LTP at these synapses appear to be triggered by the influx of calcium through NMDA recptors (Barrionuevo et al., 1983).

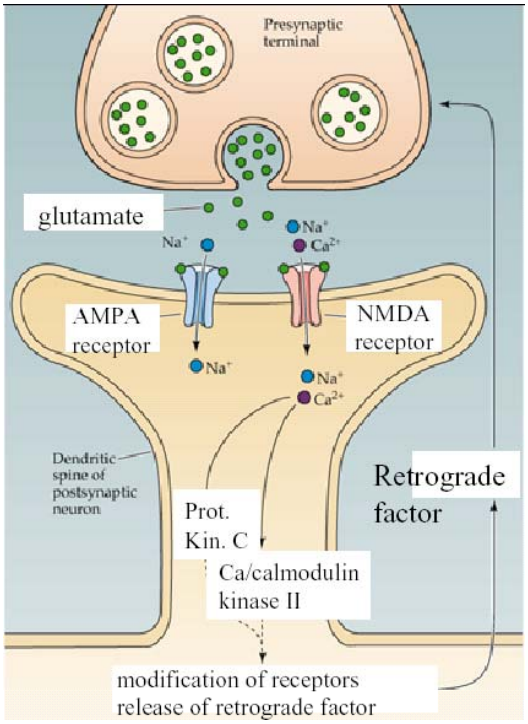


Figure 1.13: The molecular basis of LTP. NMDA receptor opening and calcium influx are necessary for initiating LTP. Activating protein kinases is necessary for maintaining LTP. Phosphorylation of AMPA receptors increases their sensitivity to glutamate (Kandel et al., 1991).

1.6.7. Extracellular matrix molecules and hippocampal synaptic plasticity

Tissue type plasminogen activator (tPA) and urokinase type plasminogen activator (uPA) convert the ubiquitous zymogen plasminogen to plasmin (Plow et al., 1995) which in turn functions to degrade extracellular matrix components (Werb, 1997). The localized expression of PAs in the spinal cord and migrating granule cells during neuronal development suggests that plasmin mediated proteolysis facilitates neuronal outgrowth and cell migration

(Sumi et al., 1992, Ware et al., 1995). The tPA deficient mice have an elevated threshold for seizures indicating that degradation of the ECM by the PA-plasmin system is a critical event in the exitotoxic neuronal death (Chen and Strickland; 1997). Degradation of laminin by plasmin resulted in impaired LTP in organotypic hippocampal cultures suggesting that laminin mediated cell-ECM interaction may be necessary for the maintenance of LTP (Nakagami et al., 2000). Tenascin-R and chondroitin sulfate proteoglycans are essential components of hippocampal extracellular matrix co-localised in perineuronal nets on interneurons. Modification of ECM by enzymatic removal of chondroitin sulfate and lack of tenascin-R showed a two-fold reduction of long-term potentiation induced by theta-burst stimulation of Schaffer collaterals in the stratum radiatum of the CA1 region of the hippocampus, as compared to wild-type mice (Bukalo et al., 2001). The ECM molecule, reelin, expressed by Cajal-Retzius cells is required for the correct layering of cortical neurons and formation of the radial glial scaffold in the hippocampus. Reelin deficient mice have pronounced defects in memory formation and external application of reelin greatly enhances LTP in hippocampal slice cultures (Weeber et al., 2002).

Proteolytic disassembly of ECM is a complex process using the regulated actions of specific extracellular proteinases called matrix metalloproteinases (MMPs). The unique expression pattern of neuronal MMP-9 induced by enhanced synaptic activity and limbic system reorganization suggest that MMPs may be involved in activity-dependent regulation of the peridendritic environment with possible effects on synaptic physiology (Szklarczyk et al., 2002). Dystroglycan-mutant mice have severely reduced hippocampal long-term potentiation with electrophysiologic characterization indicating that dystroglycan might have a postsynaptic role in learning and memory (Moore et al., 2002). Mice with reduced expression of α3, α5, and α8 integrins are also defective in hippocampal LTP and spatial memory as analysed by the Morriz water maze (Chan et al., 2003).

1.7. The nidogen knockouts

To understand the importance of nidogen in basement membranes, mouse knockouts of both nidogen-1 and -2 were made. Surprisingly, basement membrane structures appeared normal in most tissues in the nidogen-1 null mice, though the mice developed neurological defects (Murshed et al., 2000). The mice displayed seizure-like symptoms and over time, a

loss of muscle control in hind legs. Thinning and discontinuity of the basement membrane was observed in brain capillaries and in the lens capsule (Dong et al., 2002). Nidogen-2 was upregulated and the protein re-distributed in heart and skeletal muscle basement membranes where it is normally found in scant amounts explaining the lack of an overt basement membrane phenotype in these tissues (Murshed et al., 2000). However, there is no nidogen-2 staining in the inner limiting membrane of both wild type and nidogen-1 knockout eyes, electron microscopy indicating a disruption in this structure in the nidogen-1 knockout (Smyth, personal communication).

Nidogen-2 deficient mice showed no abnormalities, were fertile and basement membranes appeared normal by ultrastructural analysis and immunostaining, strongly suggesting that both isoforms can compensate for each other (Schymeinsky et al., 2002).

Loss of function mutants in *C. elegans* lacking the single nematode nidogen isoform support the neurological phenotype observed in the nidogen-1 null mice as basement membrane formation occurs here as well. Nidogen is localised to body wall basement membranes and is required to direct longitudinal nerves dorsoventrally and to direct axons at the midlines (Kim and Wordsworth, 2000). However, type IV collagen localization is not affected in *nid* mutants (Kang and Kramer, 2000). A recent report also indicates that *nid* and collagen type XVIII double mutants have distinct defects in the organisation of the neuromuscular junction (Ackley et al., 2003).

Mice lacking both alleles of nidogen-1 and -2 die within a few hours of birth. They have multiple phenotypes with gross abnormalities in heart, lungs, kidneys and brain, bleeding into the eye and brain ventricles and syndactly in limbs with fusion of skeletal and soft tissues (Smyth, personal communication).

1.8. Aim of the study

The purpose of this project was to study the biochemical, electro-physiological and neurological aspects of the nidogen knockout mice, to gain a further insight into the function of this protein family. Both nidogen-1 - -/nidogen-2 + + (N1 - -/N2 + +) and nidogen-1 - -/nidogen-2 + - (N1 - -/N2 + -) mice were analysed.

The neurological defects mentioned in the nidogen-1 null mice (Dong et al., 2002) were mainly reports of observations of spontaneous seizure-like locomoter activity and behavioural abnormalities. Electrophysiological evidence for seizure activity, or indeed of neuronal hyperexcitability was not reported. Mice lacking both alleles of nidogen-1 and one more allele of nidogen-2 show severe neurological disorders with more frequent seizures and ataxia, specially of the hind limbs. In this study, an analysis of the seizure pattern in both nidogen knockouts and its effect on network function in the brain was initiated.

Earlier studies have stressed the importance of nidogen in the supramolecular organisation of basement membranes. Hence, it was surprising when ultrastructural analysis failed to reveal differences in morphology and basement membrane structure in allmost all tissues in the nidogen-1 deficient mice (Murshed et al., 2000). This phenotype was merely explained by the compensatory effect of nidogen-2 in basement membrane assembly in the nidogen-1 deficient mice. To understand the contribution of nidogen-1 and nidogen-2, to the formation and stability of basement membranes, a biochemical study was planned. Both ES cells and tissues from the nidogen knockout mice were used for the analysis.

2. Results

Part 1: Nidogen knockouts - A neurobiological study

To study neurological defects, experiments were conducted on several levels. First, to quantify effects in the whole animal, a behavioural test for judging motor function and an *in vivo* test for monitoring brain electrical activity were performed. Second, for more specific understanding of mechanisms underlying the behavioural and electrographic abnormalities, *in vitro* studies were conducted.

2.1. Rotarod analysis

Motor co-ordination and balance are measured by performance on the rotarod (Jones and Roberts, 1968). The rotarod is a rotating cylinder, approximately 3 cm in diameter when used to test mice. The mice have to walk forward continuously to keep from falling off the rotating cylinder. The rotarod test was used to quantify motor co-ordination in the N1 - - /N2 + + and N1 - - /N2 + - mice. Adult mice were trained and accustomed to movement on a rotarod for two days. The rotarod apparatus was set at 10 rotations per minute. The time taken for each mouse to fall from the rotarod was recorded. Latency to fall off is thus a measure of ability to maintain balance, as the requirements for running forward are gradually increasing. Measurements were made three times for six mice each and the longest time was recorded.

Performance on the rotarod task was significantly decreased in both N1 - -/N2 + + and N1 - - /N2 + - mice ($p<0.001$) when compared to wild type mice (figure 2.1).

This sharp drop in perfomance demonstrates concurrent motor dysfunction. However, it does not indicate whether the underlying neurological defect is central or peripheral.

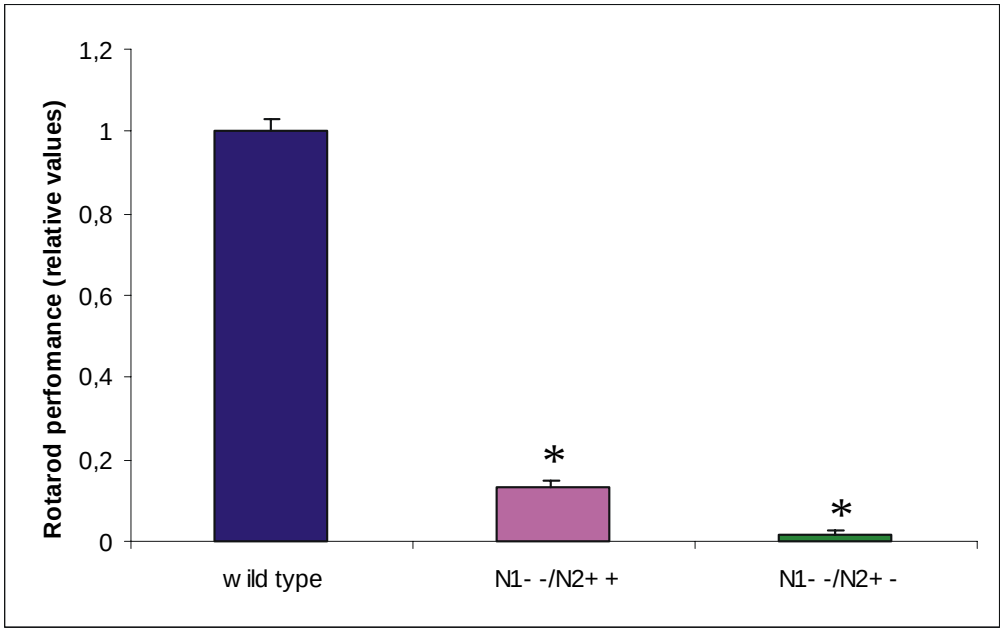


Figure 2.1: Rotarod analysis. The performance has been depicted as relative values which indicate the latency of fall off the rotating cylinder. An average performance rate from 6 individual mice tested for wild type, N1 - -/N2+ + and N1 - -/N2 + -. Both nidogen knockouts performed poorly ($p<0.001$) on the rotarod task when compared to the wild type.

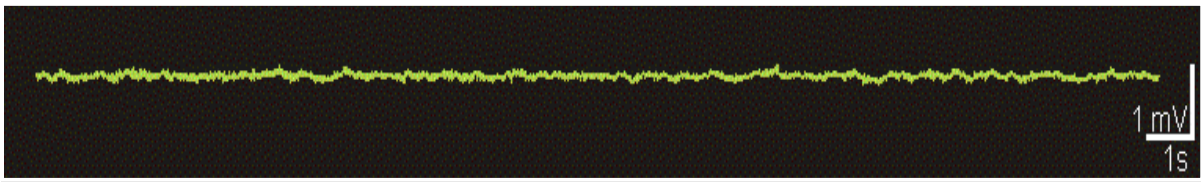
2.2. Electroencephalogram (EEG)

An EEG reflects changes of electrical activity of large ensembles of neurons in the brain. Specifically, it correlates with the extracellular current flow due to the summed synaptic activity of many individual neurons. Figure 2.2 shows EEG in awake mice obtained from epicortical electrodes. In part A of the figure, an EEG of a control mouse is displayed, consisting of low voltage, high frequency waves in the α (8-12 Hz) and β (13-30 Hz) range. The EEG recordings from N1 - -/N2 + + mice (B) and N1 - -/N2 + - mice (C) additionally show abnormal high amplitude discharges riding on slow negative waves (depicted with the symbol '↑').

During these episodes, the animals showed behavioural abnormalities like grooming and sudden involuntary movements that resembled a seizure. Jerking of the head was followed by bowing motions of the upper body, as the animals staggered around on their hind legs. Such episodes ceased abruptly and the animals resumed their normal behaviour. These observations have also been reported in a similar study on the nidogen-1 null animals (Dong

et al., 2002). Hence, the discharges observed in both nidogen knockouts, very likely, represent the EEG correlate of the behavioural seizures.

A



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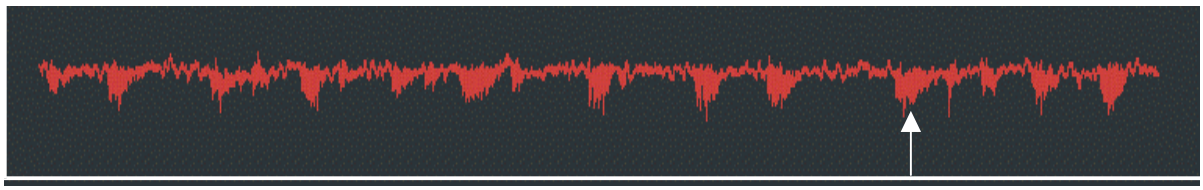

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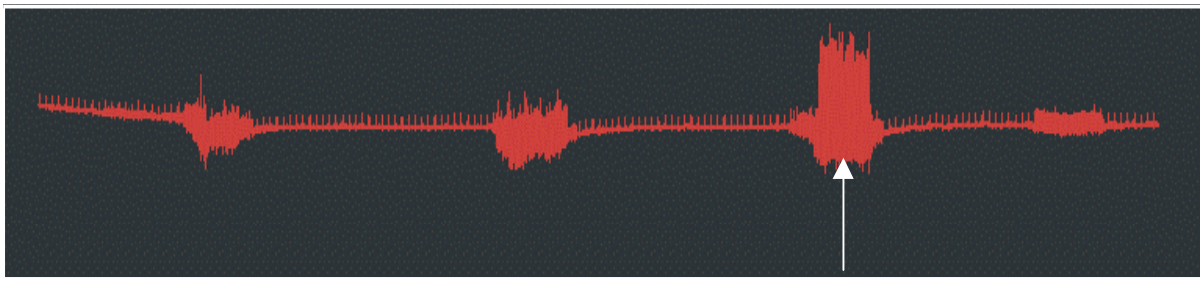


Figure 2.2: EEG recordings from A) wild type, B) N1 - -/N2 + + and C) N1 - -/N2 + - mice. The high amplitude discharges could be observed in all affected animals (n = 4) and not in wild type animals (n = 2). As these discharges appeared often (as indicated by white arrows), in short time intervals ranging from a few seconds to a few minutes, they resemble focal, seizure-like activity.

2.3. Spontaneous activity

As the epileptiform activity shown on the EEG and behavioural changes (grooming and involuntary head jerking movements) indicated an involvement of the hippocampus in several studies (Jeltsch et al., 2001; King and La Motte, 1989; Jiang et al., 1998), this brain area was next investigated. To this end, recordings were performed on the hippocampus *in vitro*. This approach allows a more detailed and subfield specific analysis that would be possible *in vivo*. The first question addressed was whether spontaneous discharges, as in the EEG recordings would also persist *in vitro*, as shown, for example, in chronically epileptic

human brains (Cohen et al., 2002; Kohling et al., 1998). In epileptic brains, spontaneous activity reflects abnormal synchronous activity of neuronal clusters. In hippocampal slice recordings, recording electrodes were placed in CA1 and CA3 regions, dentate gyrus and neocortex and monitored for spontaneous activity for about 20 minutes. Spontaneous epileptiform activity was seen in two out of six cases in the CA3 region and dentate gyrus of both N1 - -/N2 + + and N1 - -/N2 + - mice (figure 2.3).

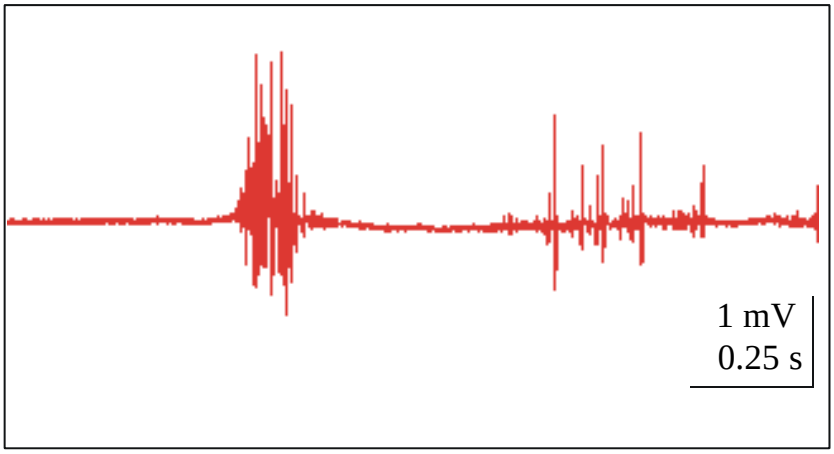


Figure 2.3.1: An example of spontaneous epileptiform activity in CA3 region of the hippocampus of a N1 - -/N2 + + mouse

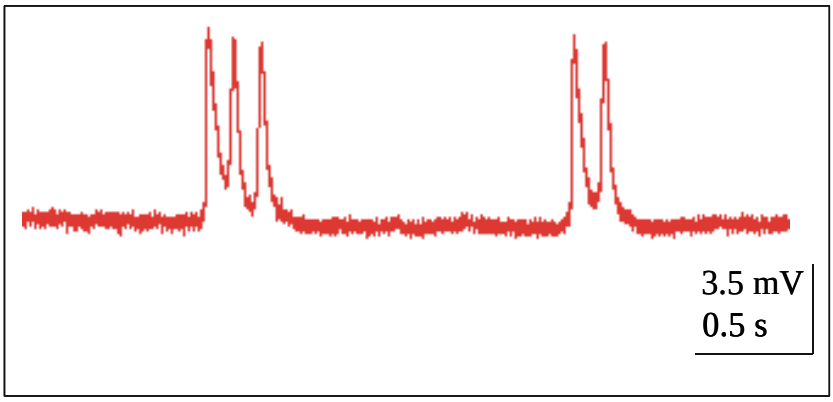


Figure 2.3.2: An example of spontaneous epileptiform activity in the dentate gyrus of a N1 - -/N2 + - mouse.

2.4. Evoked epileptiform field potentials

The next question addressed was whether synaptic responses would be abnormal in the absence of spontaneous epileptform activity. To test this, responses were evoked in the CA1 area by double electrical stimuli applied to the afferent fibres, the Schaffer collaterals. In four out of six cases, responses consisting of two single population spikes were recorded in N1 - -/N2 + - (figure 2.4.1A) and in all wild type slices. However, in two N1 - -/N2 + - slices, multiple epileptiform population spikes appeared (figure 2.4.2B). This response was graded in that, with increasing stimulation intensity, both the size and the number of population spikes increased (figure 2.4.2).

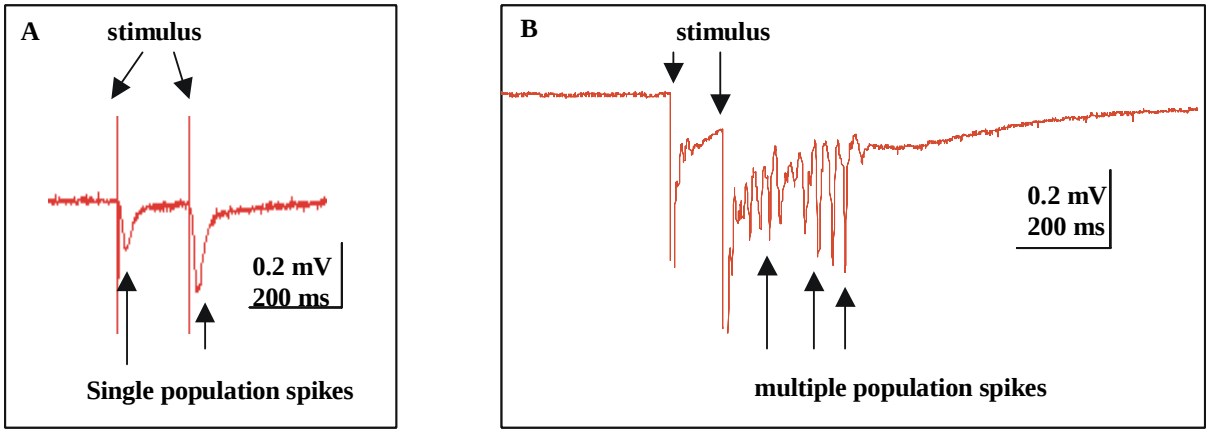


Figure 2.4.1: Evoked field potential recording in CA1 region of the hippocampus. A) Normal responses in N1 - -/N2 + - and wild type slices. B) Epileptiform responses in two out of six slices from N1 - -/N2 + - mice.

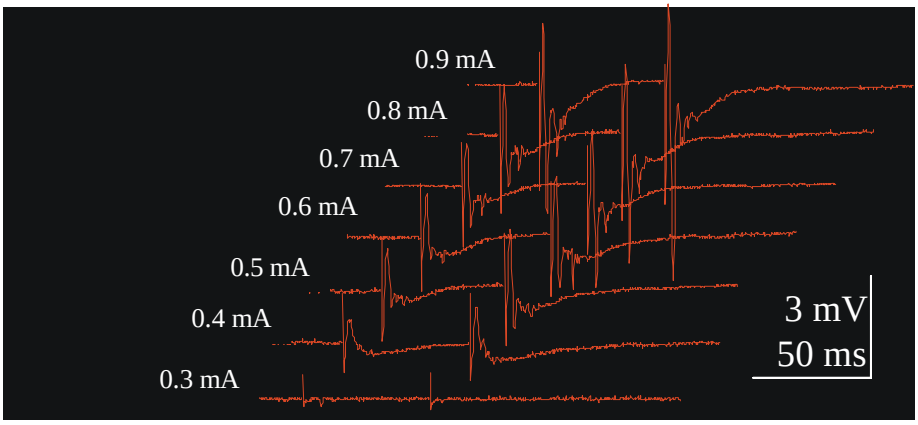


Figure 2.4.2: Evoked epileptiform field potential recording in CA1 region of the hippocampus. At an initial stimulus intensity of 0.3 mA there was no response. From 0.4 mA stimulation till 0.9 mA stimulation, an increase in epileptic responses followed.

2.5. Analysis of baseline synaptic function

To test for general deficits in synaptic function, input-output relationships of different synapses were examined in response to single electrical stimuli. If graded stimuli would result in disproportionally large responses in the nidogen knockout slices, then it would be indicative of increased excitability. For this purpose, both the input region (dentate gyrus) and the output region (CA1) of the hippocampus as well as the neocortex were chosen. After placement of stimulating and recording electrodes in specific areas of a hippocampal slice, the stimulus strength was turned down until no excitatory postsynaptic potential (fEPSP) was evoked. The stimulus was then increased in increments, and the responses collected at each increment. The stimulus current was increased until the size of the resulting fEPSP was clearly saturated. Data were averaged across six animals in each group (wild type, N1- -/N2 + + and N1- -/N2 + - mice) to construct input-output curves.

To examine potential presynaptic influences in the absence of nidogen, paired pulse facilitation (PPF) was examined in slices from wild type and both nidogen knockouts. PPF occurs when two stimuli are delivered to synapses in rapid succession, resulting in the facilitation of the synaptic response to the second stimulus. Paired pulse induced fEPSPs were evoked with various interstimulus intervals ranging from 10-500 ms.

PPF is represented as the ratio of the response to the second stimulus divided by the response to the first. PPF is a measure of presynaptic function, thought to result from an increase in transmitter release probability caused by calcium influx into the presynaptic terminal during the response to the first stimulus. It may be involved in the pathologic process of increasing seizure susceptibility since a persistent increase in an *in vitro* PPF of the EPSP was observed after partial hippocampal kindling or a single infant seizure (Kamphuis et al., 1988; Zhao and Leung, 1993).

2.5.1. Field potential recordings in the dentate gyrus

2.5.1.1. Input-output relationships

The dentate gyrus is composed of a discrete granule cell layer with each granule cell arranged similar to the next. The layer containing the dendrites of these cells is the molecular

layer (as shown in figure 1.10B) which contains three sublayers: an inner molecular layer, a middle molecular layer and an outer molecular layer. The lateral perforant path (LPP) and medial perforant path (MPP) form synapses in adjacent portions of the molecular layer. LPP synapses are located more distally on the dendritic tree, whereas MPP synapses are located more proximally, (figure 1.12), (Steward, 1976; Witter, 1993). Stimulation electrode was placed in the lateral perforant path and recording electrode in the outer molecular layer. Though input-output curves were increased slightly in the N1- -/N2 + - mice and decreased in the N1- -/N2 + - mice, there were no significant differences when compared to wild type (figure 2.5.1.1). However, input-output relationships varied significantly between both nidogen knockouts ($p<0.05$), indicating changes brought about by an altered gene dosage. The dentate gyrus in the more severe knockout N1 - -/N2 + - , shows an increasing trend towards excitability.

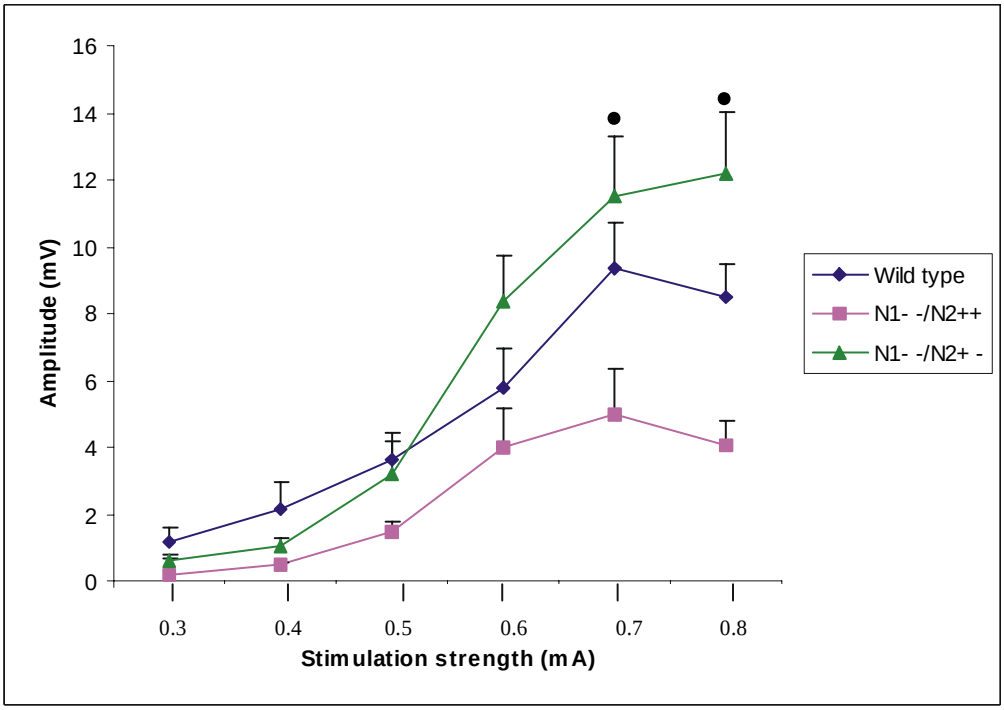


Figure 2.5.1.1: Input-output curves of field excitatory postsynaptic potentials (fEPSPs) in the outer molecular layer after lateral perforant path stimulation. No significant changes were observed between both nidogen knockouts and the wild type. However, a significant change in the input-output relationship was observed when the N1 - - /N2 + - mice was compared with that of the N1 - -/N2 + + mice ($p<0.05$) from 0.7 mA stimulation onwards (depicted as symbol '•'). Values are the mean standard error for 6 slices.

2.5.1.2. Paired pulse ratio

Paired pulse ratio was significantly increased in the N1 - -/N2 + - mice between 20-50 ms interpulse interval. No significant changes were observed in PPF in N1 - -/N2 + + mice when compared to wild type (figure 2.5.1.2).

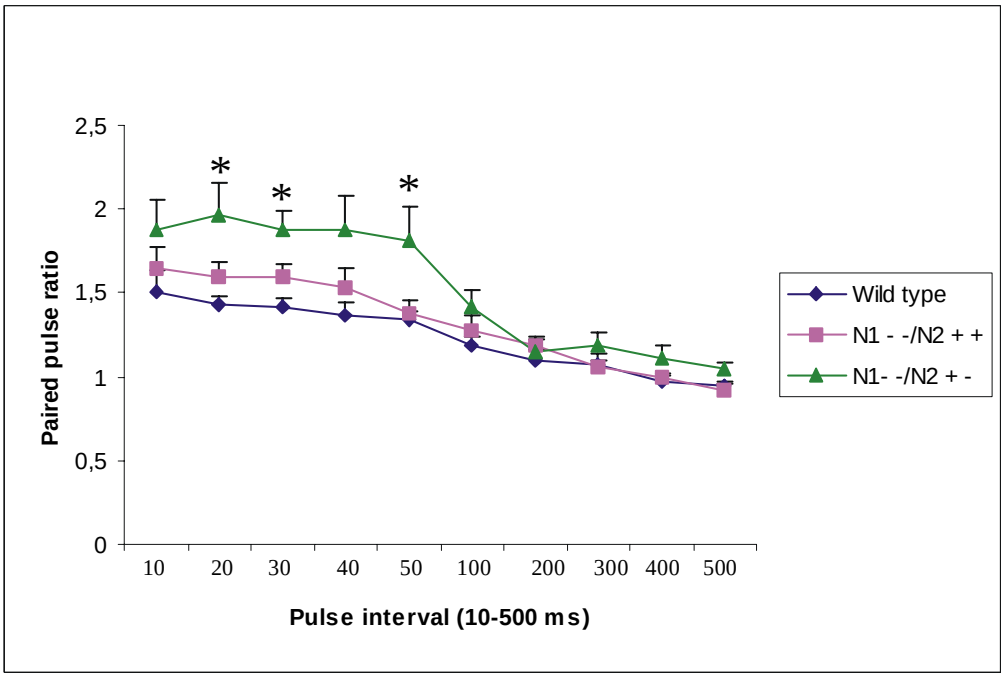


Figure 2.5.1.2: Paired pulse profiles of fEPSPs in outer molecular layer after lateral perforant path stimulation. A significant increase in the paired pulse ratio was observed in N1 - -/N2 + - mice ($p<0.05$) when compared to the wild type. Values are the mean standard error for 6 slices.

2.5.2. Field potential recordings in CA1 region

2.5.2.1. Input-output relationships

The hippocampal allocortex has been subdivided into three layers (section 1.6.1, figure 1.10 B). On the sheet of alvear white matter of the hippocampus, lies layer III, the stratum oriens, a layer of polymorph, rather loosely arranged cells. On top of stratum oriens lies layer II, the stratum pyramidale or pyramidal layer. In layer I, of the the allocortex, is the stratum radiatum, next to the pyramidal layer, containing mostly the apical dendrites of the pyramidal cells. Input-output curves were obtained by stimulating Schaffer collaterals (figure

1.12) and the recording electrode was placed in stratum pyramidale, representing pyramidal layer synaptic activation (figure 2.5.2.1) in area CA1 of the hippocampal slice. A significant increase in input-output curves in both nidogen knockouts as compared to the wild type indicated hyperexcitability in area CA1.

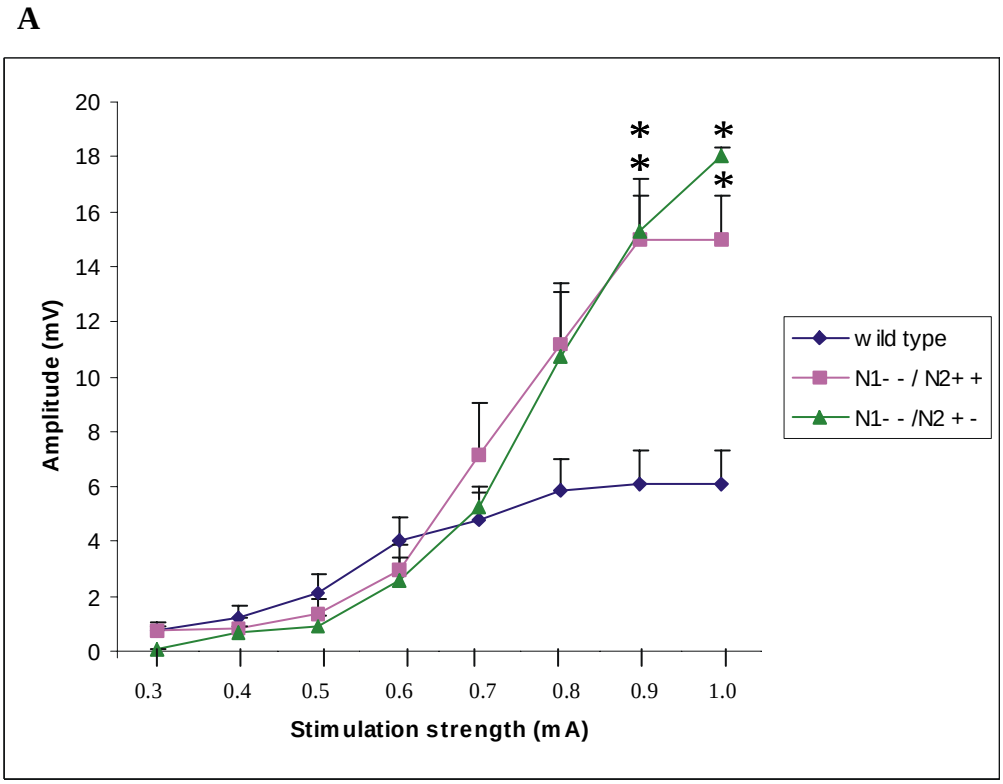


Figure 2.5.2.1: Input-output curves of field excitatory postsynaptic potentials (fEPSPs) in the CA1 region after Schaffer collateral stimulation. Recording electrode placed in stratum pyramidale. A significant increase in the amplitude of responses was observed in both nidogen knockouts ($p<0.05$) when compared to the wild type, from 0.9 mA stimulation onwards. Values are the mean standard error for 6 slices.

2.5.2.2. Paired pulse ratio

Results obtained from recordings in stratum pyramidale (figure 2.5.2.2A) and stratum radiatum (figure 2.5.2.2B) showed a similar picture. In stratum pyramidale, N1- -/N2 + + slices showed an increasing trend in PPF. However, in stratum radiatum, significantly larger PPF was observed at intervals from 10-500 ms in N1- -/N2 + + slices, when compared to wild type slices. In contrast, PPF from N1- -/N2 + - slices showed no significant difference from that of wild type at all intervals.

A

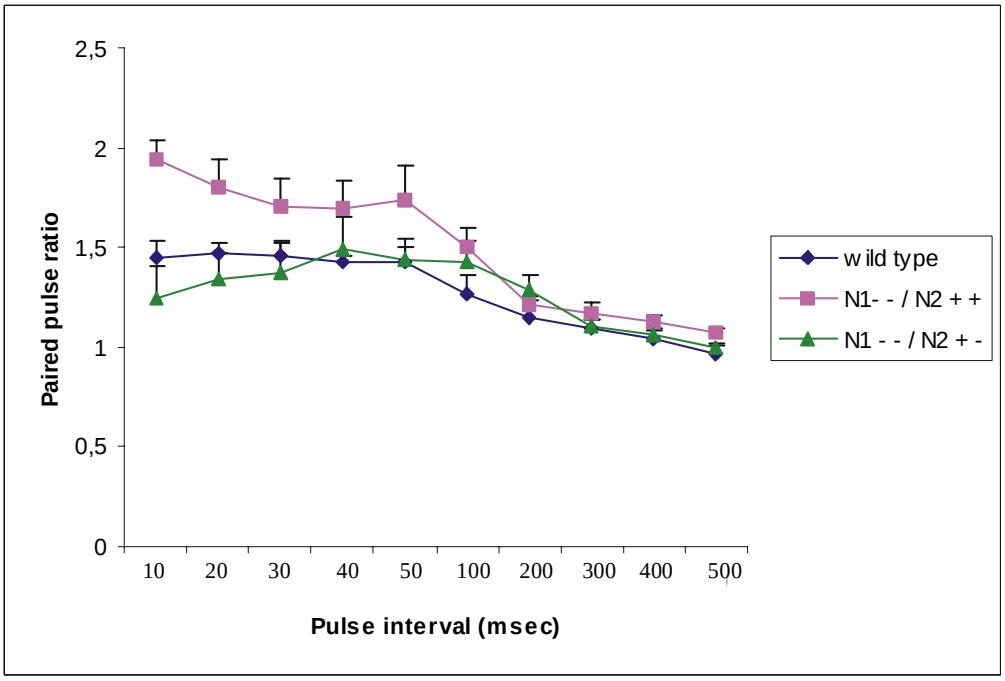


Figure 2.5.2.2A: Paired pulse profiles of fEPSPs in CA1 after Schaffer collateral stimulation. Recording electrode placed in stratum pyramidale. There were no significant differences in the paired pulse ratio between wildtype and both nidogen knockout mice. However, the PPR from N1 - -/N2 + + mice showed an increasing trend. Values are the mean standard error for 6 slices.

B

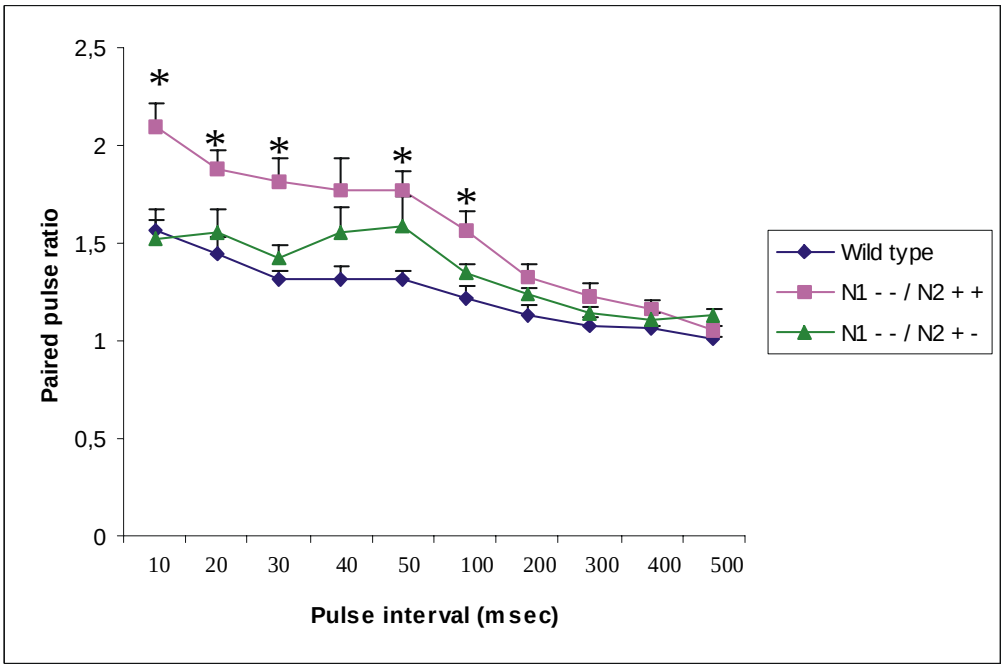


Figure 2.5.2.2B: Paired pulse profiles of fEPSPs in CA1 after Schaffer collateral stimulation. Recording electrode placed in stratum radiatum. A significant increase in the paired pulse ratio was observed in the N1- -/N2 + + mice ($p<0.05$) between 10-100 ms interpulse intervals. There were no significant differences in the paired pulse ratio of the fEPSP slope in N1 - -/N2 + - and wild type mice. Values are the mean standard error for 6 slices.

2.5.3. Field potential recordings in the neocortex

2.5.3.1. Input-output curves

The structure of the temporal neocortex has been described in figure 1.10 A. The small pyramidal cells of layers II and III give rise to cortico-cortical axons that provide connections to other cortical areas and to limbic structures (Meissirel et al., 1991). The horizontal axons in layer I are terminal branches of cortico-cortical afferents or collaterals of axons ascending from deep cortical layers. These parallel fibres have an estimated tangential spread of about 5 mm and establish excitatory connections (Szentagothai, 1975). Stimulation of parallel fibres and assessment of fEPSPs in layers I and II of the neocortex resulted in a significant decrease in input-output curves in N1 - -/N2 + + mice when compared to the wild type, though N1- -/N2 + - mice showed a decreasing trend as well (figure 2.5.3.1).

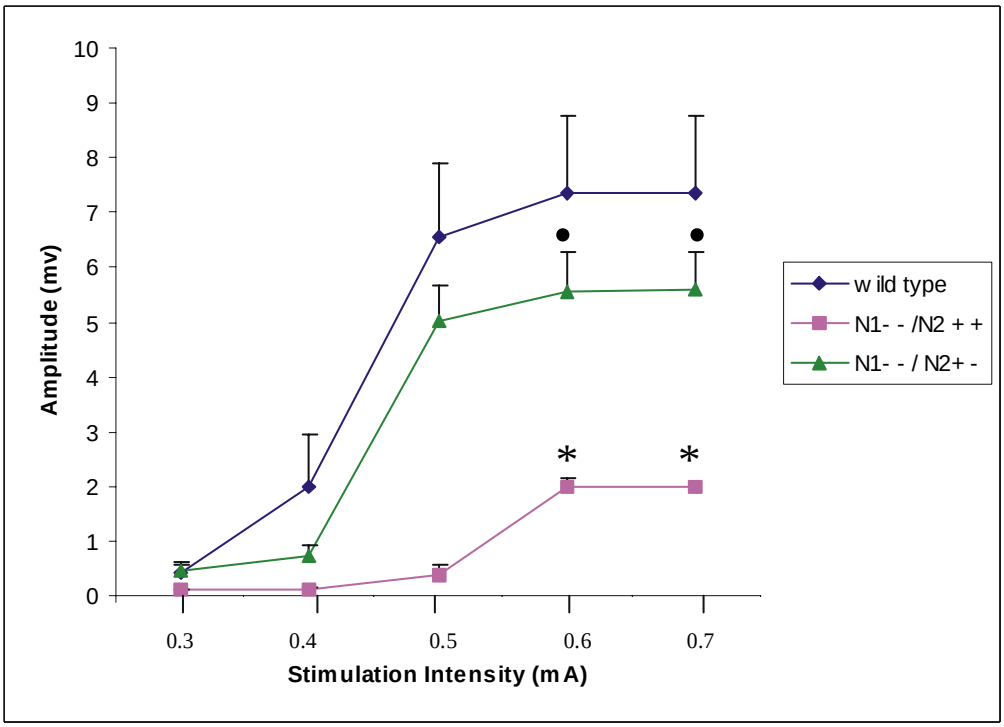


Figure 2.5.3.1: Input-output curves of field excitatory post-synaptic potentials (fEPSPs) in the neocortex after stimulation of parallel fibres. A significant decrease in the amplitude of responses was observed in N1 - -/N2 + + mice when compared to the wild type ($p<0.05$) from 0.6 mA stimulation onwards. A significant change in the input-output relationship was also observed when the N1 - - /N2 + - mice was compared with that of the N1 - - /N2 ++ mice ($p<0.05$) from 0.6 mA stimulation onwards (depicted as symbol '•'). Values are the mean standard error for 6 slices.

Strangely, synapses in the N1 - -/N2 + + mice seemed to be more drastically affected than the N1 - -/N2 + - mice. Again, a variation in gene dosage between both nidogen knockouts seems to have different effects in the neocortex as well. Overall, the results indicate very low excitability in synapses in the neocortex.

2.5.3.2. Paired pulse ratio

In line with the input-output relationships, a significant reduction in paired pulse facilitation was observed at intervals from 10-100 ms interval in N1 - -/N2 + + mice when compared to the wild type (figure 2.5.3.2). No significant changes were observed in the PPF between wild type and N1 - -/N2 + - mice.

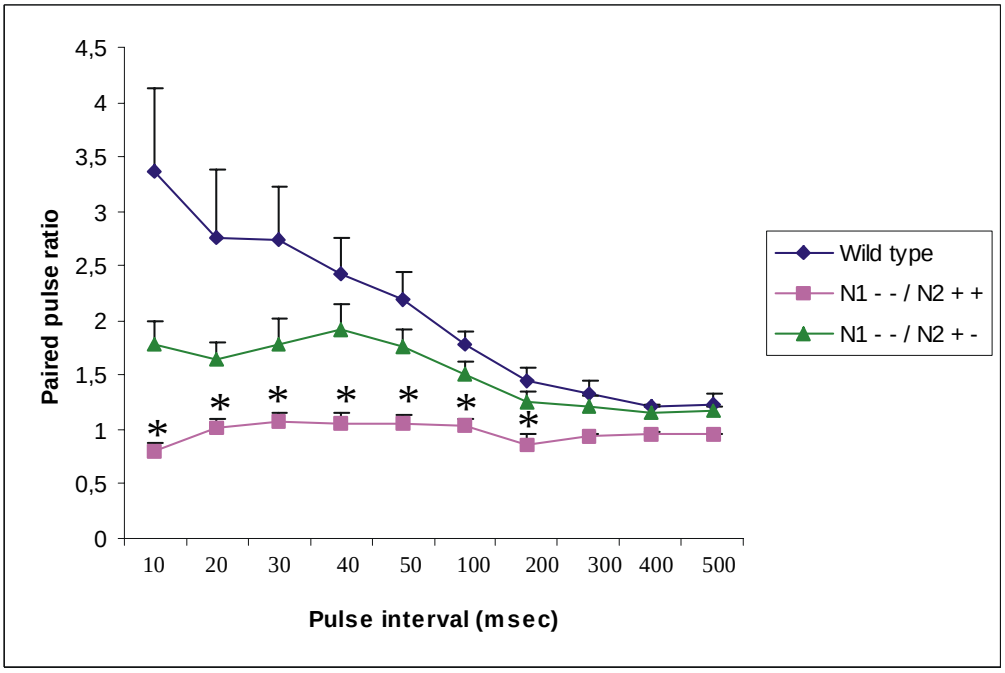


Figure 2.5.3.2: Paird pulse profiles of fEPSPs in the neocortex after stimulation of parallel fibres. A significant decrease in the paired pulse ratio was observed in N1 - -/N2 + + mice ($p<0.05$) between 10-100 ms interval. Values are the mean standard error for 6 slices.

A summation of the data obtained from input-output relationships and paired pulse profiles reveals that 1) the input region of the hippocampus, the dentate gyrus has mixed synaptic responses between the two nidogen knockouts, 2) the output region, CA1 is hyperexcitable in both nidogen knockouts and 3) the neocortex is far less excitable in the single knockout of nidogen, N1 - -/N2 + +.

2.6. Long term potentiation (LTP)

An important issue next addressed, was whether the epileptiform discharges and increased excitability observed in *in vivo* and *in vitro* recordings would be accompanied by changes in neural activity over long periods of time. LTP is the sustained increase in synaptic strength obtained after a high-frequency conditioning stimulus (called tetanus), and is a compelling model for a synaptic mechanism underlying some forms of learning and memory (Madison et al., 1991). Similarities have been reported between the responses of specific cells to stimulus that produce LTP and the responses of the same specific cells to a limbic seizure. One example is the increase in immediate early gene expression and neurotropins by both a tetanus used to elicit LTP and seizures that involve the dentate gyrus (Bramham et al., 1996). Similarities in postulated mechanisms of epileptogenesis and LTP have been described as well (Morris et al., 2000).

Stimulation of lateral perforant path evoked fEPSPs that were recorded in the outer molecular layer of hippocampal slices prepared from wild type and nidogen-1 knockout mice. At least 15 minutes of stable baseline transmission was obtained, testing once every 30 seconds. Following the baseline period, tetanic stimulation was applied. Immediately after the tetanus, testing of synaptic strength was resumed by returning to the test stimulus for at least 60 minutes. The fEPSP responses observed prior to and after tetanus in wild type, N1 - -/N2 + + and N1 - -/N2 + - slices have been shown in figure 2.6.1. Finally, L-AP4, a drug that acts on metabotropic glutamate receptor (mGluRIV) was washed in at the end of the experiment.

LTP, on an average from 6 experiments, was significantly impaired in both nidogen knockouts as compared with wild type animals. L-AP4 successfully reduced LTP formation in both wild type and nidogen knockouts (figure 2.6.2) confirming the fact that the lateral perforant path had been stimulated.

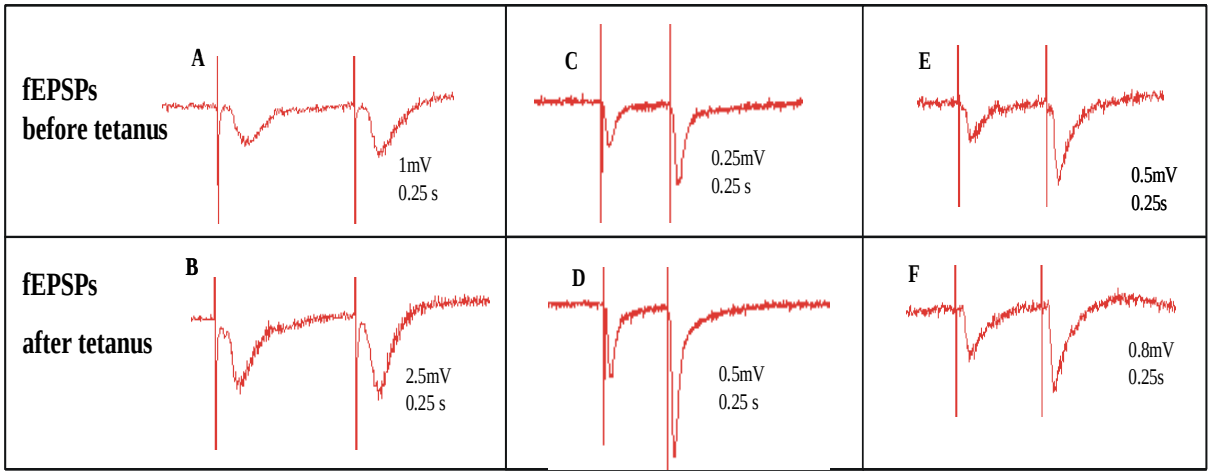


Figure 2.6.1: Representative field potential recordings before and after tetanus (A) wild type pre-tetanus fEPSP (B) wild type post-tetanus fEPSP (C) N1 - -/N2 + + pre-tetanus fEPSP (D) N1 - -/N2 + + post-tetanus fEPSP (E) N1 - -/N2 + - pre-tetanus fEPSP (F) N1 - -/N2 + - post-tetanus fEPSP.

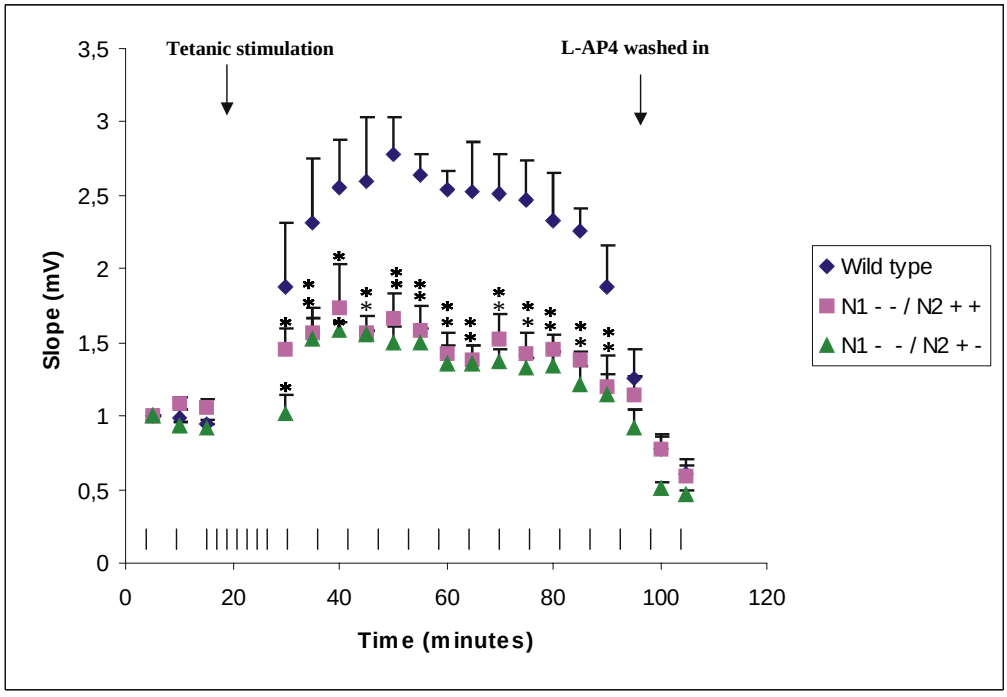


Figure 2.6.2: LTP is impaired in slices from both nidogen knockouts ($p<0.05$). LTP, recorded for 60 minutes post-tetanus in wild type and both nidogen knockouts, following a 15 minutes baseline. Data points represent the mean standard error of 5 wild type and 6 nidogen knockout slices.

2.7. Distribution of mossy fibres determined with Timm's stain

Structural changes in the dentate gyrus have often been described after seizures which involves the growth of new axon collaterals or 'sprouting'. The most widely studied example is the sprouting of mossy fibres (Ben-Ari and Cossart, 2000; Babb et al., 1991). The axons of the granular cells, the mossy fibres establish synaptic contacts with interneurons in the inferior region and with the proximal part of the apical dendrites of giant CA3 pyramidal neurons. At the EM level, the mossy fibre synapses appear as multi invaginated boutons (up to 10 μm in diameter) establishing multiple contacts with giant dendritic spines (Ben-Ari and Represa, 1990).

In humans and in various animal species, mossy fibre terminals contain the highest concentrations of zinc found in the brain (ie. 150-250 pm zinc). Zinc is found in large dense core vesicles and is taken up and released from mossy fibres. The function of zinc in mossy fibres has not been elucidated; however it is believed to block NMDA channels, reduce GABAergic responses and calcium currents. The Timm's stain which reveals zinc, can be used to study innervation, as noticed in some severe cases of epilepsy (Haug, 1973; Ibata and Otsuka, 1969). However, this is not a necessary condition in many chronic cases of epilepsy (Raol et al., 2003; Mathern et al., 1996; Ebert, 1995).

Timm's silver sulphide stains with a cresyl-violet counterstain. The brownish-black staining can be seen throughout the hilus of the dentate gyrus and the whole depth of the stratum pyramidale. Timm's staining was similar in both nidogen knockouts to that of the wild type (figure 2.7).

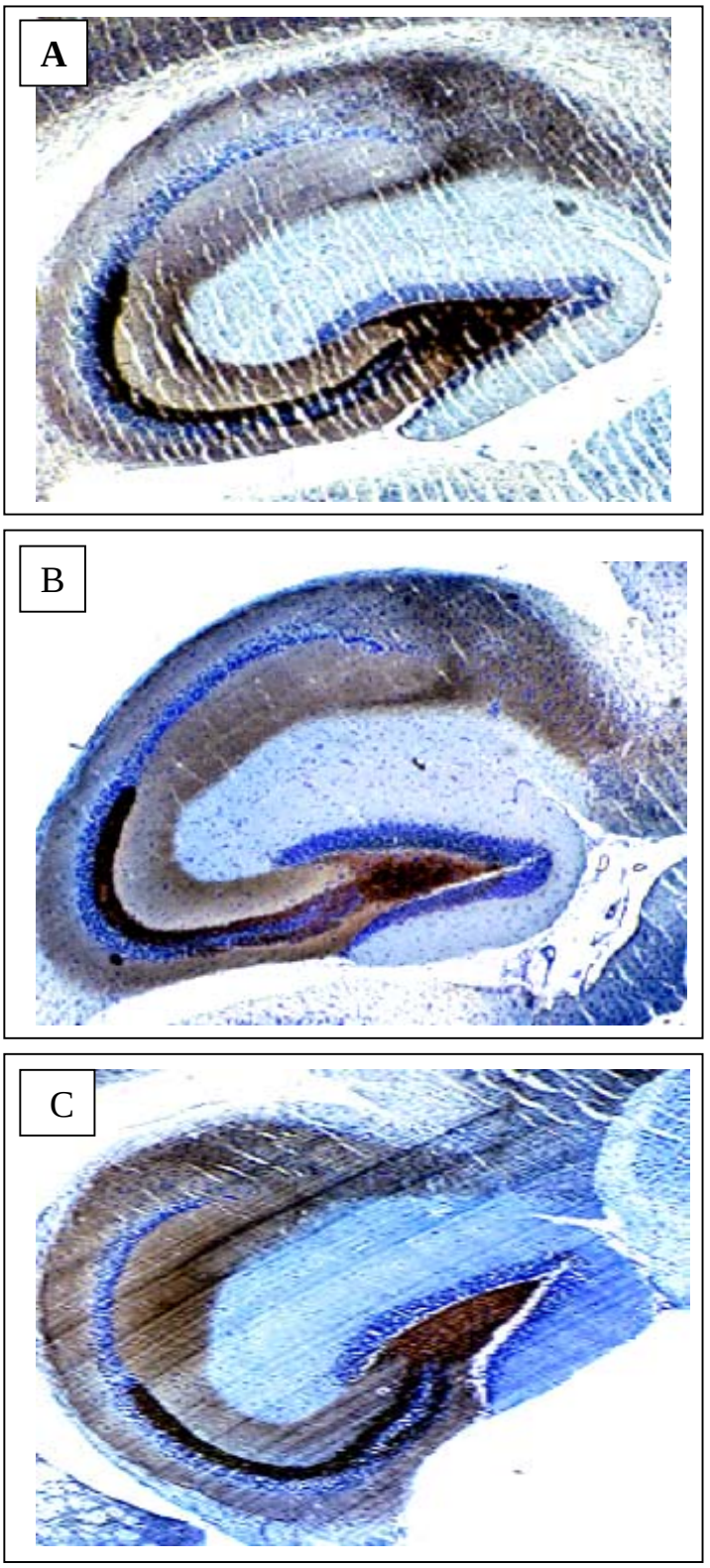


Figure 2.7: Timm's staining: Hippocampal slices from A) wild type, B) N1 - -/N2 + + and C) N1 - -/N2 + - mice.

2.8. Nidogen-1 and laminin γ1 expression patterns in hippocampal slices

Hippocampal slices (300 μm) were immunostained with polyclonal antibodies against nidogen-1 and laminin γ1. In wild type slices, antibodies clearly labelled the walls of blood vessels (figure 2.8A and C). This result was consistent with previous studies which reported the same pattern of immunoreactivity seen throughout the whole brain (Niquet and Repressa, 1996). However in nidogen-1 knockout slices, laminin γ1 immunoreactivity was reduced in CA3 region and denate gyrus (figure 2.8D).

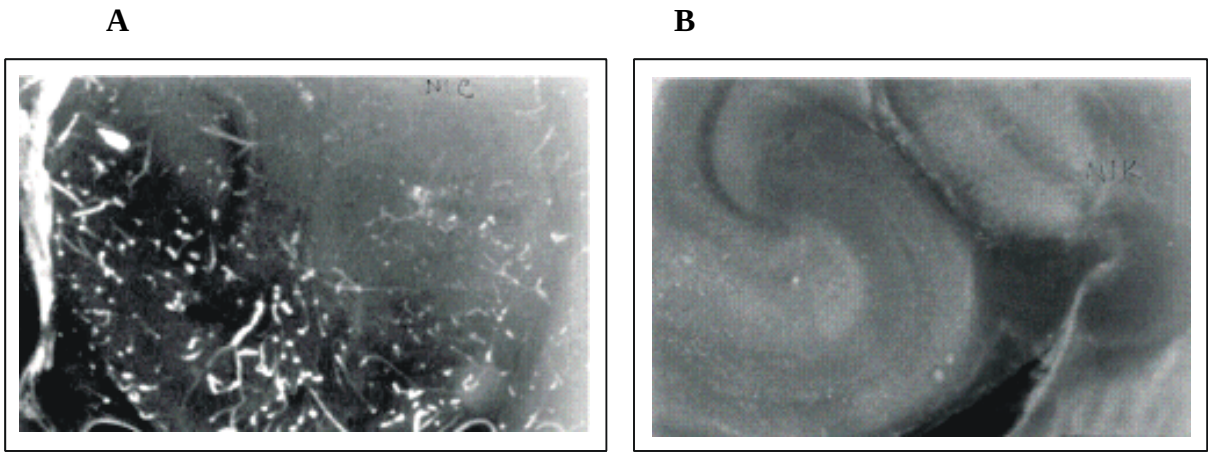


Figure 2.8: Immunofluorescence for nidogen-1. Hippocampal slices from A (wild type) and B (N1 - -/ N2 + +) animals were stained with rabbit polyclonal antibody against nidogen-1.

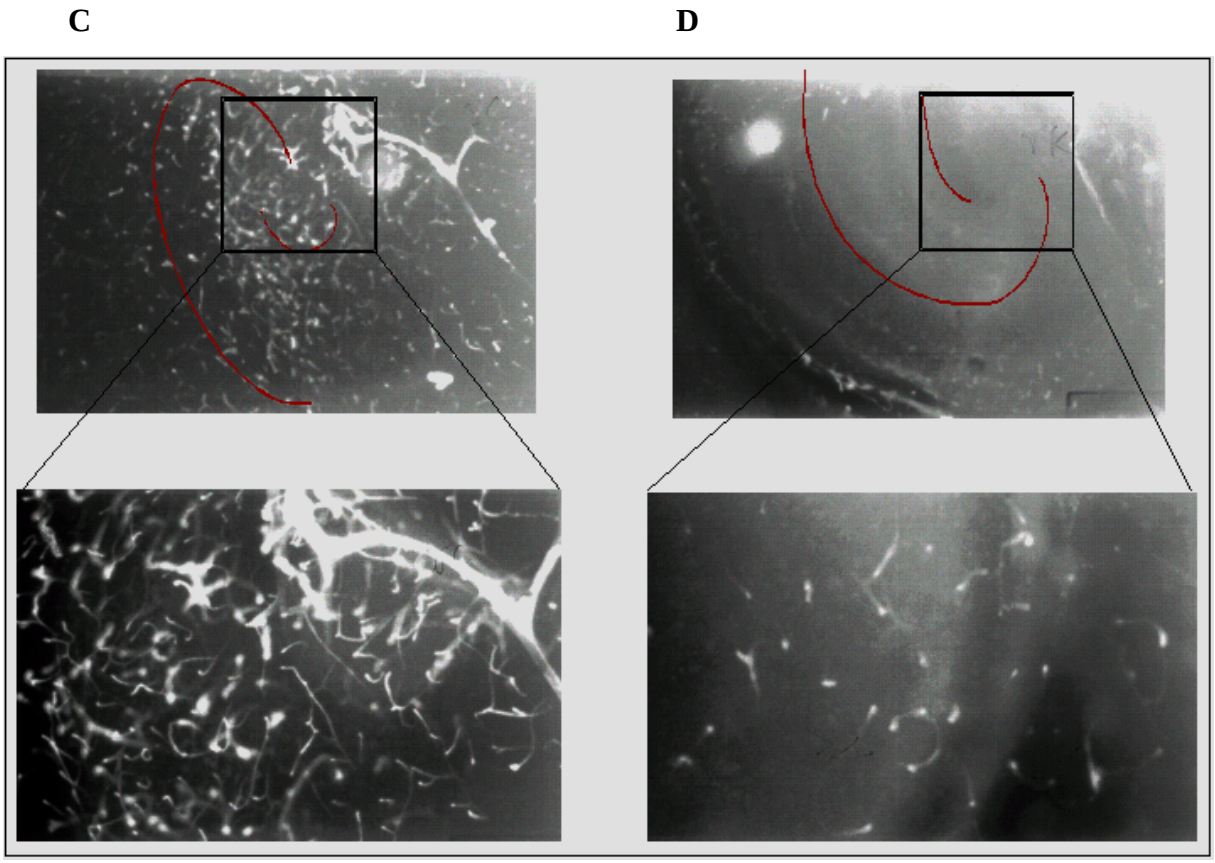


Figure 2.8: Immunofluorescence for laminin γ1. Hippocampal slices from C (wild type) and D (N1 - -/ N2 + +) animals were stained with rabbit polyclonal antibodies against nidogen-1 (A and B) and laminin γ1 (C and D) respectively. The rectangular boxes mark the CA3-dentate gyrus region. Laminin γ1 staining is decreased in the CA3 region and dentate gyrus in N1 - -/ N2 + + animals. Images were taken at 10x magnification

2.9. Confocal laser images of nidogen-1 and laminin γ1 in the hippocampus

A co-staining of nidogen-1 and laminin γ1 in hippocampal slices confirmed the previous result. A similar expression pattern for nidogen-1 and laminin γ1 was observed in CA1 in both wild type and N1 - - /N2 + + slices (figure 2.9.1). However, in both CA3 (figure 2.9.2) and in dentate gyrus (figure 2.9.3), laminin γ1 expression seemed to be drastically reduced in the N1 - - /N2 + + slices when compared to the wild type.

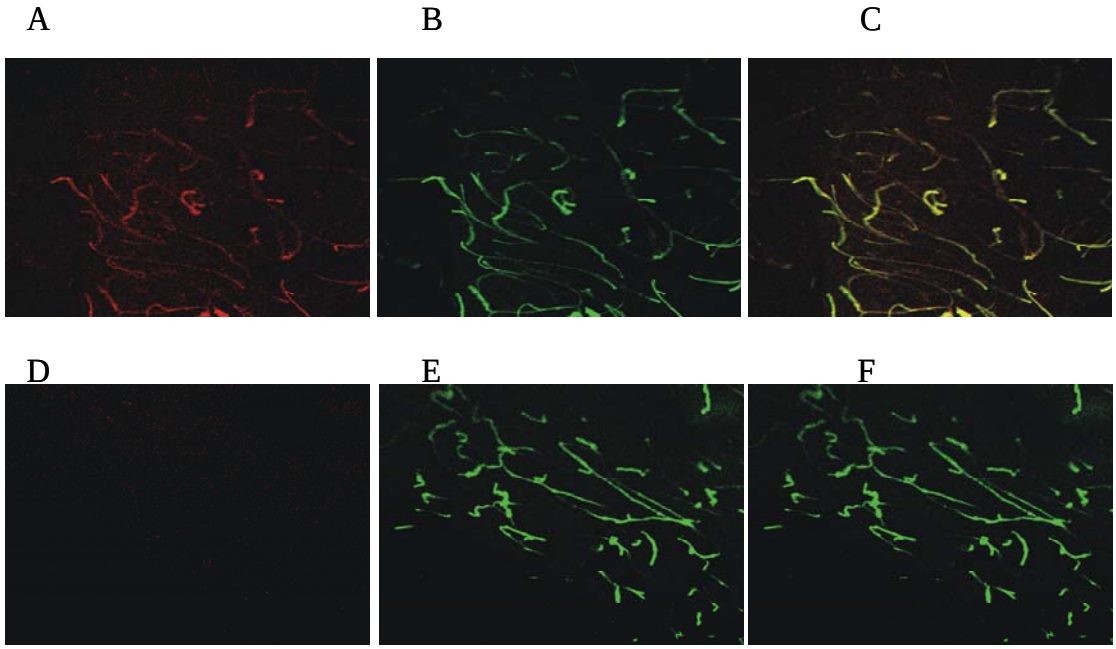


Figure 2.9.1: Double immunofluorescence for nidogen-1 and laminin γ1 in the CA1 region. Hippocampal slices were incubated simultaneously with a guinea pig polyclonal antibody against nidogen-1 (red) and a rabbit polyclonal antibody against laminin γ1 (green). A, B and C are wild type images and D, E and F are N1 - -/N2 + + images. Images were taken at 20x magnification.

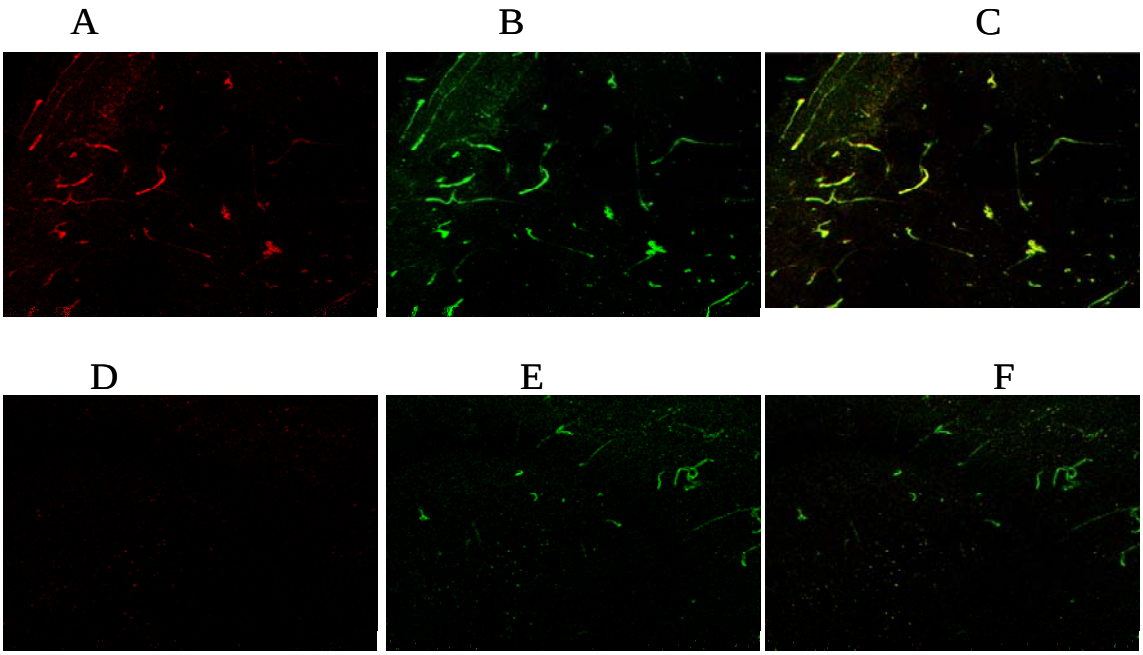


Figure 2.9.2: Double immunofluorescence for nidogen-1 and laminin γ1 in the CA3 region. Hippocampal slices were incubated simultaneously with a guinea pig polyclonal antibody against nidogen-1 (red) and a rabbit polyclonal antibody against laminin γ1 (green). A, B and C are wild type images and D, E and F are N1 - -/N2 + + images. Images were taken at 20x magnification.

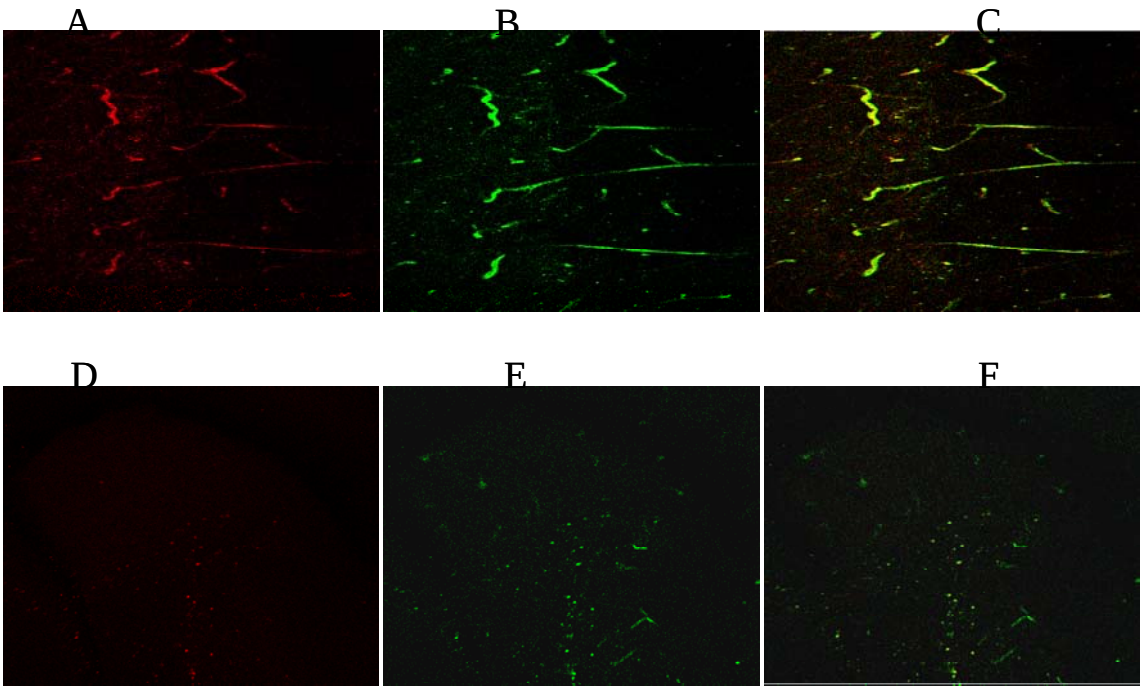


Figure 2.9.3: Double immunofluorescence for nidogen-1 and laminin γ1 in the dentate gyrus. Hippocampal slices were incubated simultaneously with a guinea pig polyclonal antibody against nidogen-1 (red) and a rabbit polyclonal antibody against laminin γ1 (green). A, B and C are wild type images and D, E and F are N1 - -/N2 + + images. Images were taken at 20x magnification.

Part 2: Nidogen knockouts - A biochemical study

The decreased expression of laminin γ1 in the hippocampus of the nidogen-1 null animals lead to further biochemical studies, wherein, a characterization of laminin-1 in the whole animal was initiated. Laminins have been very well characterized in embryoid bodies (Mayer et al., 1998) and in mouse heart tissue (Paulsson and Saladin, 1989). As both these tissues have been shown to give high yields of laminin in analytical extractions, they were chosen as a preparative source. As laminin self-aggregation is Ca^{2+} dependent (as shown in figure 1.5), chelating agents like EDTA allow the selective extraction of laminin from tissues (Yurchenco et al., 1985; Paulsson, 1988).

2.10. Analysis of laminin from embryoid bodies

Wild type and nidogen-1 null ES cells were used to produce embryoid bodies, using the cell spin system (Integra-Biosystems), (Tunggal et al., 2003). The embryoid body system, over a period of 10-21 days offers an excellent model of basement membrane deposition (Cooper et al., 1983; Howe and Solter, 1980; Alonso et al., 1991). About 5000 wild type and nidogen-1 null embryoid bodies were homogenised and extracted with 0.05 M Tris buffer, pH 7.4, to remove loosely associated proteins and those present from the serum. They were subsequently extracted with the same buffer containing EDTA. The EDTA extracts were subject to chromatography on a Sepharose CL-4B column.

A high yield (100 μg/ml) of laminin with nidogen was obtained from wild type embryoid bodies (figure 2.10A). Surprisingly, the yield of laminin only from nidogen-1 null embryoid bodies was extremely poor and could be detected only by an immunoblot (figure 2.10B).

These results suggested two possibilities. Firstly there might be changes in the production of laminin in nidogen-1 null embryoid bodies or alternatively the laminin was being lost out at some stage during culture, extraction or purification steps. To determine if a technical error had occurred, the next set of experiments were performed.

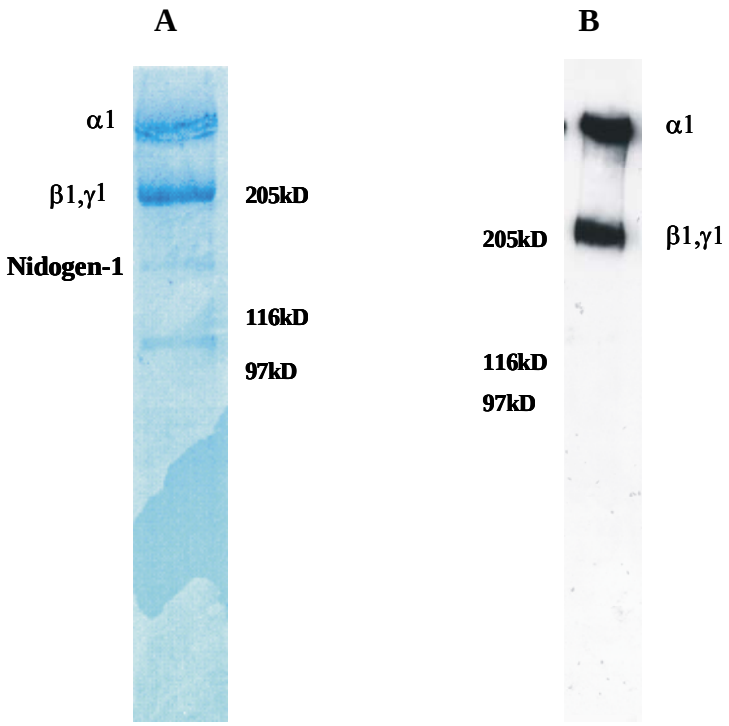


Figure 2.10: Laminin purified from embryoid bodies by gel filtration. (A) Coomassie staining of a 3-15% polyacrylamide gel loaded with purified laminin from wild type embryoid bodies. The band between 97kD and 116kD is a degradation product. (B) Immunoblot analysis with laminin-1 antibody under reducing conditions identifies laminin from nidogen-1 knockout embryoid bodies. The laminin-1 antibody reacts with the laminin α1, β1, γ1 chains and nidogen-1.

2.11. A stepwise extraction of laminin from wild type and nidogen-1 knockout embryoid bodies (at 7 days of growth)

An equal wet weight of both wild type and nidogen-1 null embryoid bodies were subjected to a two step extraction procedure. The first step involved a brief homogenisation of embryoid bodies in tris-buffered saline (TBS) to disrupt the structure to allow easier extraction and to remove loosely associated proteins, which are mainly of serum and intracellular origin. In the second step, the embryoid bodies were supplemented with 10 mM EDTA and extracted for about two hours, to release laminin held in the Ca^{2+} dependent network of the basement membrane. Equal concentrations of extracted protein were loaded on to a 4-10% SDS-polyacrylamide gel, blotted on to a nitrocellulose membrane and probed with anti-laminin-1 antibody. The initial TBS wash extracts from nidogen-1 null embryoid bodies (figure 2.11B) contained a significant quantity of laminin when compared to wild type

embryoid bodies (figure 2.11A). This indicates a change in the extractability of laminin in the nidogen-1 null embryoid bodies. In the absence of nidogen, laminin seems to be detached more easily from the basement membrane, the signal being thereby picked up in the TBS-wash extract. In the EDTA extract, less laminin is seen in the nidogen-1 null embryoid bodies (figure 2.11D) when compared to wild type embryoid bodies (figure 2.11C). Total protein extracts were made by extracting embryoid bodies in 0.05 M Tris buffer, pH 7.5, containing in addition, 10 mM EDTA and 1% Triton. Laminin expression did not seem to be altered in these extracts in nidogen-1 knockout embryoid bodies (figure 2.11F) when compared to the wild type (figure 2.11E)

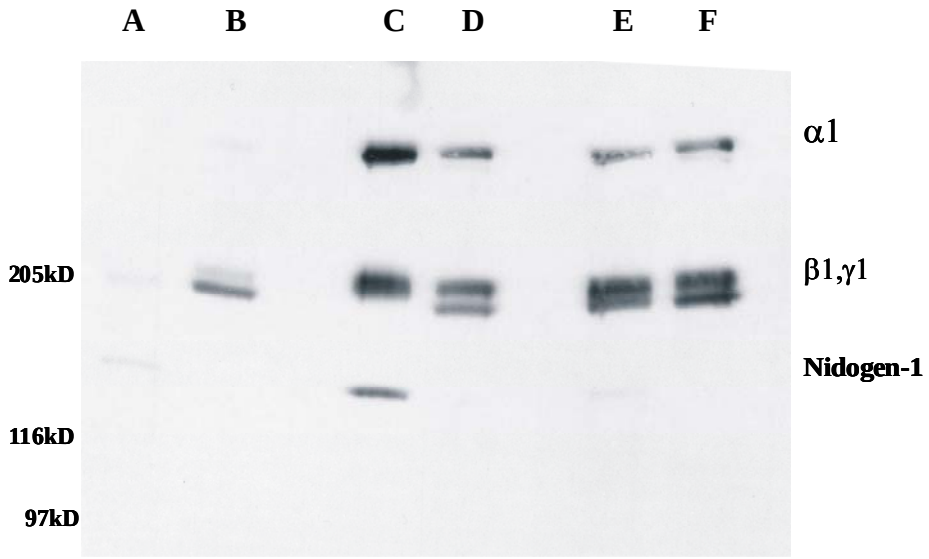


Figure 2.11: Analysis of extractability of laminin from embryoid bodies. Immunoblotting for laminin-1 in TBS extracts (A and B), EDTA extracts (C and D) and total protein extracts (E and F) from embryoid bodies. A, C and E are extracts from wild type embryoid bodies and B, D and F are extracts from nidogen-1 knockout embryoid bodies. Equal loading for each tissue extract was controlled by ponceau staining.

The next question addressed was whether these changes in extractability of laminin in nidogen-1 null embryoid bodies could be rescued in any way. ES cells offer an excellent system as basement membrane deposition can be experimentally manipulated by the addition of exogenous proteins (Murray and Edgar, 2000). To achieve this, both nidogen-1 and nidogen-2 proteins were recombinantly expressed in human embryonic kidney 293 cells.

2.12. Cloning of nidogen-1 and nidogen-2

Full length mouse nidogen-1 and human nidogen-2 cDNAs, lacking the native signal peptide sequence, cloned in bluescript vector (kindly provided by Nischt R, Dept. of Dermatology) were re-cloned into the NheI-NotI site of pCEP-Pu (figure 2.12). The human CMV enhancer-promoter was used to drive the expression of the recombinant proteins. To enable secretion of the expressed proteins into the extracellular space and their detection and purification, sequences encoding the BM-40 signal peptide was placed downstream of the promoter and fused in frame with a histidine tag (Yurchenco et al., 1997; Mayer et al., 1993). To aid cloning, the cDNA was amplified by PCR and a 5' NheI and a 3' NotI site introduced into the sequence. However, sequencing of the construct indicated an error at position 2663 where the amino acid histidine had been mutated to arginine. To remove this error, the original mouse nidogen-1 cDNA in bluescript was restriction digested with BamHI (position 1004) and StuI (position 2924). This new fragment was inserted into the new mouse nidogen-1 construct in pCEP-Pu. All inserts and borders were verified by sequencing.

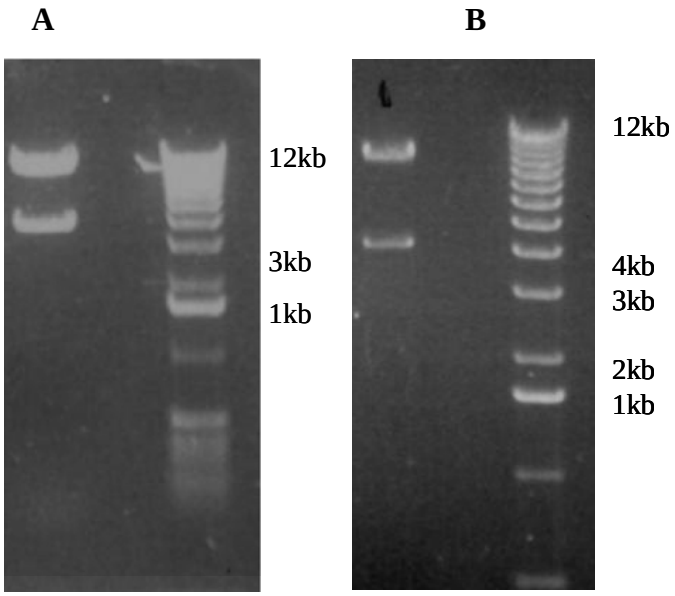


Figure 2.12: Restriction digest of both nidogen constructs in pCEP-Pu. (A) NheI/NotI restriction yields two fragments, the empty vector pCEP-Pu of 12kb and mouse nidogen-1 of 3.9kb, separated on a 0.7% agarose gel. (B) NheI/NotI restriction yields a 4.8kb fragment of human nidogen-2 along with that of pCEP-Pu.

2.13. Expression of nidogen-1 and 2 in 293-EBNA cells

EBNA–293 cells were transfected by electroporation with mouse nidogen-1 and human nidogen-2 constructs and selected for puromycin resistance. Serum-free supernatant was collected, TCA precipitated and tested for expression of the recombinant protein. Cells expressing both constructs were then grown on a large scale. The proteins were secreted at significant levels (0.5 kg/l) into serum free culture medium and were purified by chelating affinity chromatography (Talon metal affinity resin, Clontech). Nidogen-1 could be eluted with a linear gradient of 0-250 mM imidazole and nidogen-2 between 2.5-250 mM imidazole.

Electrophoretic analysis of purified nidogen-1 showed a band of 150kD (figure 2.13A) while the apparent molecular mass of nidogen-2 was 200kD (figure 2.13B).

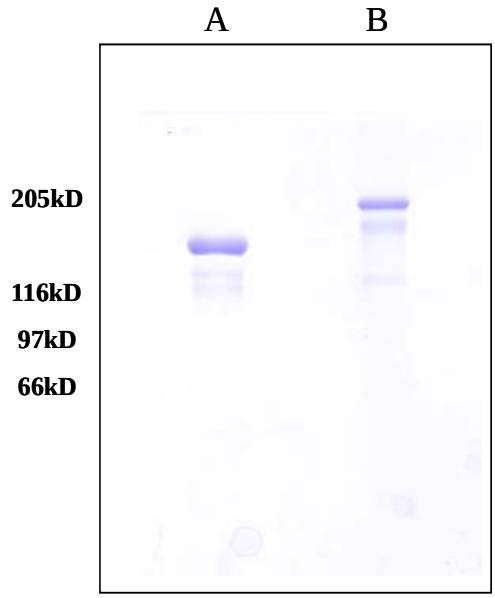


Figure 2.13: Expression of both nidogen constructs in 293-EBNA cells. Coomassie staining of (A) mouse nidogen-1 and (B) human nidogen-2 on a 8% polyacrylamide gel. The moderate degradation observed is characteristic for preparations of the highly protease – sensitive nidogens.

2.14. Rescue of extractability of laminin in nidogen-1 null embryoid bodies

To test whether the extractability of laminin in nidogen-1 null embryoid bodies could be rescued by exogenous addition of recombinant proteins, nidogen 1 -/- ES cells were

incubated separately with recombinant mouse nidogen-1, and human nidogen-2 proteins (20 μg/ml), added to the culture media. Wild type ES cells were grown as control. A complete rescue of laminin in nidogen-1 null embryoid bodies was seen by immunoblotting, within four to six days after addition of recombinant mouse nidogen-1 and a partial rescue was observed with human nidogen-2 (figure 2.14). This indicates that nidogen-1 and in its absence nidogen-2 can modulate laminin deposition in embryoid bodies.

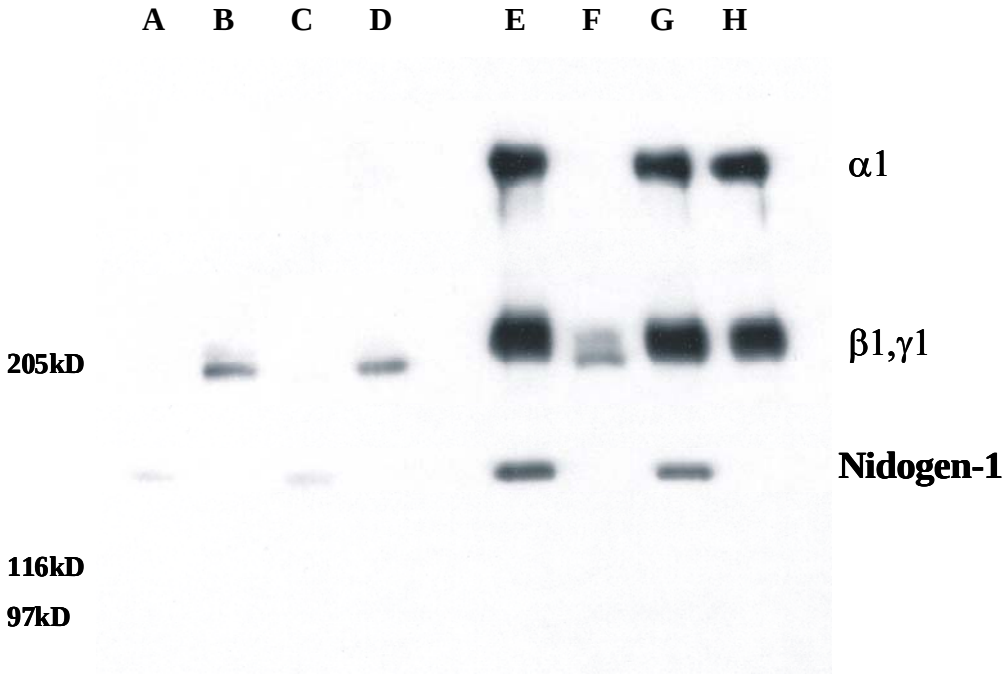


Figure 2.14: Rescue of laminin in nidogen-1 knockout embryoid bodies by exogenous addition of nidogen proteins. Immunoblot analysis of laminin-1 from TBS wash extracts (A-D) and EDTA extracts (E-H). A and E are wild type embryoid body extracts, B and F are nidogen-1 knockout embryoid body extracts, C and G are extracts from nidogen-1 knockout embryoid bodies grown in the presence of recombinant nidogen-1 and D and H are extracts from nidogen-1 knockout embryoid bodies grown with addition of recombinant nidogen-2. Equal loading for each tissue extract was controlled by ponceau staining. The laminin-1 antibody reacts with the laminin α1, β1, γ1 chains and nidogen-1.

2.15. Analysis of laminin from wild type and nidogen-1 knockout embryoid bodies at later stages of growth

As embryoid bodies were grown for a longer period of time, a change in kinetics of laminin synthesis was observed in the nidogen-1 knockout. Laminin was still released into the TBS wash extract of nidogen-1 knockout embryoid bodies. However, the yield of laminin in

the EDTA extracts increased significantly in nidogen-1 knockout embryoid bodies as they grew up to 14 days (figure 2.15). Total protein extracts from nidogen-1 knockout embryoid bodies also indicated higher expression levels of laminin when compared to wild type.

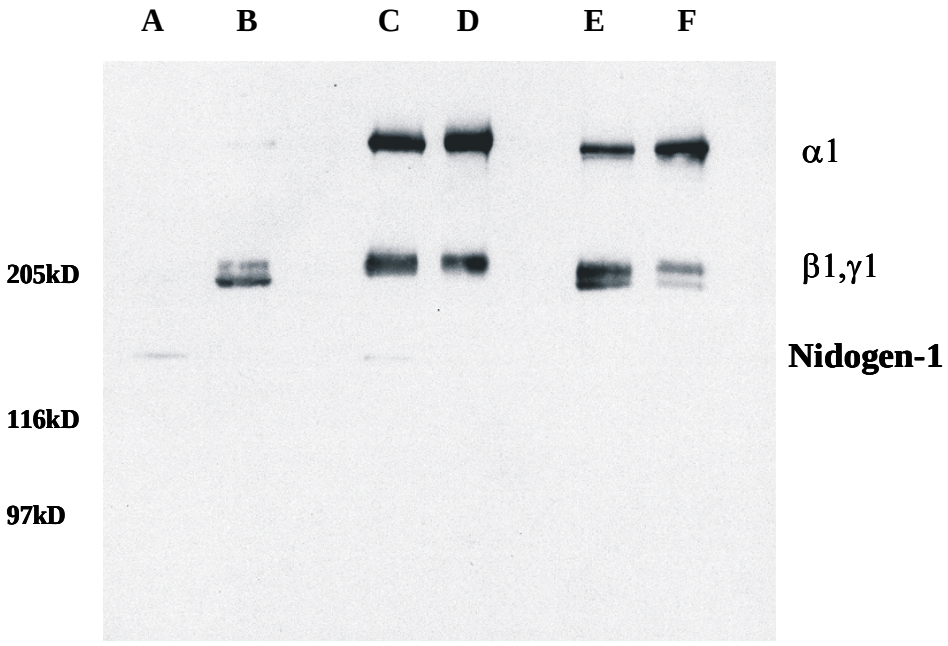


Figure 2.15: Analysis of laminin yield from embryoid bodies at 14 days of growth. Immunoblotting for laminin-1 on TBS wash extracts (A and B), EDTA extracts (C and D) and total protein extracts (E and F) from embryoid bodies. A, C and E are wild type embryoid bodies and B, D and F are nidogen-1 knockout embryoid bodies. Equal loading for each tissue extract was controlled by ponceau staining.

2.16. A stepwise extraction of laminin from mouse heart

The results obtained with embryoid bodies prompted an analysis of tissue extracts from wild type and nidogen-1 null mice. Heart tissue was chosen for the experiment as it is known to be a rich source of laminin. Further, it has a particularly high level of nidogen-1 when compared to nidogen-2. In a similar experiment as performed with embryoid bodies, both wild type and nidogen-1 null heart tissues were subject to the two step extraction procedure - the first short TBS wash, the second longer EDTA extraction. Once again, the results obtained by immunoblotting showed a similar alteration as in embryoid bodies and reconfirmed the biochemical changes in laminin extractability and yield in the nidogen-1 null

animals (figure 2.16). Heart laminin contains the α2β1γ1, α2β2γ1, α4β1γ1 and α4β2γ1 isoforms and not the α1β1γ1 isoform found in EHS tumour which is restricted to epithelial structures during development (Leivo and Engvall, 1988; Paulsson et al., 1991; Frieser et al., 1997; Timpl et al., 1979). Hence, the laminin-1 (α1β1γ1) antibody picks up a signal only for the β1, γ1 chains and nidogen-1.

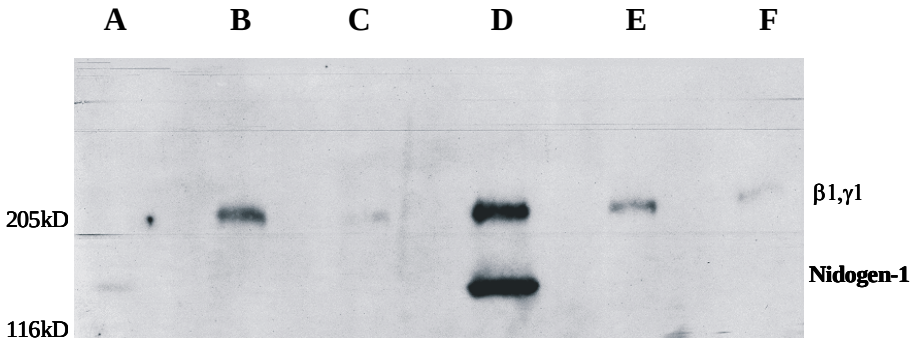


Figure 2.16: Immunoblot analysis of heart tissue extracts from -/- animals and their +/+ littermates. A, B and C are TBS extracts and D, E and F are EDTA extracts, resolved on a 4-10% polyacrylamide gel under reducing conditions. A and D are extracts from wild type mice, B and E are extracts from N1- -/N2 + + mice and C and F are extracts from N1 - -/N2 + - mice. Equal loading for each tissue extract was controlled by ponceau staining.

2.17. Extraction of mouse heart with 6M Guanidinium HCl (Gu HCl)

The next question addressed was whether, in addition to the changes in extractability of laminin in both nidogen knockouts, there was a change in expression or deposition of laminin in these mice. To test this, equal wet weight heart tissue from wild type, N1 - -/ N2 + + and N1 - -/N2 + - mice were subject to a harsh extraction with 6M Gu HCl to extract total laminin. The net yield of laminin decreased progressively in both nidogen knockouts when compared to the wild type by immunoblotting (figure 2.17).

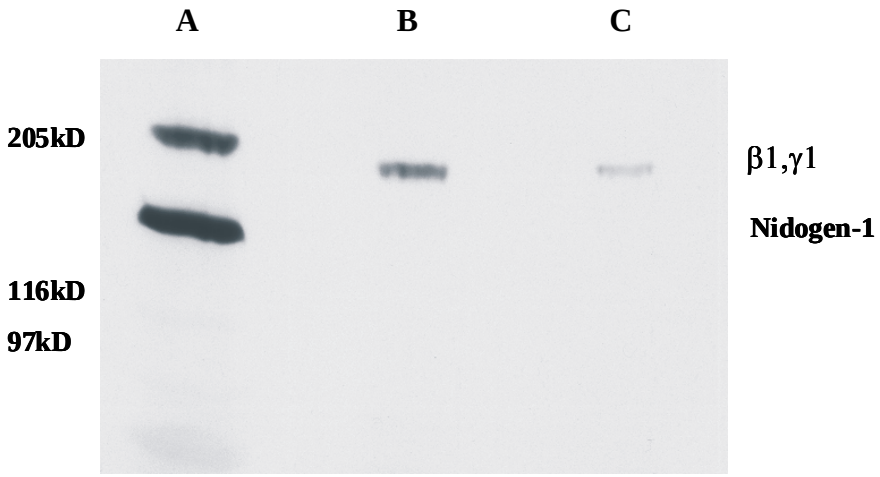


Figure 2.17: Extraction of total laminin from heart tissue. Heart tissues from wild type mice (A), N1- -/N2 + - mice (B) and N1 - -/N2 + - mice were extracted with 6 M Gu HCl and run on a 4-10% polyacrylamide gel under reducing conditions. Equal loading for each tissue extract was controlled by ponceau staining.

2.18. Analysis of β1 integrin

Cell adhesion to laminin is mediated by members of the β1 integrin family (Condic and Letourneau, 1997). These receptors signal the presence of laminin on the cell surface to the inside of the cell, thereby initiating cellular responses. As previous studies report that β1 integrin null embryoid bodies fail to develop basement membranes and that α1 laminin expression is down regulated (Fassler et al., 1995; Aumailley et al., 2000), it follows that they are essential receptors for basement membrane assembly (Lohikangas et al., 2001; Raghavan et al., 2000). β1 integrin is a predominant β subunit expressed in the heart (Keller et al., 2001), and its spatial expression during cardiac morphogenesis suggests that it has a role in the stable interaction of cardiac muscle cells with laminin (Fassler et al., 1996). Hence heart tissues as well as embryoid bodies were used for β1 integrin expression analysis. An equal wet weight of embryoid bodies and heart tissues from wild type and nidogen-1 null mice were extracted with a cocktail mix containing Tris buffered saline, EDTA, SDS and detergent, electrophoresed, blotted and probed with a monoclonal antibody that recognises the C-terminal cytoplasmic domain of β1-integrin. No significant differences were observed in β1 integrin expression in nidogen-1 knockout embryoid bodies or heart tissues when compared to the wild type (figure 2.18). Hence changes in laminin expression and extractability in N1 - -/ N2 + + and N1 - -/N2 + - mice did not initiate changes in receptor expression.

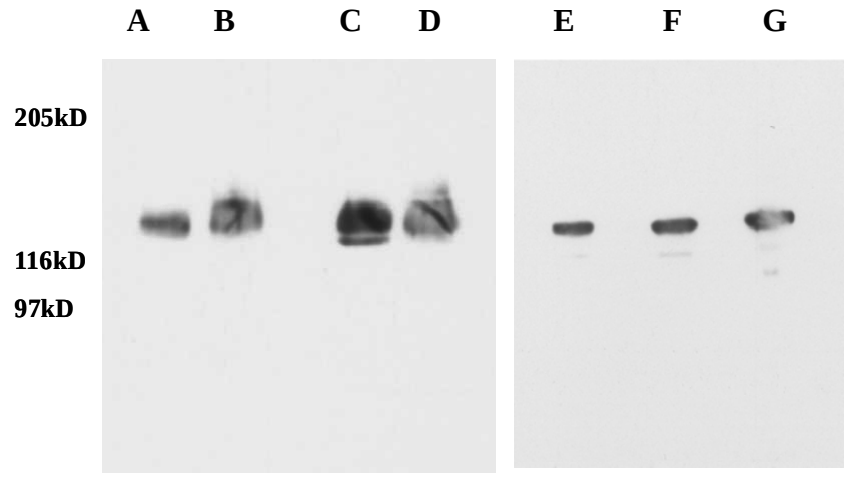


Figure 2.18: Immunoblot analysis of total protein extracts from embryoid bodies and heart tissues of nidogen-1 knockout and wild type animals. A (+/+) and B (-/-) are embryoid body extracts at 7 days of growth, C (+/+) and D (-/-) are embryoid body extracts at 14 days of growth. E, F and G are total protein extracts from wild type, N1 - -/N2 + + and N1 - -/N2 + - mice respectively. Equal loading for each tissue extract was controlled by ponceau staining.

2.19. Analysis of RNA in embryoid bodies and tissues

To understand whether there were changes in β1 integrin and laminin expression occurring at a transcriptional level, with a possible higher protein turnover, RT-PCR and northern blots were performed. The mRNA levels of α1 and β1 chains of laminin were comparable in wild type and nidogen-1 null embryoid bodies and tissues as quantified by RT-PCR (figure 2.19.1). Only a 20% down regulation of β1 integrin and the γ1 chain of laminin was observed in nidogen-1 null embryoid bodies and heart tissues when compared to the wild type in northern blots, as quantified by densitometry (figure 2.19.2).

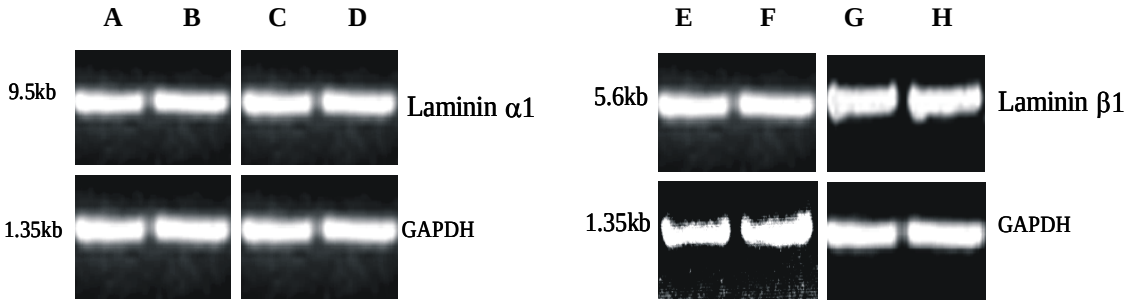


Figure 2.19.1: Analysis of mRNA levels of laminin α1 and β1 chains. Total RNAwas prepared from embryoid bodies (A, B, E and F) and heart tissues (C, D, G and H), reverse transcribed with primers for laminin α1 and β1

and separated on a 1% agarose gel. A, C, E and G are wild type embryoid body levels and B, D, F and H are from nidogen-1 null aggregates. GAPDH levels showed that comparable amounts of RNA had been analysed.

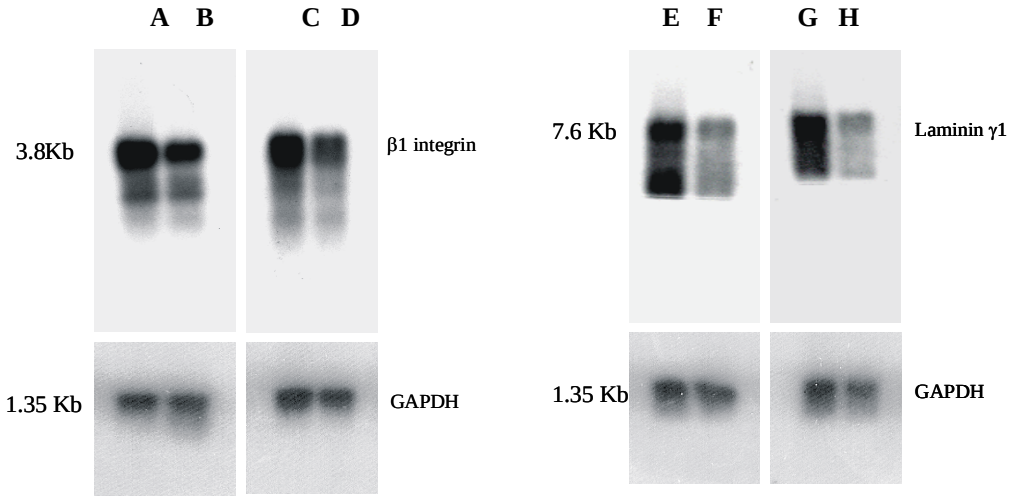


Figure 2.19.2: Analysis of mRNA levels of β1 integrin.and laminin γ1. Poly A RNA was prepared from embryoid bodies (A, B, E and F) and heart tissues (C, D, G and H), separated on a 1% agarose gel and hybridised with a cDNA probe for β1 integrin and laminin γ1. A, C, E and G being mRNA from wild type embryoid bodies and B, D, F and H being nidogen-1 knockout embryoid bodies. Probing for GAPDH message showed that comparable amounts of poly A RNA had been loaded.

3. Discussion

Neuro – physiological characterization of the nidogen – deficient mice

The knockout of nidogen-1 is the first report of the removal of a basement membrane gene that results in epilepsy. Among the several causes of epilepsy, the one linking the basement membrane and epilepsy is the blood brain barrier. In the cerebral microvasculature, the basement membrane surrounds the endothelium and adjacent pericytes. The composition and structure of the basement membrane affects the permeability of the vessel. Unique capillary endothelial cell structure, tight junctions between cells, collagen containing basement membrane and associations with astrocyte foot processes are important for the blood brain barrier (Rubin et al., 1991). The function of astrocytes involve cell development, tight junction modulation and phagocytosis; without astrocyte projections on the basement membrane, the blood brain barrier would fail resulting in epilepsy (Arthur et al., 1987; Janzer et al., 1987). However, initial experiments have indicated that there is no leakage in the blood brain barrier in the nidogen-1 knockout mice (Smyth, personal communication).

It is essential to understand the origin and nature of the 'epileptic' focus, for further understanding of the cascade of events that have undergone a change in the absence of nidogen-1. Such neurobiological changes that have been investigated through behavioural, *in vivo* and *in vitro* studies have been dicussed below.

3.1. Behavioural Study

Many mouse models of epilepsy show motor abnormalities. For example, the 'tottering', 'stargazer' and 'lethargic' mice have aberrant motor manifestations in addition to spontaneous seizures (Frankel, 1999). Motor behaviour is the final common behavioural output. A large number of genes, working at many sites, influence motor behaviour. A defect in a gene mediating a single aspect of the neuronal cell body, axon, dendrite, neurotransmitter, receptor, transducer, muscle or skeleton may impair motor functions. Motor co-ordination as well as motor learning are thought to require functional integration of frontoparietal and motor cortex, cerebellum, and striatal circuitry (Carter et al., 1999). The loss of motor coordination is therefore a common characteristic of many neurological disorders. Motor co-ordination

and balance are measured by perfomance on the rotarod (Jones and Roberts, 1968). Both nidogen knockouts showed significant impairment in motor perfomance when compared to the wild type. Whether this defect involves the central or peripheral nervous system or both, cannot be deduced from this test alone. Sciatic nerve from nidogen-1 - -/nidogen-2 + - mice however, showed disruption in the basement membrane and perturberances in myelination when observed by electron microscopy (Miosge, personal communication). An important role for the single isoform nidogen in *C. elegans* has been recently reported in the formation of the neuromuscular junction. Both nidogen and collagen type XVIII are associated with axons and particularly enriched near synaptic contacts. Collagen type XVIII has been shown to affect cell motility and axon guidance via the NC1/endostatin domains (Ackley et al., 2001). Nidogen and collagen type XVIII mutants exhibit movement defects and altered responses to acetylcholinesterase inhibitor, indicating changes in synaptic function (Ackley et al., 2003). Recently, the conditional knockout of laminin γ1 in Schwann cells, the myelinating cell in the peripheral nervous system has been described. The mice exhibit motor defects leading to hind leg paralysis and tremor. Without laminin γ1, Schwann cells were incapable of differentiating into a myelinating phenotype, and therefore unable to sort and myelinate axons. Sciatic nerve regeneration after injury was impaired as well (Chen and Strickland, 2003). Taking into consideration, all these changes, it is necessary to further analyse this functional defect observed in the nidogen knockout animals.

3.2. *In vivo* and *in vitro* studies

3.2.1. Epileptiform activity

In vivo recordings were done to examine the sporadic seizure-like movements in the nidogen knockouts. The activity level of the brain can be gauged by the electroencephalogram (EEG). In the EEG, both non-specific changes such as general alterations of the EEG frequency, and specific ones such as spikes and sharp waves indicative of epileptic activity can be discerned. In both nidogen knockouts, short (<500 ms), recurrent negativities with superimposed high-frequency spikes could be observed, reminiscent of series of interictal spikes with additional high-frequency components (ususally not visible in the EEG due to low-pass filtering in conventional recordings). Such spikes are important diagnostic features of epilepsy (Dichter and Ayala, 1987). In this sense, the discharges found in the experiments suggest that the knockout mice are indeed epileptic. Whereas it is generally considered that

the origin of an epileptic seizure, i.e. the focus, arises from the sites where spikes or sharp waves in the EEG originate, the epileptic focus need not be localized superficially in neocortical areas as suggested by the epicortical recordings. The relationship between spike discharges and seizure onset is not a clear-cut one, in that interictal spikes may be generated in areas not responsible for seizure onset (Spencer et al., 1992, Spencer et al., 1999; Dichter and Ayala, 1987), or indeed in that spikes can be the result of projected activity whose focus is inaccessible to the EEG recording. The use of depth electrode recordings could help clarify this issue.

It is interesting to note, however, that both behavioural and electrographic findings similar to the ones observed in the present study were seen in animal models with hippocampal seizures, indicating that, in these nidogen knockouts, the hippocampus may be involved. For instance, in the stargazer mice, hippocampal network hyperexcitability has been found along with normal neuronal intrinsic properties (Buchhalter, 1993). The El mouse, which is a model of focal epilepsy shows hippocampal abnormalities (King and LaMotte, 1989) and mutations of the E6-Ap ubiquitin ligase gene, a mouse model of Angelman's syndrome, produced mice with poor perfomance on the rotarod and impaired synaptic function in the hippocampus (Jiang et al., 1998). In models with hippocampal seizures, activity is often thought to invade the dentate gyrus and propagate via the trisynaptic circuit to CA3 and CA1, and finally to extrahippocampal areas, or to originate within the hippocampus proper (Scharfman et al., 2002). *In vitro* hippocampal slice recordings were therefore performed for a careful investigation of this particular area.

Spontaneous epileptiform activity was observed in both nidogen knockouts in CA3 region of the hippocampus and the dentate gyrus. Spontaneous activity has been described in brain slices from epileptic patients, demonstrating that the general ability of such tissue to produce spontaneous field potentials is not lost *in vitro* (Kohling et al., 1998; Cohen et al., 2002). As spontaneous field potentials generally displayed pointed peaks and lasted for less than 200 ms, they can, in principle be described as sharp waves on the EEG (International Federation of Society for Electroencephalography and Clinical Neurophysiology, 1974). Though they do not resemble typical epileptiform field potentials elicited in *in vitro* models, they are definitely indicative of epileptic activity. For example, they reflect activity surrounding an epileptic focus and an increased synchronization of neural networks (Kohling et al., 1998). In patients with temporal lobe epilepsy, such discharges seems to be generated

by a minority of subicular neurons including interneurons and a subset of pyramidal cells. Both glutamatergic and GABAergic signalling are involved in causing such activity (Cohen et al., 2002). Calcium currents have also been reported to play a crucial role in the generation of spontaneous activity (Kohling et al., 1994). Pharmacological manipulations suggest that CA3 possess's different mechanisms for the generation of such activity which depends on gap junctions, presumably in conjunction with recurrent excitation (Traub et al., 1994). Moreover, the ability of CA3 pyramidal cells to generate intrinsic bursts may support epileptiform activity in this region (Miles et al., 1991). Burst-firing pyramidal cells that are spontaneously active serve as pacemakers for the rest of the neuronal population, bursters and no-bursters alike. Therefore such neurons should discharge prior to the general neuronal population (Yaari and Beck, 2002). In the dentate gyrus, spontaneous activity can be associated with alterations in glutamate transporters (Gorter et al., 2002). Spontaneous activity is usually monosynaptic in origin, causing a relatively limited spillover of transmitter to extra-synaptic receptors or neighbouring post-synaptic sites (Dalby and Mody, 2003). Taking these observations together, the next question addressed was whether in addition to epileptiform activity, there would be changes in synaptic plasticity in the neocortex or hippocampus of the nidogen knockouts.

3.2.2. Synaptic plasticity

The hippocampus is connected directly and indirectly, in both afferent and efferent directions (cortical and subcortical) with various other parts of the brain and can thus be considered the centre of a more extended system. Information, after being processed in the neocortex is finally channelled into the core of the limbic system, the hippocampus and the amygdala. The messages to the hippocampus are funnelled through the subiculum and the entorhinal cortex which are known as the gate keepers of the hippocampus (Amaral, 1987), (as shown in figure 1.12). The entorhinal cortex also receives important connections from the amygdala (Aggleton, 1986). Both subiculum and entorhinal cortex are also the mediators of hippocampal outputs to the neocortex and many subcortical regions. Signals enter the entorhinal cortex almost exclusively through layers II and III from where mainly through the perforant path, they enter the trisynaptic loop with relays in the dentate gyrus, hippocampal sectors CA3, CA2 and CA1 and subiculum before returning to the entorhinal cortex (Amaral, 1987).

Changes in synaptic plasticity can be seen in both nidogen knockouts as observations are made from the neocortex, through the dentate gyrus to the CA1 region of the hippocampus. Input-output relationships in the nidogen-1 - -/nidogen-2 + + mice were significantly lower than that of the nidogen-1 - -/nidogen-2 + - mice and wild type. Even the nidogen-1 - -/nidogen-2 + - mice showed a decreasing trend, suggesting low excitability in the neocortex. However, a change in the pattern was observed as recordings were made in the dentate gyrus where input-output relationships in the nidogen-1 - -/nidogen-2 + - mice were increased, indicating an increasing trend towards excitability. In nidogen-1 - -/nidogen-2 + + mice, the reduction in excitability as observed in the neocortex continued to occur. Differences in cell density or density of synapses might account for these contrasting observations in the dentate gyrus between both nidogen knockouts. Moving on to the CA1 region, evoked epileptiform field potentials were observed in two out of six slices from the nidogen-1 - -/nidogen-2 + - animals and in the remaining slices, a significant increase in input-output relationships occurred, indicating that this area was hyperexcitable in both nidogen knockouts. Schaffer collateral stimluation evoked discharges in CA1 closely mimicked the spontaneously occuring discharges in CA3 in relative latency. Events evoked by stimulation can be polysynaptic and may be due to the simultaneous activation of neighbouring release sites, causing considerable spill-over, activating extra-synaptic receptors or neighbouring synapses (Dalby and Mody, 2003). Such evoked epileptiform responses can be produced by e.g. gradually blocking $GABA_A$ and $GABA_B$ receptors, suggesting that impaired $GABA_A$ and $GABA_B$ receptor-mediated inhibition of pyramidal cells (Steffensen & Henriksen, 1991) might contribute to these changes. The hyperexcitability in CA1 region of both nidogen knockouts also suggests that this region might have an increased cell density, an increased density of synapses and that individual synapses in a population are of altered strength (Usdin et al., 1999).

The next examined factor was paired pulse plasticity. This is of functional interest as it decides the short-term computational properties of the synapse. In its simplest form, this plasticity can be seen as paired pulse facilatation (PPF) or paired pulse depression in which the second afferent stimulation produces more/less synaptic action than the first one (Katz and Miledi, 1968; Thomson, 2000). Mechanisms both on the presynaptic and postsynaptic side, may contribute to paired pulse plasticity (Wang and Kelly, 1997). Two factors decide initial release probability: the number of immediately releasable vesicles (preprimed pool) and the vesicle release probability (Pves). Depletion and replenishment of

the pre-primed pool, an alteration in Pves and densensitization of post-synaptic receptors can contribute to paired pulse plasticity (Hanse and Gustafsson, 2001). A significant increase in paired pulse ratio has been reported in some cases of brain tissue surgically removed from patients with epilepsy, of genetic models of epilepsy and of chemical or stimulation induced epilepsy in animals (Fueta et al., 2002). 'Weak' synapses with high failure rates exhibit paired pulse facilitation whereas 'strong synapses' with low failure rates express a reduction of the response to the second stimulus ie. paired pulse depression.

In the neocortex, paired pulse ratio was decreased in both nidogen knockouts correlating with the observations in the input-output relationships and confirming the low excitability of synapses in this area. This result suggests that the epileptiform activity seen on the EEG of both nidogen knockouts probably came from the hippocampus. Severely decreased paired pulse ratio might have several reasons like changes in calcium handling mechanisms of the pre-synaptic terminal, alterations in cycling of synaptic vesicles to the release site, increase in synaptic inhibition of interneurons etc. (Usdin et al., 1999). In the dentate gyrus, significantly increased paired pulse facilitation was observed in the nidogen-1 - -/nidogen-2 + - animals, keeping with the earlier observed increasing trend in input-output relationships. A decrease in GABA receptor activation and an increase in NMDA receptor activation have been reported to increase the paired pulse ratio in the dentate gyrus (Fueta et al., 2002). In fact, a decrease in GABAergic inhibition has been suggested as a powerful hypothesis explaining hyperexcitability in epilepsy (De Deyn et al., 1990). Paired pulse ratio in nidogen-1 - -/nidogen-2 + + mice was comparable to the wild type. In these animals, it is possible that synaptic and extra-synaptic NMDA receptors are differentially regulated. The dentate gyrus has been reported to defend itself against hyperexcitability during kindling for eg. by lowering the initially increased density of postsynaptic NMDA receptors (Kamphuis et al., 1995). However, the CA1 region showed opposite results between the two nidogen knockouts to the dentate gyrus in paired pulse profiles. Here the nidogen-1 - -/nidogen-2 + + mice showed increased paired pulse facilitation while there was no difference in the nidogen-1 - -/nidogen-2 + - animals when compared to the wild type. A number of possible mechanisms might underlie the lack of change in plasticity in the more severe nidogen-1 - -/nidogen-2 + - animals in this area. Seizures often activate homeostatic mechanisms that dampen tissue excitability and help to terminate the seizure. Less neurotransmitter might be released from activated axons. Extracellular calcium concentrations decrease during seizure

activity (Heinemann et al., 1977; Stringer and Lothman, 1989) and reducing extracellular calcium concentration impairs synaptic transmission (Dingledine and Somjen 1981).

Even though differences in short-term plasticity were observed between the two nidogen knockouts in the hippocampus, the effects on long term plasticity as analysed in the dentate gyrus was similar in both knockouts. LTP was significantly impaired in both nidogen knockouts when compared to the wild type. Impaired LTP in the denate gyrus has been reported in kindling and kainic acid evoked models of epilepsy (Cain, 1993; Lynch, 2000) and even in slices from epileptic patients (Beck, 2000). It would have been interesting to analyse hippocampal-dependent learning and memory tasks in the Morris water maze, but as the nidogen knockout animals have significant motor impairment, such behavioural studies cannot be done.

The lateral perforant path to the dentate gyrus displays both opioid-receptor-dependent and NMDA-receptor-independent LTP. The granule cells in the dentate gyrus receive complex excitatory and inhibitory synaptic inputs from various sources (Amaral and Witter, 1989). Hyperexcitability in the dentate gyrus can be regulated by κ opioid peptides like dynorphins.. Endogenous dynorphins present in dense-core vesicles can be released from granule cell dendrites after high-frequency synaptic activation (Drake et al., 1994). Dynorphin release is calcium-dependent and requires activation of L-type calcium channels (Simmons et al., 1995). The released peptides activate κ opioid receptors present on perforant path terminals and inhibit excitatory amino acid release, thereby inhibiting neurotransmission and LTP at this synapse (Simmons et al., 1994). κ opioids have been suggested to have therapeutic effects in certain forms of temporal lobe epilepsy (Terman et al., 2000).

The metabotropic glutamate receptor (mGlu) is implicated in LTP induction (Manzoni et al., 1994). Selective activation of either medial or lateral perforant pathways has revealed that micromolar concentrations of (L)-2-amino-4phosphonobutyric acid (L-AP4) reduces fEPSPs in lateral but not medial perforant path, suggesting involvement of group III (mGluR4) at the lateral perforant pathway. The effect of L-AP4 at the lateral perforant path synapse is mediated by a pre-synaptic mechanism, whereby glutamate release is reduced and hippocampal excitatory transmission is suppressed (Macek et al., 1996). As L-AP4 successfully inhibited LTP in wild type and knockout mice, it confirms an involvement of mGluR4 as well.

3.2.3. Structural changes after seizures

No obvious structural changes were observed in the hippocampus or neocortex of both nidogen knockouts so far. However, this is still under investigation in that whole brain sections are being examined for changes in cell number, layering defects etc. In many models of epilepsy, a variety of structural changes occur in the dentate gyrus, some of which involve the growth of new axon 'collaterals' or 'sprouting'. The most widely studied example is the sprouting of mossy fibres after seizures because the new collaterals can be extensive, and they can target a very specific area, the inner molecular layer of the dentate gyrus. Mossy fibre sprouting has been described after kainic acid administration, pilocarpine–induced seizures and kindling (Tauk and Nadler, 1986) and even in patients with human temporal lobe epilepsy (Sutula et al., 1989). However, Timm's staining showed no such structural differences in both nidogen knockouts. This is not a criterion in epileptic brains as many other animal and human models of epilepsy do not show these structural changes (Raol et al., 2003; Mathern et al., 1996; Ebert, 1995). Hence hippocampal cell loss and mossy fibre sprouting may occur after early-life status epilepticus but are not necessary prerequisites for epileptogenesis.

3.2.4. Expression of nidogen-1 and laminin γ1 in the hippocampus

The immunoflurorescence studies revealing nidogen-1 and laminin γ1 expression patterns in hippocampal slices need to be continued in whole brain sections. Until now it could be shown that nidogen-1 stains the walls of blood vessels in hippocampal slices. Nidogen-1 is produced by endothelial cells and mesenchymal cells of the developing nervous system. Astrocytes in culture have also been reported to synthesise nidogen-1 (Grimpe et al., 1999). A stronger nidogen-1 expression has been observed in immature than in adult brain, suggesting a role for nidogen-1 in developmental and reactive angiogenesis (Niquet and Represa, 1996). Nidogen-2 expression in endothelia is higher than that of nidogen-1 (Schymeinsky et al., 2002). It would be interesting to examine nidogen-2 staining in the hippocampus and neocortex of nidogen-1 null animals.

In the nervous system, laminin is mainly produced by Schwann cells (Cornbrooks et al., 1983), astroglia (Liesi, 1990), olfactory-ensheathing glia and neurons (Hagg et al., 1997). Basement membrane independent laminin γ1 chain has been reported to have an important

role in axon regeneration in the mossy fibre pathway in the hippocampus (Grimpe et al., 2002). A decreased expression of the laminin γ1 chain was observed in slice stainings of hippocampus from nidogen-1 knockout animals in the CA3 region and dentate gyrus. Both regions have shown spontaneous epileptiform activity. No differences were observed between wild type and nidogen-1 knockout in the CA1 region. The role of laminin in the development and function of synapses on hippocampal pyramidal neurons is now under investigation by other research groups where it has been shown that laminin may be involved with the regulation of LTP (Nakagami et al., 2000). In addition laminin has been shown to have prominent enhancing effects on the motility of dendritic spines (Siel,1998) for which the dynamic interactions with axonal growth cone filopodia may be essential during axonal outgrowth and synapse formation (Halfter, 1996, Prange and Murphy, 2001).

3.3. Conclusion

My results indicate the epileptic nature of the mice in the absence of nidogen-1. Epileptic activity seems to originate in the hippocampus as neocortical areas seem to be far less excitable. Alterations in synaptic plasticity and network function in the nidogen-1 null animals are indicative of a novel role for a nidogen-1, a protein found only in basement membranes. Synapse formation is a very complicated process that requires many dynamic interactions between neurons and target cells. The removal of nidogen-1 seems to have altered a pathway required for normal brain development. One of the molecules in the cascade affected seems to be laminin. Therefore, a biochemical study on laminin in the nidogen-1 null animals was carried out.

Biochemical consequences of nidogen depletion

As nidogen-1 interacts with all the important basement membrane components and mediates the connection between the two independently forming networks of collagen IV and laminin with high affinity (Fox et al., 1991), it was believed to have a structural function. Moreover, it is only when nidogen-1 becomes present that embryonic basement membranes have a fully developed ultrastructural architecture (Miosge et al., 2000). *In vitro* experiments in the presence of antibodies that interfered with the laminin-nidogen interaction perturbed basement membrane formation and inhibited epithelial development in kidney, salivary gland and lung organ cultures (Ekblom et al., 1994; Kadoya et al., 1997). Disruption of basement membranes by overexpression of the nidogen-binding site of laminin in F9 embryoid bodies (Tunggal et al., 2003) is also evidence of a role for nidogen in basement membrane organisation and assembly. The inactivation of the nidogen-1 gene in mice, however contradicted these earlier observations. The results suggested that nidogen-1 has a limited structural role in the basement membrane or that its absence is compensated for by nidogen-2 (Murshed et al., 2000). Even the nidogen binding site deletion (Willem et al., 2002) where basement membrane defects were confined to certain tissues indicated that the laminin-nidogen interaction is not needed for the formation of all basement membranes.

Information that specialised basement membranes may regulate nerve positioning came from *C. elegans* where the single nidogen gene was required for specific axons to switch from circumferential to longitudinal migration (Kim and Wadsworth, 2000). The results obtained from loss of function mutants in *C. elegans* have lead to the suggestion that nidogen or the nidogen-laminin complex might act directly as a ligand for axon-guidance receptors. Also, it might be possible that nidogen creates a different basement membrane configuration that allows new interactions between the migrating axon and the basement membrane of the body wall muscle in *C. elegans* (Kim and Wadsworth, 2000). Furthermore, with collagen VIII, nidogen has been shown to be important for the formation of the neuromuscular junction in nematodes (Ackley, 2003). This neurological phenotype was in correlation with the nidogen-1 knockout mice which show spontaneous seizure-like activity. The nidogen-1 animals were thus ideal for a careful study of basement membrane proteins interaction to understand the molecular mechanisms leading to the neurological defects.

During brain development, both neuronal migration and axon guidance are influenced by extracellular matrix molecules present in the environment of the migrating neuronal cell bodies and nerve fibres. Developing neurons preferentially attach to glial laminin. Since laminin is deposited along the radial glial fibers and along developing nerve pathways in a punctate form, the punctate assemblies may be one of the key factors in routing the developing neurons *in vivo* (Liesi, 1990). Proteolytic treatment of the basement membrane can cause growth cones to retract in the peripheral nervous system of grasshopper embryos (Condic and Bentley, 1989). Another indication of how ECM molecules can influence growing axons came from experiments on identified cells isolated from the leech central nervous system and grown in culture. Substrates that contained laminin not only supported rapid extension of neurites from leech neurons, but also influenced the pattern of neurite outgrowth and the distribution of calcium channels on the cells (Masuda-Nakagawa and Nicholls, 1991).

The major reason as to why biochemical studies on laminin have been extensively performed in cell culture or tumour systems is the insolubility of basement membranes. This problem was overcome by the use of denaturing solvents like guanidinium HCl (Ohno et al., 1986) and then later by extraction with EDTA (Paulsson et al., 1987). The protocol of extraction of laminin with EDTA was later applied to embryoid bodies and mouse heart, to solubilize laminin in a native form and well characterized. Similar steps were taken to characterize laminin from nidogen-1 null ES cells and heart tissue and compared to the wild type.

3.4. Analysis of laminin in ES cells

3.4.1. Purification of laminin

When cultured as aggregates, ES cell lines form embryoid bodies, structures that recapitulate early stages of implantation development, including the formation of endoderm on the surface of the inner cell mass (ICM), differentiation of columnar epithelium and formation of a central cavity (Martin et al., 1977; Martin, 1980). A biochemical basement membrane containing laminin-1, type IV collagen, nidogen and perlecan assembles (at 3-4 days) between the outer visceral endoderm and the inner columnar ectoderm (Li et al., 2002).

The appearance of extracellular laminin also coincides with α1 chain expression and a linear immunofluorescence staining (Aumailley and Smyth, 1998). Therefore, embryoid bodies are a perfect model for analysing the function of the laminin-nidogen interaction and the roles of nidogen. For their biochemical characterization, embryoid bodies were washed in Tris buffered saline, then extracted with EDTA-containing buffer followed by purification by gel filtration. As previously shown for the EHS tumour (Paulsson et al., 1987), chelating agents like EDTA allow the selective extraction of laminin from a variety of tissues (Paulsson and Saladin, 1989). It confirms that divalent cation-dependent interactions such as laminin self-aggregation (Yurchenco et al., 1985, Paulsson, 1988) is critical for anchorage of laminin in normal vertebrate tissues. Electron microscopy of purified laminin obtained from wild-type embryoid bodies have shown reasonable amounts of typical cross-shaped particles with indications of bound nidogen (Mayer et al., 1998). The efficient formation of the laminin-nidogen complex in wild-type extracts could be seen from the high and pure yield of laminin and nidogen as seen on a coomassie stained gel. The ratio of nidogen to laminin is close to the one in the EHS tumour (Paulsson et al., 1987), but due to the susceptibility of nidogen to proteolytic degradation, many laminin preparations contain sub-stoichiometric amounts of nidogen (Dziadek et al., 1985).

However, the yield of laminin alone from nidogen-1 null embryoid bodies was comparatively low and though undegraded, was detectable only by immunoblotting. Interestingly, immunoflurorescence studies on nidogen-deficient embryoid bodies had shown a linear deposition of laminin, nidogen-2 and collagen IV below the endodermal cell layer in an identical pattern as in wild type embryoid bodies typical of a forming basement membrane (Murshed, 2000). The method used was, however, non-quantitative and the embryoid bodies had been treated with paraformaldehyde for fixation, causing protein-crosslinking. So the drastic reduction in yield of laminin from nidogen-1 null embryoid bodies suggested that there might either be biochemical changes in the properties of laminin in the absence of nidogen, a down-regulation in laminin synthesis or that laminin had been lost out at some stage during culture, extraction or purification. The cell-spin system used to culture embryoid bodies required about 250-300 ml of culture medium. Both ELISA and immunoblot analysis of culture medium failed to show significant amounts of laminin within the medium. A careful biochemical approach to analyse the extractability of basement membrane proteins from wild type and nidogen-1 null embryoid bodies was then undertaken.

3.4.2. A stepwise extraction of laminin

Wild type and nidogen-1 null embryoid bodies were maintained under similar conditions in the cell spin system for 7 days and showed similar size and shape by light microscopy. The conventional hanging drop technique which had been used to study wild type and nidogen-1 null embryoid body sections (Murshed, 2000) at 8 days had yielded identical staining patterns with TROMA-1, indicating that endodermal cell differentiation is not hampered by the absence of nidogen-1. The first Tris-buffer saline 'wash' extract removed loosely associated proteins. However, significant quantities of laminin was released into the wash extracts of the nidogen-1 null embryoid bodies indicating that in the absence of nidogen, though basement membranes form, their stability is decreased. Binding of laminin to other basement membrane proteins is weaker and hence it is easily detached from the basement membrane. The complex interactions between the individual components, laminin, collagen IV, perlecan and nidogen, result in the specialized network of the basement membrane that serves as both a structural barrier and as a substrate for cellular interactions (Erickson and Couchman, 2000). The genetic inactivation of most of the major components of the basement membrane have given evidence that each of the proteins perform specific functions. Allmost all mutations interfere with basement membrane integrity, resulting in either major embryonic defects or else appearing later during development or adulthood. For instance, perlecan-null mice develop normally before they die of cardiac defects at 10.5 dpc as a result of mechanical stress induced instability in basement membranes (Costell et al., 1999). Hence, interactions between basement membrane molecules have a unique significance of their own. The discovery that nidogen-1 can mediate the formation of ternary complexes between laminin and collagen IV *in vitro* (Fox et al., 1991), had lead to the initial hypothesis that it was crucial for basement membrane assembly. Though the redundancy of nidogen isoforms disproved this hypothesis (Murshed et al., 2000), yet the results of the experiments present here brings to light the fact that interactions between basement membrane components are indeed strengthened in the presence of nidogen-1. When the laminin-nidogen interaction was blocked in F9 embryoid bodies, a re-distribution of nidogen-1 was noticed which was more significant than actual loss from the embryoid bodies. This data suggests that laminin-free nidogen-1 might be able to interact with other binding partners using an independent site (Tunggal et al., 2003). It would be interesting to analyse whether other basement membrane proteins are also released into the wash extract as easily as laminin in nidogen-1 null embryoid bodies. Also, this result brings forth the question as to whether

basement membranes in the nidogen-1 knockout mice would be able to withstand intense physical activity or stress or if during a period of mechanical stress, a breakup of basement membranes and release of laminin can be prevented by the presence of nidogen-2.

In the EDTA extracts, distinctly less laminin was observed in nidogen-1 null embryoid bodies as a significant amount had already been lost into the wash extract. This explained the reason for the inability to purify enough laminin from the EDTA extracts of nidogen-1 null embryoid bodies. Indeed, the results of these experiments bring further questions about the role Ca^{2+} ions play in arranging the configuration of basement membranes. It is known that binding of laminin to cells is insufficient for generation of a differentiating signal during early embryogenesis. Laminin molecules have to self-assemble to form a nascent basement membrane (Cheng et al., 1997, Li et al., 2003). Ca^{2+} ions are a requirement for this self-assembly into a polymer which occurs by the formation of a heterotrimeric laminin-N terminal complex by the reversible, cooperative interaction of the $\alpha\beta\gamma$ arms (Yurchenco et al., 1985; Paulsson, 1988; Yurchenco and Cheng, 1993). When laminin γ1-null embryoid bodies were treated with laminin-1, basement membranes formed, but when treated with polymer inhibiting fragments, they did not, although laminin was seen as punctate staining on the cell surface. Hence, laminin polymerisation is crucial for basement membrane formation and its downstream developmental consequences (Li et al., 2002). The observations that EDTA is not required for extraction of part of the laminin in nidogen-1 null embryoid bodies suggests that not merely the laminin, but the laminin-nidogen complex is important for polymerisation in response to Ca^{2+} ions. There is strong evidence which indicates that the G3 domain at the carboxyl end of nidogen-1 interacts with laminin leaving the N-terminus region free to interact with other molecules (Mann et al., 1988; Chakravarti et al., 1990; Takagi et al., 2003). The N-terminal segment of nidogen-1 (residues 15-26 and 250-261) and two of the EGF-like repeat units contain potential Ca^{2+} binding sequences (Durkin et al., 1988). The Ca^{2+} binding properties of nidogen-1 were demonstrated with peptide sequences made from its N-terminal region as well as with the intact molecule (Yurchenco, 1985; Paulsson, 1988). Overall, these results suggest that the heterotrimeric concept of laminin N-terminal polymeristion may be more complicated, requiring other interactions and other molecules to form a stable network.

As single step total protein extraction, did not show any difference in laminin expression in both wild type and nidogen-1 null embryoid bodies, it indicated that production of laminin had not been altered in the absence of nidogen. At the mRNA level, only a 20%

downregulation of laminin γ1 was observed in the nidogen-1 null embryoid bodies. No changes were observed in the laminin α and β chains. This indicates that the changes at the protein level are more severe than at the mRNA level.

3.4.3. Rescue of extractability of laminin in nidogen-1 null embryoid bodies

As embryoid bodies can incorporate exogenously added proteins, they can be successfully manipulated to study deposition and organisation of basement membrane proteins in their extracellular matrix (Murray and Edgar, 2000). Embryoid bodies were cultured in bacterial plates instead of the cell spin system which would otherwise require an extremely high quantity of recombinant proteins. The α1 chain is missing in the EDTA extract of the nidogen-1 null embryoid bodies. Probably, nidogen-1 null embryoid bodies behave differently in bacterial dishes when compared to the cell spin system which is more ideal for growth and differentiation. An alternative α chain might have been secreted which is not detectable by the laminin-1 antibody. The presence of an α chain is of importance as it drives the secretion of the laminin heterotrimer (Yurchenco et al., 1997), so it is assumed that some α isoform is expressed. It might also be that the secreted α chain is not well stabilized and is lost into the culture media. However, addition of recombinant nidogen proteins is able to rescue α1 chain expression as well.

Addition of recombinant nidogen-1 to nidogen-1 null embryoid bodies prevented the release of laminin into TBS extracts. The yield of laminin in EDTA extracts increased dramatically to match control levels indicating a rescue that is complete. Interestingly, it has been reported that the attachment of cells to nidogen was in part mediated by the integrin RGD peptide sequence located in one of the EGF-type repeats (Chakravarti et al., 1990). An ECM protein thrombospondin can bind Ca^{2+} ions by its RGD sequences (Lawler et al., 1988). Binding of calcium at the RGD site of nidogen might be involved in stabilizing the RGD site in a cell surface receptor compatible conformation as suggested for thrombospondin (Lawler et al., 1988). Addition of recombinant nidogen-2 also increased the yield of laminin in EDTA extracts though some of it was still released into the wash indicating that the rescue was only partial. This might be due to that recombinant human nidogen-2 had been used in the experiment. Human nidogen-2 binds with 50-100 fold lesser affinity than nidogen-1 to the mouse laminin γ1 chain (Schymeinsky et al., 2002). However, this experiment proves that

even with the far lower binding affinity human nidogen-2 can still affect the interactions of proteins in the basement membrane and improve the extractability of laminin in nidogen-1 null embryoid bodies. The expression levels of nidogen-2 in nidogen-1 null embryoid bodies had been analysed only by immunofluorescence where a linear deposition of the protein was seen below the endodermal cell layer (Murshed, 2000). The current characterization of the domain structure of mouse nidogen-2, its binding to other basement components and cells, and the expression studies emphasize the similarities of the mouse nidogens (Salmivirta et al., 2002). In the perturbation experiment where the nidogen-binding site was overexpressed in F9 embryoid bodies, an irregular deposition of laminin and both nidogens were seen, revealing the presence of laminin molecules not bound by either nidogen-1 or 2 (Tunggal et al., 2003). Exogenously added nidogen-2 to nidogen-1 null embryoid bodies seems to behave in almost the same way as nidogen-1. Two Ca^{2+} binding consensus sequences have been described in EGF modules of the rod domain of nidogen-2 (Kohfeldt et al., 1998). The cell attachment and Ca^{2+} binding sequences in nidogen-2 provide the basis for defining further biological functions of nidogen-2 and the importance of calcium mediated laminin polymerisation for organisation of basement membranes. There is data suggesting that nidogen-2 can also bind to laminin-1 through at least a second epitope not related to γ1III4 which means that the affinity of these additional interactions must be examined and precisely mapped to individual modules of laminin-1 (Kohfeldt et al., 1998).

3.4.4. Extraction of embryoid bodies at 14 and 21 days of growth

In reports on embryoid body studies, experiments are performed on cultures within 12 days of growth (Tunggal et al., 2003; Murray and Edgar, 2000; Li et al., 2002) indicating that they are best studied at early stages of differentiation. But when nidogen-1 null embryoid bodies were maintained in culture for 14 and 21 days, an increase in laminin synthesis was observed. Though a significant quantity of laminin was still released into the TBS extracts of nidogen-1 null embryoid bodies, the yield of laminin in EDTA extracts increased as well.

Cells in aggregate in embryoid bodies change protein expression involved in basement membrane formation and epithelial polarity formation during differentiation (Grover and Adamson, 1985). The processes of morphological and functional differentiation are intimately related at the single cell level (Folkman and Moscona, 1978; Roskelley et al., 1994;

Schischmanoff et al., 1997). In the cell spin sytem, embryoid bodies are under continuous stress and agitation. Since nidogen-1 knockout embryoid bodies have unstable basement membranes as shown in earlier experiments, they possibly require 3 dimensional cell-cell interactions for growth. Hence, in suspension cultures, they undergo morphological changes. Indeed, the embryoid bodies from nidogen-1 -/- cells exhibit altered growth characteristics upon long tissue culture. Alternative approaches that might reduce such changes would be to culture these cells in a monolayer on an artificial substrate such as microcarrier beads or a microporous membrane (Miki, 1999). The only disadvantage would be that these cultures would involve muliple unknown factors which might make it difficult to interpret results.

However, the ready leakage of laminin into the wash extract maintains the fact that the basement membrane in nidogen-1 null embryoid bodies remains unstable even at later stages of growth. It would be interesting to see if exogenously added recombinant nidogen can rescue the changes in morphology observed in these embryoid bodies at such late stages. In laminin-γ1-null and β1-integrin-null embryoid bodies, the absence of basement membrane formation was accompanied by a significant decrease in nidogen and collagen IV expression. Both proteins, however accumulated in the conditioned media, suggesting that inability to form laminin polymers resulted in a failure to sequester nidogen and collagen IV into the basemnt membrane (Li et al., 2002). Exogenous addition of laminin-1 to laminin-γ1-null embryoid bodies resulted in a rescue of formation of basement membranes, epiblast differentiation and cavitation (Murray and Edgar, 2000). Exogenous laminin-1 was also able to restore basement formation and epiblast development in β1-integrin-null embryoid bodies indicating that the failure of laminin α1 chain expression had caused the early differentiation block (Li et al., 2002; Aumailley et al., 2000). Furthermore, as the data is suggestive of an integrin-laminin crosstalk for the assembly of basement membrane components, it became necessary to analyse β1-integrin expression in nidogen-1 null embryoid bodies, specially in the light of earlier observations about the changes in extractability of laminin.

3.5. Analysis of mouse heart laminin

To correlate the biochemical changes in laminin observed in nidogen-1 null embryoid bodies with that of adult mice, further investigations were carried out. Mouse heart was chosen as the favourable tissue for the following reasons. Laminin isolation from mouse heart

resulted in a high yield and has been well characterized (Paulsson et al., 1989). Heart tissue also contains less proteases, than in other tissues, for instance, in lung (Paulsson et al., 1991). Both nidogen-1- -/nidogen-2 + + and nidogen-1 - -/nidogen-2 + - mice were analysed. Laminin was released into the wash extracts in both nidogen knockouts. Laminin yield was lowered considerably in the EDTA extracts from the nidogen-1- -/nidogen-2 + - mice. This result indicates that when only one allele of nidogen-2 is left, then though basement membranes form, they are highly unstable and that there is a change in laminin expression in these mice. It would be worthwhile analysing ES cells from these mice. ES cells can be isolated from 3.5 day p.c expanded blastocysts or delayed blastocysts collected 4-6 days after ovariectomy. Tissue culture on these blastocysts give rise to ES cell colonies that can be subcultured to give permanent cell lines (Hogan et al., 1994).

6M Gu HCl is a denaturing agent that efficiently extracts laminin. Extraction with 6M Gu HCl is a better way of confirming that most of the laminin has been extracted rather than with the protein extraction buffer, consisting of 0.05 M Tris buffer pH 7.4 with 10 mM EDTA and 1% triton. A progressive decrease in laminin yield was observed from the nidogen-1- -/nidogen-2 + + mice to the nidogen-1- -/nidogen-2 + - mice indicating a decrease in laminin expression that increased with the more severely affected animal. The changes in extractability and yield of laminin in the nidogen-1 knockout mice resemble that seen earlier in embryoid bodies. Though both nidogens are ubiquitous components of adult mouse basement membranes, radioimmunoassays had revealed that the contents of nidogen-2 were distinctly lower than those of nidogen-1 in all tissues (Kohfeldt et al., 1998). But in nidogen-1 null animals, a 3.4-fold increase of nidogen-2 in the heart, was demonstrated by radioimmunoassays (Miosge et al., 2002), that confirmed the immunofluorescence observations indicating an upregulation of nidogen-2 in heart muscle (Murshed et al., 2000). The increased nidogen-2 levels in the nidogen-1 null heart, is therefore not able to fully rescue the biochemical changes observed, suggesting that basement membrane stability is higher in the presence of nidogen-1. In the nidogen-binding site knockout, nidogen-1 is poorely retained in majority of basement membrane structures, although the protein level determined was normal. No changes were observed in nidogen-2, laminin γ1, collagen IV and perlecan expression in these mice (Willem et al., 2002). This indicates that the laminin-nidogen-1 interaction is crucial for nidogen-1's integration into basement membranes. This phenotype in the nidogen-binding site knockout is to be considered with that observed in the nidogen-1 null mice where laminin is weakly integrated into basement membranes in the absence of nidogen-

1. A regulatory function for nidogen-1 has been earlier reported in mammary epithelial cells. Nidogen-1 secreted by mesenchymal and myoepithelial cells was shown to be incorporated into basement membrane-like structures in epithelial and mesenchymal co-cultures to ensure optimal laminin-1 mediated signals for mammary specific gene expression (Pujuguet et al., 2000). This suggests that the ability of nidogen-1 to allow basement membrane ternary complexes might have physiological consequences in specific tissues.

3.6. Analysis of β1 integrin

The deletion of β1 integrins or of dystroglycan in mice resulted in pre-implantation lethality and the embryos showed a defective morphogenesis of the endoderm or of the Reichert's membrane respectively, indicating that these receptors are required at the same developmental stage when basement membrane appears (Fassler and Meyer, 1995; Stephens et al., 1995; Williamson et al., 1997). In β1 integrin deficient embryoid bodies, laminin-1 was not secreted in the extracellular space due to a rapid switch off of laminin α1 chain synthesis which normally drives the secretion of laminin heterotrimers (Aumailley et al., 2000, Li et al., 2002). These results demonstrate that the initiation of basement membrane formation requires synthesis, secretion and deposition of laminin-1 and that the process is driven by integrins of the β1 family. However, in the absence of nidogen-1, though there is an instabilty in basement membranes and changes in extractability and yield of laminin, yet no changes in β1 integrin was noticed either in embryoid bodies or in heart tissue. This result shows that the absence of nidogen-1, despite changes induced in the basement membrane has no effect on the expression or stability of β1 integrins. In β1 integrin deficient embryoid bodies too, with the exception of nidogen secretion, all other events were absent (Aumailley et al., 2000). Dystroglycan expression has not yet been checked in the nidogen-1 null mice. However, there is data indicating that β1 integrin function is required after dystroglycan in the cell-surface-mediated laminin assembly process (Henry et al., 2001). However, this is an area open for study in the nidogen-1 null tissue. Exogenous laminin was able to rescue the defects in laminin γ1-null and β1 integrin-null differentiating embryoid bodies. This suggests that laminin secretion and polymeristion is the essential pre-requisite for the differentiation processes absent in β1 integrin-null mice and ES cells (Li et al., 2002). And since laminin assembly does occur in nidogen-1 null animals, β1 integrin expression is unaltered, even if the interaction between basement membrane components are weaker. It will be of interest to

study mice carrying the total knockout of both nidogens, where the new-born mice have severe cardio-myopathy (Smyth, personal communication). In these mice, alterations in β1 integrin expression may occur.

3.7. Conclusion

My results indicate that athough nidogen-1 is not important for basement membrane formation in most tissues, it is definitely required for its stability. Nidogen-2 compensates to some degree for the absence of nidogen-1 and aids in basement membrane assembly. Though it has an overlapping binding repertoire to nidogen-1, yet it does not seem to have an identical binding pattern. Hence, interactions between basement membrane components are not as strong as in the presence of nidogen-1. Instability in basement membranes is increased in mice lacking both nidogen-1 alleles and heterozygous of nidogen-2. Therefore, nidogen-1 does have an important role in maintaining the structural integrity of basement membranes.

3.8. Perspectives

Several factors may contribute to the propensity for the developing brain to have seizures and develop epilepsy. The relative hyperexcitabilty of the immature nervous system may contribute to the processes that create the substrate for enduring seizure disorders. The immature brain is prone to develop circuits that support neuronal hypersynchrony because of interneuronal connections at synaptic and nonsynaptic junction that the adult brain lacks (Johnston et al., 1996). The presence of seizures can be observed very early in nidogen-1 null animals. It would be interesting to make a careful study of the expression of both nidogens and laminin all through nervous sytem development in both wild type and nidogen-1 animals.

No morphological changes were observed in brain preparations from nidogen-1 knockout mice. However, there might be subtle changes in neuronal migration which is common in many cases of epilepsy. The formation of the cerebral cortex occurs in 3 major developmental stages (1) proliferation, (2) migration and (3) differentiation. Neural precursors proliferate in the ventricular zone and post-mitotic neurons migrate to establish the mature layered cortex. The cortical plate is eventually filled with subsequent waves of cells migrating past the earlier cells in an ‘inside-out’ pattern (Rakic, 1974, 1990). Migration occurs radially along glial guides as well as independent of radial glia in a tangential pattern parallel to the pail surface or laterally within the ventricular (Allen and Walsch, 1999). Although, the cellular events leading to the development of the mature cerebral cortex is well described, little is known about the biochemical and molecular signals involved. Brains of young post-natal and adult nidogen knockout mice should therefore be analysed with migration markers. The finding that there are changes in laminin expression in both nidogen knockouts, enhances the chances of migratory defects occuring in these mice.

As astrocytes have their end feet on basement membranes in capillaries in the brain, which are rich in nidogens, another area of study comes into focus. The thin lamina between the hippocampal hilus and granule cell layer, is an area of active proliferation within the adult hippocampus known to generate new neurons throughout adult life. Dividing cells are found in dense clusters associated with the vasculature. Most of the newborn endothelial cells disappear over several weeks, suggesting that neurogenesis is intimately associated with a process of active vascular recruitment and subsequent remodeling. My biochemical studies indicate an instability of basement membranes in the absence of nidogen-1. Thinning of

basement membranes in capillaries in the brain of the nidogen-1 null animals have also been reported (Dong et al., 2002). Whether this has a direct or indirect relevance in causing epilepsy is necessary to be studied.

4. Materials and Methods

4.1. EEG

The mice were anesthetised as follows. A mixture containing 2.5 ml of ketamine (10%) + 2 ml of Xylazin (2%) + 9.5 ml of sterile NaCl solution was prepared. 125μl of this mixture was injected i.p/20 g of body weight. A short cutaneous incision was made over the skull. The stereotactic coordinates were 2 mm bregma and 2 mm left and right of sagittal. The tips of silver electrodes were placed within the epidural space on the surface of the cerebral cortex and were secured with cement and glue. The skin incisions over the skull were closed with careful sutures. Brain electrical potentials were recorded continuously throughout the day. Spiking activity in the EEG was confirmed as not to be movement artefacts.

4.2. Hippocampal slice preparation and chemicals

Mice were anesthetised with ketamine/xylazin mixture, perfused with cold artificial cerebrospinal fluid (ACSF) and decapitated. The brain was gently removed and dipped in cold ACSF (pH 7.4) saturated with an O_2/CO_2 mixture (95%:5%). The composition of the ACSF in mM was: NaCl, 124; KCl, 3; NaH_2PO_4,1.25; $CaCl_2$, 1.6; $MgSO_4$, 1.8; $NaHCO_3$, 26; and D-glucose, 10. Coronal slices of 400 μm thickness were cut in cold ACSF with a vibrating microtome (Leica). All slices containing hippocampi were saved which yielded five to seven slices per brain. Slices were then transferred to a holding chamber containing oxygenated ACSF, gradually brought to 32°C, and allowed to equilibrate under these conditions for at least one hour prior to recording. For the LTP experiments, the ACSF was supplemented with the drug L-AP 4 (10 μM).

4.3. Stimulation and recordings

For electrophysiological recording, a slice was gently transferred to a nylon mesh in an interface–type chamber having a humidified gas atmosphere, and perfused at a rate of 1-2 ml/min with ACSF (32°C). The stimulating glass electrode, filled with ACSF, enclosed

a well chlorinated silver wire and the recording electrode consisted of a borosilicate glass electrode (1 MΩ) filled with extracellular solution. For all experiments, the recording electrode was placed at an optimal distance of 250-300 μm from the stimulating electrode. The initial depth of the electrodes was 150-200 μm below the surface of the slice, however fine adjustments were made in the depths of both stimulating and recording electrodes to optimise the responses.

A single presynaptic stimulus can gives rise to a complex waveform (figure 1) comprising of:

A) the stimulus artefact
B) the presynaptic volley (the action potential evoked in the presynaptic fibres)
C) the population excitatory post synaptic response (negative-going in extracellular DC recordings)
D) a positive-going population spike, if the evoked excitatory post-synaptic potential is sufficiently large to reach threshold for action potential initiation in postsynaptic neurons.

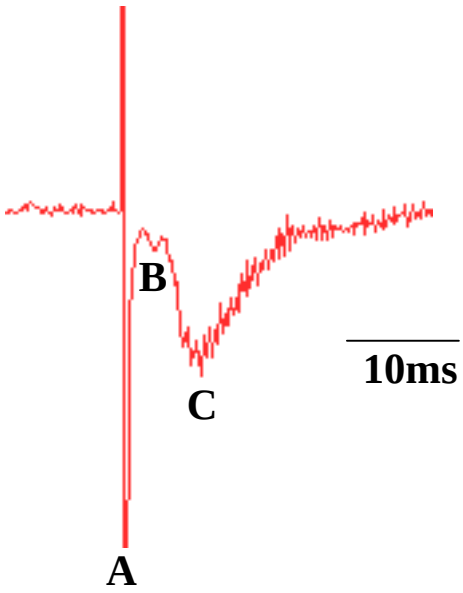


Figure 1: Extracellular recording of the synaptic response recorded from CA1 hippocampal pyramidal neurons. A) stimulus artefact, B) presynaptic volley C) excitatory postsynaptic response, normally 1-2 mV in amplitude.

An input-output function was generated for each slice by varying stimulus intensity in ten equal steps from 0.1 mA to 1 mA. This range of stimulus intensity was sufficient to cover the response range from just-threshold to maximum response in all slices reported herein. In paired pulse experiments, pairs of identical pulses were delivered at stimulus intensity when the amplitude of the fEPSP was half maximal. The ten values of interpulse interval that were tested for each slice were 10, 20, 30, 40, 50, 100, 200, 300, 400 and 500 msec For the LTP experiment, tetanic stimulation consisted of ten stimulus trains of 20 impulses at 100 Hz (interpulse interval 10s). The stimulus intensity was adjusted to double the baseline value.

4.4. Analysis

To study the input-output curve, a mean of the amplitude of each fEPSP was taken. To analyse paired pulse responses, calculation of the paired-pulse ratio (PPR) was done as follows:

PPR of fEPSP = 2nd fEPSP slope / 1st fEPSP slope.

For LTP analysis, a mean of 10 fEPSP slopes from 10 responses corresponded to each data point on the graph.

Statistical significance was evaluated by the Student's *t*-test and ANOVA for the difference between both nidogen knockouts and the control mice.

4.5. Timm's staining

Cryosectioned material was stained through a non-perfusion Timm's staining protocol. Slides were placed in a rack and put into a desiccation chamber overnight also containing a beaker with 100 ml of a 0.1% Na_2S solution that had been adjusted to pH 7.3 with 1.75 ml of 1N HCl to form H_2S gas. The following day, the slides were rinsed in 95% EtOH for 15 minutes, then 2 minutes each in 70% EtOH, 50% EtOH, & dH2O. Following rehydration, the slides were placed in the Timm's staining solution (30 ml Gum Arabic, 5 ml 2M Citrate Buffer, 15 ml 0.5 M Hydroquinone, and 0.25 ml $AgNO_3$) for approximately 3 hours, rinsed in running tap water for 10 minutes, and dipped into dH20 for 1 minute. They

were then placed in a 5% Sodium Thiosulfate solution (to stop the reaction, decrease background, and fix), rinsed again in dH2O, counterstained with Cresyl Violet, dehydrated & coverslipped with Permount.

4.6. Immunostaining

Hippocampal slices (300 μm) were fixed with 4% paraformaldehyde for 10 minutes and blocked with normal goat serum (NGS) in PBS for 30 minutes. The primary antibodies used were anti-rabbit nidogen-1 antibody (produced using the recombinant nidogen-1 described in 2.13) and anti-rabbit laminin γ1 antibody (a kind gift of Uwe Odenthal, University of Cologne) at 1:4000 dilution. After 4 hours of incubation with the primary antibody and 4 washes with blocking solution, the slices were incubated with secondary antibody for 1 hour and then washed and mounted in fluorescent mounting medium (DAKO). Immunoreactivity was detected with a Cy3-conjugated goat anti-rabbit IgG antiserum (Jackson Immuno Research Laboratories).

4.7. Confocal Microscopy

Hippocampal slices (150 μm) were fixed with 4% paraformaldehyde for 10 minutes and blocked with normal goat serum (NGS) in PBS for 30 minutes. A co-staining was done with primary antibodies: anti-guinea pig nidogen-1 antibody (produced using the recombinant nidogen-1 described in 2.13) and anti-rabbit laminin γ1 antibody at 1:4000 dilution. After 4 hours of incubation with primary antibodies, 4 washes were made with blocking solution. The slices were incubated with Cy3 -conjugated goat anti-guinea-pig IgG antiserum (Jackson Immuno Research Laboratories) and Alexa 488 (1:1000, Molecular Probes) for 1 hour. Finally, the slices were washed thrice with 1x PBS and mounted with fluorescent mounting medium (DAKO). The immunoflurescence labelling was analysed by confocal laser-scanning microscopy (Leica, Germany).

4.8. Molecular Cloning

4.8.1. Bacterial Cell Culture

The bacterial strain DH5α was grown following the instructions of Sambrook et al. (1989). The method of Hanahan (1983) was applied for transformation and production of competent *E. coli* cells.

4.8.2. DNA preparation

Small amounts of plasmid DNA (10 μg) were isolated from 2 ml *E. coli* cultures following the protocol of Birnboim (1983). For production of up to 100 μg of plasmid DNA the Midi-Prep kit (Macherey and Nagel) was used. The DNA concentration was measured photometrically at 260 nm and calculated on the basis of the assumption that an optical density of 1 equals a concentration of 50 μg/ml double stranded DNA. DNA gel elctrophoresis with 1kb DNA ladder (GIBCO BRL^{TM}) was performed in 1x TAE buffer as described in Sambrook et al (1989).

4.8.3. Polymerase Chain Reaction (PCR)

Oligonucleotides were ordered at MWG-Biotech AG in highly purified salt free quality and primer concentration was determined photometrically at 260 nm assuming that an optical density of 1 equals 37 μg/ml single stranded DNA. Expand High Fidelity PCR System (Roche) was used for amplification of DNA fragments. Using the RoboCycler® Gradient 40 (Stratagene) the template DNA was denatured at 95°C for 2 minutes followed by 35 amplification cycles each comprising 1 minute incubation at 95°C, 1 minute at annealing temperature of 52°C and 2 minutes at 72°C. Per kb fragment length in an incubation time of 1 minute was calculated and the annealing temperature was defined by addition of 3°C to the lowest melting temperature $T_m = 4x(G+C) + 2x(A+T)$.

The sense primer used for the PCR on mouse nidogen-1 was CGA **GCT AG**C ACT GAA TCG CCA GGA GCT C and the antisense primer was ATT **GCG GCC GC**T CAT

TTC CGT TCA ATG CA containing a **NheI-NotI** site respectively. The sense primer for the PCR on human nidogen-2 was CGA **GCT AGC** ACT GCA CCC AGA CCG AGC TC and the antisense primer was ATT **GCG GCC GC**T TAC TTT CTT CCT GTT GG.

4.8.4. Recombinant techniques

All DNA modifying enzymes (e.g. restriction enzymes, T4 DNA ligase) were obtained from New England Biolabs and used following the manufacturers instructions. DNA sequences were determined in the service laboratory of the ZMMK (Center for Molecular Medicine Cologne) using ABI Prism^{TM} 377 DNA Sequencer (Applied Biosystems). Sequences were analysed using the Wisconsin Sequence Analysis Package, Version 8.1 (Genetics Computer Group, Inc.)

4.9. Culture and maintenance of tissue culture cell lines

4.9.1. ES cells and embryoid bodies

Wild-type and nidogen 1 -/- ES cells (Murshed et al., 2000) were cultured on mitomycin treated embryonic fibroblast feeder cells in gelatinised tissue culture dishes. The cells were maintained in knockout^{TM} SR medium (Life Technologies) supplemented with 15% (vol/vol) serum replacement, 0.1 mM β mercaptoethanol, 1 mM-L-glutamine, 1 mM penicillin, 0.1 mM non-essential aminoacids, 0.1 mM sodium pyruvate and 1000 U/ml of LIF (ESGRO; Gibco-BRL). ES cells were then trypsinised, depleted of fibroblasts by re-plating and allowing them to attach for 15 minutes and then transferred to the cell spin system (Integra Biosciences). After one day, when small aggregates had formed, more growth medium (DMEM, Gibco-BRL) supplemented with 10% FCS, 1 mM penicillin and 1 mM glutamine was added up to a volume of 250 ml and changed daily. In this system embryoid bodies are maintained for up to 21 days under continuous agitation by magnetic stirring which guarantees optimal nutrition and oxygen support.

For the rescue experiment, ES cells were trypsinised and replated into bacterial petridishes, under which conditions, the ES cells remained in suspension and formed

aggregates. 20 μg/ml of recombinant protein was added to the culture medium, the following day and replated every second day until harvesting.

4.9.2. Human embryonic kidney cell line

293-EBNA cells (Invitrogen) were cultured in DMEM-F12 containing 200 U/ml penicillin, 200 μg/ml streptomycin, 20 mM L-glutamine, 10% FCS and 50 μg/ml G418 (GIBCO BRL^{TM}) at 37°C in a humified incubator with a 5% CO_2 atmosphere. After transfection, to maintain expression in 293-EBNA cells transfected with both nidogen constructs, G418 was removed and the cells were treated with 0.5 μg/ml puromycin (Sigma-Aldrich).

4.9.3. Establishment of EBNA transfected cell lines

$1x10^6$ cells were electroporated with 5 μg circular plasmid DNA in 800 μl growth medium using the Gene Pulser ® II and 0.4 cm cuvettes (Biorad). Resistance and voltage were set to 500 μF and 230V, respectively and time constants of approximately 7 sec were obtained. Selection with puromycin was initiated 48 hours after electroporation.

4.9.4. Thawing and freezing cells

Frozen cells were quickly transferred from liquid nitrogen into a 37°C waterbath, thawed and washed with prewarmed growth medium and seeded on tissue culture plates (Greiner). For the purpose of freezing, cells were trypsinised, resuspended in growth medium supplemented with 20% fetal calf serum (GIBCO BRL^{TM}) and 10% DMSO (Sigma-Aldrich) and slowly cooled to -80°C. After incubation at -80°C, cells were transferred to liquid nitrogen for long term storage.

4.10. Purification of laminin from embryoid bodies

About 5000 embryoid bodies were homogenised in 10 volumes (1 ml/ 0.1g of wet weight) of 0.15 M NaCl, 0.05 M Tris/HCl pH 7.4, containing 2 mM Phenyl methane sulphonyl fluoride and 2 mM N-ethylmaleimide as protease inhibitors. After stirring in the cold room for 15 minutes, the insoluble material was removed by centrifugation (8000 rpm, 15 minutes) and the pellet homogenised in the same buffer which contained in addition, 10 mM EDTA. After extraction for 2 hours with stirring, the supernatant was clarified by centrifugation (10,000 rpm, 30 minutes). The EDTA extraction was repeated twice more. The combined EDTA extracts were concentrated to ¼ of its original volume by filtration through an Amicon ultra-filtration membrane (dia:44.5mm, NMLW-100,000). This sample was then passed over a sepharose CL-4B column (140 × 6 cm) equilibriated in the same buffer as above and eluted at the rate of 0.3 ml/min. All purification steps were done at 4°C.

Selected fractions were analysed by SDS-polyacrylamide gel electrophoresis, (Laemmli et al; 1970), on a 4-10% gradient gel. Laminin was detected with Coomassie Brilliant Blue R or by immunoblotting after transfer to nitrocellulose. The primary antibody was a polyclonal antiserum against EHS laminin-1 and the secondary antibody was horse radish peroxidase (HRP) conjugated immunoglobulin from swine anti-rabbit (P0399; DAKO).

4.11. Tissue extraction and antibodies used

For the laminin analysis from heart tissue, 0.1g of tissue was extracted with 1ml of 0.05 M Tris HCl, pH 7.4, 0.1 M NaCl, 2 mM PMSF and 2 mM NEM for 10 minutes at 4°C. The insouble material was removed by centrifugation (8000 rpm, 15 minutes), called the ‘wash’, and then the pellet was homogenised in the same buffer which contained in addition, 10 mM EDTA. After extraction for 2 hours with stirring, the supernatant was clarified by centrifugation (10,000 rpm, 30 minutes), (Paulsson et al.,1987).

For the total protein extracts, 0.1g heart tissue was extracted with 1ml of 0.05 M Tris HCl, pH 7.4, 0.1 M NaCl, 10 mM EDTA, 1% triton, 2 mM PMSF and 2 mM NEM for 2 hours at 4°C. For the Gu HCl extracts, 0.1g of tissue was extracted with 1 ml of 0.05 M Tris HCl, pH 7.4, 0.1 M NaCl, 6M Gu HCl, 2 mM PMSF and 2 mM NEM for 1 hour. The

supernatant was subject to an overnight ethanol precipitation. The protein content of the extracts were determined using the BCA protein assay (Biorad Laboratories). During immunoblotting of tissue extracts (10 μg), a polyclonal antibody against laminin-1 (1:1000) was used.

For the β1 integrin analysis, heart tissue and embryoid bodies were extracted with 0.05 M Tris HCl, pH 7.4, 0.1 M NaCl, 10mM EDTA, 2mM PMSF and 2mM NEM with the addition of 1% Nonidet P-40, 0.5% deoxycholate and 0.1% SDS. A rat monoclonal antiserum (no. 553715, BD Biosciences, Pharmingen) that recognises the cytoplasmic tail of β1 integrin was used.

4.12. RNA preparation, RT-PCR and northern blots

Total RNA was extracted from embryoid bodies and freshly isolated cardiac tissue with Trizol reagent (Life Technologies). The concentration was calculated from the optical density at 260 nm assuming that an OD value of 1 corresponds to 40 μg/ml RNA. For the RT-PCR, 1 μg of total RNA was reverse transcribed with AMV reverse transcriptase (Stratagene). For second strand synthesis, 2 μl of the reaction mixture was used in PCR reactions. After 35 cycles, PCR products were analysed on a 2% agarose gel. Control reactions were carried out without reverse transcription.

The primers used for RT-PCR are as follows:

1) Laminin α1 chain RT primer: 5′ TGC TTG AGT TCA ACA AAC TGC 3′.
2) Laminin α1 chain upstream primer: 5′ TTC TAA CAA TGA TAC TGT CG 3′.
3) Laminin α1 chain downstream primer: 5′ ATT GGC TAA ATC GGC ATG G 3′.
4) Laminin β1 chain RT primer: 5′ GTT ATT TGT AGA TGA CTG CC 3′.
5) Laminin β1 chain upstream primer: 5′ GTA ACT GCA AAT GAA CAT TCC 3′.
6) Laminin β1 chain downstream primer: 5′ CAA CCA TAC GGG TCT TCA GC 3′.
7) GAPDH RT primer. 5′ AGG GAG ATG CTC AGT GTT GG 3′.
8) GAPDH upstream primer: 5′ AAC GGA TTT GGC CGT ATT GG 3′.
9) GAPDH downstream primer: 5′ TTC AGC TCT GGG ATG ACC 3′.

Poly A plus RNA was then prepared with the Micro Poly (A) Purist kit (Ambion). 2 μg of Poly A plus RNA was run on a denaturing formaldehyde gel of 1% agarose and transferred on to a nylon membrane (Hybond N; Amersham). After baking for 2 hours at 80°C, to crosslink the RNA to the membrane, it was hybridised with a 50% formamide containing buffer at 42°C against cDNA probes for laminin γ1, β1 integrin and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNAs. DNA probes were labelled with [α^{32}P]dCTP (Hartmann Analytic) by random priming as reported by Feinberg and Vogelstein (1983) using the Ladderman^{TM} Labelling Kit (TaKaRa Shuzo Co.,Ltd.). After high stringency washing, the blots were exposed to an autoradiographic film.

The probe for the γ1 mRNA was a Bam H1-EcoR1 fragment between 2959 and 4163 in the protein coding area (Sasaki and Yamada, 1987). The β1 integrin probe recognises the transmembrane region (1896-2390 bp). The primer for reverse transcription was: 5′ CAG AAA TTC ACA TGC AAG G 3′. The upstream primer for PCR was 5′ ACA TGT CAG ACC TGC CTT GG 3′ and the downstream primer was 5′ CTC ATA CTT CGG ATT GAC 3′.

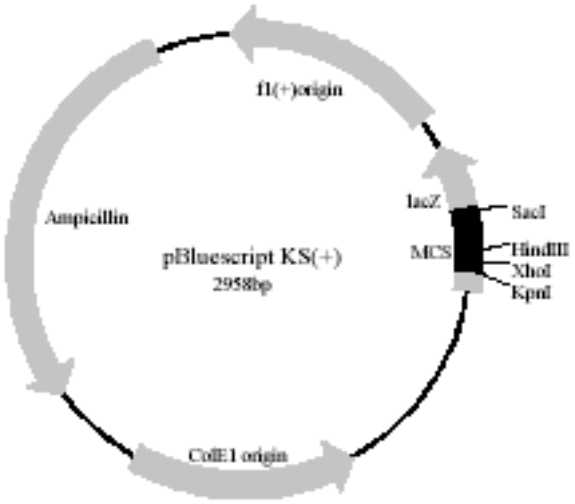
5. Appendix

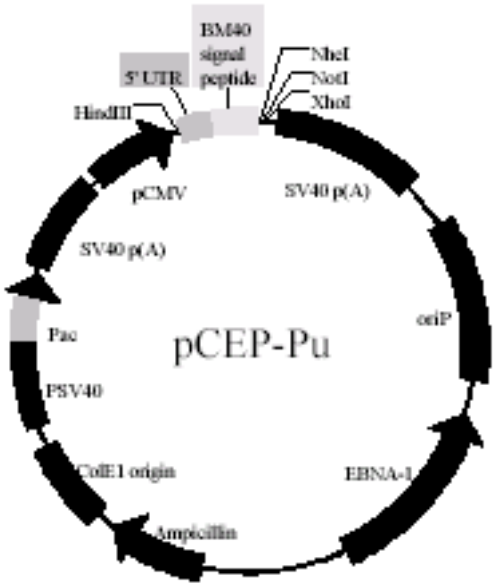
5.1. Abbreviations

ACSF	artificial cerebrospinal fluid
BM40	basement membrane protein 40kD
cDNA	complementary DNA
dCTP	deoxycytosintriphosphate
DMEM	Dulbecco’s modified Eagle medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNTP	deoxyribonucleictriphosphate
EBNA	Epstein Barr nuclear antigen
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
EGF	epidermal growth factor
EHS	Engelbreth-Holm-Swarm
FCS	fetal calf serum
G418	geneticin
GAPDH	glycerinaldehydephosphate dehydrogenase
HRP	horse radish peroxidase
kB	kilobases
kD	kilodalton
LE	laminin EGF-like
LTP	long term potentiation
MCS	multiple cloning site
mRNA	messenger RNA
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPD	paired pulse depression
PPF	paired pulse facilitation
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate

Taq	Thermophilus aquaticus
TBS	Tris buffered saline
TCA	trichloric acid
T_m	melting temperature

5.2. Vectors





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