Universität Hamburg

Synaptic plasticity in mice (*Mus musculus L.*, 1758) deficient in cell adhesion or extracellular matrix molecules: in vivo and in vitro electrophysiological analysis

Dissertation

zur Erlangung des naturwissenschaftlichen Doktorgrades des Fachbereichs Biologie der Fakultät für Mathematik, Informatik und Naturwissenschaften an der Universität Hamburg

vorgelegt von Luminita Stoenica

Hamburg, Februar 2008

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Genehmigt vom Department Biologie
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an der Universität Hamburg
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Weiterer Gutachter der Dissertation:
Herr Professor Dr. K. WIESE
Tag der Disputation: 25. April 2008

Hamburg, den 11. April 2008

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I. ABSTRACT

The extracellular matrix (ECM) is a complex network of macromolecules including glycoproteins, polysaccharides and proteoglycans. In the nervous system, cell adhesion molecules (CAMs) and ECM components mediate cell-cell and cell-matrix interactions, regulating cell migration, survival, differentiation, axonal pathfinding and synapse formation.

Previously published studies have revealed that recognition molecules, such as tenascin-R (TN-R), tenascin-C (TN-C) and polysialylated neural cell adhesion molecule (NCAM) are involved in cognitive functions and modulation of hippocampal synaptic transmission and plasticity. All these electrophysiological studies have been conducted *in vitro* and not verified *in vivo*. In order to fill the gap between *in vitro* recordings of synaptic plasticity and behavioral analysis, we recorded long-term potentiation (LTP) induced by theta-burst stimulation in anesthetized mice deficient in TN-C, TN-R or NCAM and its polysialylation enzymes, polysialytransferases ST8SiaII /STX and ST8SiaIV / PST. The recordings were performed at perforant path - dentate gyrus synapse since it is the most convenient for identification and induction of LTP *in vivo*.

The neural cell adhesion molecule, NCAM, plays important roles in synaptic plasticity in the CA1 and CA3 regions of the hippocampus *in vitro*: in CA1, LTP depends on polysialic acid (PSA) produced by the polysialtransferase ST8SiaIV / PST, mostly involved in polysialylation of NCAM in mature neurons. In CA3, mossy fiber LTP is not dependent on PSA. Here we report that basal synaptic transmission in the dentate gyrus, measured as the slope of field excitatory postsynaptic potentials, was reduced strongly in mice lacking ST8SiaII / STX, the enzyme involved in polysialylation of NCAM in immature granule cells. Mice deficient either in the NCAM glycoprotein or ST8SiaIV / PST had normal basal synaptic transmission. On the other hand, mice deficient in NCAM, although not in ST8SiaIV / PST or ST8SiaII / STX, were impaired in LTP induced by theta-burst stimulation, suggesting that LTP in the dentate gyrus depends on the NCAM glycoprotein alone rather than on its associated PSA. As also patterns of synaptic activity during and immediately after induction of

LTP were abnormal in NCAM deficient mice, it is likely that induction of LTP requires NCAM. Since there is ongoing neurogenesis in the dentate gyrus of adult mice, LTP in this regions has two components: synaptic changes in immature neurons, induction of which is dependent on activity of NR2B subunit-containing NMDA glutamate receptors and does not require disinhibition of dentate gyrus *in vitro* and synaptic changes in mature neurons, which have opposing properties. Recording of *in vitro* LTP in immature neurons did not reveal a difference between NCAM deficient and wild-type neurons, suggesting that abnormalities in *in vivo* LTP are due to abnormalities in synaptic plasticity in mature granule cells. These data are the first to suggest that (1) independently of PSA expression, NCAM is necessary for induction of synaptic plasticity in mature granule cells *in vivo* and (2) polysialylated NCAM expressed by immature granule cells in the dentate gyrus supports development of basal excitatory synaptic transmission in this region.

Our *in vivo* recording of synaptic plasticity in mice deficient in the extracellular matrix glycoprotein TN-C did not reveal any abnormalities in basal synaptic transmission or synaptic plasticity at the perforant path – dentate gyrus connections. The situation was different for another investigated member of the tenascin family, TN-R. Deficiency in TN-R caused abnormal paired-pulse facilitation and impaired synaptic plasticity. Since morphological analysis of TN-R deficient mice revealed a high ratio of inhibitory to excitatory cells in the dentate gyrus, we performed experiments to rescue the impairment in LTP by blocking the GABAergic inhibition in the area of recording. Indeed, presence of $GABA_A$ antagonist, picrotoxin, in the recording pipette normalized the levels of LTP in TN-R deficient mice to those recorded in wild-type controls. To extend analysis of major excitatory synapses in the hippocampus of TN-R-deficient mice, we also studied synaptic plasticity in the CA3 region. Since from a technical point of view it is difficult to record synaptic responses in the CA3 area *in vivo*, these experiments were performed *in vitro*. Recordings of LTP in the CA3 subfield revealed no differences between TN-R and wild-type genotypes, fact which is in agreement with the morphological data, showing normal synaptic coverage of CA3 principal cells by synapses of parvalbumin-expressing interneurons.

In summary, our results provide novel evidence that NCAM and TN-R molecules are essential for normal hippocampal structure and synaptic function *in vivo*.

II. ZUSAMMENFASSUNG

Die extrazelluläre Matrix (ECM) besteht aus einem komplexen Netz verschiedener Makromoleküle, darunter Glykoproteine, Polysaccharide und Proteoglykane. Innerhalb des Nervensystems vermitteln die ECM und Zelladhäsonsmoleküle (CAMs) Zell-Zell und Zell-Matrix Interaktionen, und regulieren so die zelluläre Differenzierung, das Überleben, axonales Wachstum und Synapsenbildung. In früheren Arbeiten konnte nachgewiesen werden, dass Zellerkennungsmoleküle wie TN-R, TN-C und das polysialylierte neurale Zelladhäsionsmolekül NCAM eine Rolle bei kognitiven Funktionen und der Modulation hippokampaler synaptischer Plastiziät spielen. Die bisherigen elektrophysiologischen Arbeiten wurden allerdings alle in *in vitro* Systemen durchgeführt ohne *in vivo* bestätigt zu werden.

Aus diesem Grund wurde Langzeitpotenzierung (LTP) in anästhetisierten Mäusen, defizient in TN-R, TN-C sowie NCAM und dessen Polysialytransferasen ST8SiaII / STX und ST8SiaIV / PST induziert, um diese Lücke zwischen *in vitro* Experimenten und verhaltensbiologischen Analysen zu schließen. Die Langzeitpotenzierung wurde durch repetitive Reizung des Tractus perforans in der Theta-Frequenz erreicht. Für die einzelnen oben aufgeführten Teilprojekte wurden folgende Ergebnisse erzielt: NCAM spielt eine wichtige Rolle in der synaptischen Plastizität in der CA1 und CA3 Region des Hippokampus *in vitro*: Die Polysialylierung von NCAM in der CA1 Region des adulten Hippokampus, katalysiert von der Polysialyltransferase ST8SiaIV / PST, ist unabdingbar um LTP zu induzieren. Die basale synaptische Transmission im Gyrus dentatus, ausgedrückt durch die Steigung des exzitatorischen Feldpotentials war stark reduziert in ST8SiaII / STX defizienten Mäusen. Dieses Enzym vermittelt die Polysialylierung von NCAM in unreifen Körnerzellen. NCAM oder ST8SiaIV / PST defiziente Tiere dagegen besitzen normale synaptische Transmission. Die Langzeitpotenzierung, hervorgerufen durch Stimulation in der Theta-Frequenz, ist selektiv verringert in NCAM defizienten Tieren. Dies legt nahe, dass LTP im Gyrus dentatus lediglich von der Anwesenheit des NCAM Proteins, nicht jedoch von dessen Polysialysierung abhängt. Da die elektrophysiologische Aktivität während und nach der

LTP Induzierung in NCAM defizienten Tieren verändert ist, wird dieses Molekül vermutlich bereits für die Induktion der LTP benötigt. Aufgrund der ständigen Neurogenese innerhalb des Gyrus dentatus besteht die Langzeitpotenzierung aus zwei verschiedenen Komponenten. Eine davon ist die synaptische Veränderung in unreifen Neuronen. Diese wird von NMDA-Rezeptoren, welche die Untereinheit NR2B enthalten vermittelt, und benötigt keine gleichzeitige Unterdrückung der Inhibition des Gyrus dentatus *in vitro*. Die zweite Komponente hat gegenteilige Eigenschaften und resultiert aus den synaptischen Veränderungen maturierter Neurone. Da die *in vitro* Langzeitpotenzierung keine Unterschiede zwischen NCAM defizienten Tieren und Kontrolltieren zeigt, sind die *in vivo* beobachteten Unterschiede vermutlich auf Veränderungen der Plastizität in maturierten Körnerzellen zurückzuführen. Mit diesen Ergebnissen wird erstmal ein Beleg dafür präsentiert, dass NCAM unabhängig von seiner Polysialylierung notwendig für die Induktion der Langzeitpotenzierung in maturierten Körnerzellen *in vivo* ist, und dass das polysialylierte NCAM der unreifen Granularzellen im Gyrus dentatus eine wichtige Rolle für die Entwicklung der basalen exzitatorischen Transmission dieser Zellen spielt.

Die *in vivo* Untersuchung der basalen Transmission und der synaptischen Plastizität in Tieren defizient für das ECM Glykoprotein TN-C haben keinerlei Unterschiede and der tractus perforans-DG Synapse gezeigt. Ein anderes Bild zeigt sich bei dem verwandten Molekül TN-R. TN-R Defizienz führt zu veränderter Doppelpulsverstärkung und verringerter synaptischer Plastizität. Morphologische Untersuchungen dieser Tiere ergaben ein erhöhtes Verhältnis inhibitorischer zu exzitatorischer Neurone. Daher sollte eine Blockierung der GABAergen Inhibition zu einer Wiederherstellung der Langzeitpotenzierung führen. Tatsächlich führte die Applikation des $GABA_A$-Rezeptor Antagonisten Picrotoxin durch die aufnehmende Elektrode zu einer Normalisierung der Langzeitpotenzierung in TN-R defizienten Tieren. Um diese Analyse auf die wichtigsten exzitatorischen Synapsen des Hippokampus zu erweitern, wurde auch eine Analyse in der CA3 Region der TN-R defizienten Tiere durchgeführt, aus technischen Gründen jedoch ausschließlich *in vitro*. LTP Untersuchungen dort zeigten keine Veränderungen, was dem Ergebnis morphologischer Untersuchungen entspricht, die keine Unterschiede in der Innervation der CA3 Pyramidenzellen durch die Synapsen Parvalbumin positiver Interneurone ergaben.

Zusammenfassend liefert diese Arbeit neue Anhaltspunkte dafür, dass NCAM und TN-R essentielle Moleküle für die strukturelle Entwicklung und die synaptische Plastizität *in vivo* sind.

III. ABBREVIATION LIST

AA - arachidonic acid

A/C - associational/commissural

ACSF - artificial cerebrospinal fluid

AMPA - alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

APV (AP5) - aminophosphonovaleric acid

BDNF - brain-derived neurotrophic factor

CALEB - chicken acid leucine-rich EGF like domain containing brain protein

CAM - cell adhesion molecule

CaMKII - Ca^{2+}/calmodulin kinase

cAMP - cyclic adenosine mono phosphate

CNQX - 6-cyano-7-nitroquinoxaline-2,3-dione

CNS - central nervous system

CREB - cAMP response element-binding

CSF - cerebrospinal fluid

DAG - diacylglycerol

DCGIV - 2, 3-dicarboxycyclopropyl-glycine IV

DG - dentate gyrus

DNA - deoxyribonucleic acid

EC - entorhinal cortex

ECM - extracellular matrix

EDTA - ethylenediaminetetraacetic acid

EEG - electroencephalography

EGFL - epidermal growth factor-like repeats

EGTA - ethyleneglycoltetraacetic acid

E-LTP - early phase long-term potentiation

EPSCs - excitatory postsynaptic currents

ERK - extracellular signal-regulated kinase

FAK - focal adhesion kinase

FCS - fetal bovine serum

fEPSP - field excitatory postsynaptic potentials

FGFR - fibroblast growth factor-receptor

FNIII - fibronectin repeat of the subtype III

GABA - gamma-aminobutyric acid

GFP - green fluorescence protein

GluR - glutamate receptor

GPI - glycosylphosphatidylinositol

GTPases - guanosine triphosphatases

HFS - high frequency stimulation

HNK-1 - human natural killer-1

HVA - high voltage-activated

Ig - immunoglobulin

IP - inositol phosphates

IPSPs - inhibitory post-synaptic potentials

L-LTP - long-lasting LTP

LPP - lateral perforant path

LTD - long-term depression

LTP - long-term potentiation

LVA - low voltage-activated

MAG - myelin associated glycoprotein

MAPK - mitogen-activated protein kinase

mGluR -metabotropic glutamatergic receptor

MPP - medial perforant path

MF- mossy fibers

mRNA - messenger ribonucleic acid

NCAM - neural cell adhesion molecule

Ng-CAM - neuron-glia cell adhesion molecule

NMDA - N-methyl-D-aspartatic acid

PDZ - postsynaptic density-95/Discs large/zona occludens-1

PI - protease inhibitor

PIP2 - phosphatidylinositol bisphosphate

PKA - protein kinase A

PKC - protein kinase C

PLC - phospholipase C

PN - perineuronal net

PP - perforant path

PPM - paired-pulse modulation

PPF - paired-pulse facilitation

PS - population spike

PSA - polysialic acid

PST - polysialyltransferase ST8SiaIV

PTP - post-tetanic potentiation

PV - parvalbumin

REM - rapid eye movement

SC - Schaffer collateral

SEM - standard error of mean

SGZ - subgranular zone

SHFS - short high - frequency stimulation

STP - short-term potentiation

STX - polysialyltransferase ST8SiaII

SVZ - subventricular zone

TA - temporo-ammonic

TAG - transient axonal glycoprotein

TBS - theta-burst stimulation

TEA - tetraethylammonium chloride

TN - tenascin

VASE - variable alternatively spliced exon

VDCC - voltage-dependent Ca^{2+} channels

IV. REVIEW of the LITERATURE

IV.1. Synaptic plasticity in the hippocampus

The hippocampus is a brain structure which received its name due to its resemblance to the sea horse (*hippos*, gr. = horse; *kampos*, gr. = sea animal). It has been extensively investigated since the beginning of the 20th century and nowadays is the best characterized structure of the brain. Even though a great deal of information regarding this structure was accumulated until the mid 1950, only in 1957 did it become clear that the hippocampus plays an important role in memory (Scoville and Milner, 1957). Although its exact function still remains to be defined, we now know more about the type of information that hippocampus processes. For example in humans it seems that particular cells respond to certain words (Heit et al., 1988) and faces (Ishai and Yago, 2006), while in rodents the so called 'place cells' are encoding the spatial coordinates of the environment (O'Keefe, 1999). It is also known that hippocampus is a major player in epilepsy, having the lowest seizure threshold of all the other brain regions studied (Green, 1964).

IV.1.1. Morphology of the rodent hippocampus

Although there is a lack of consensus relating the terms describing the hippocampus and the adjacent cerebral cortex, the term *hippocampal formation* includes a group of four structures within the limbic system: dentate gyrus, hippocampus proper, 'subicular complex'(subiculum, presubiculum and parasubiculum) and entorhinal cortex (Johnston and Amaral, 2004). A cross-section taken perpendicular to the long axis (septal-temporal) will reveal the internal structure as two interlocking "Cs", one reversed in relation to the other, each with its own principle cell layer (Fig. 1). One "C" makes up Ammon's Horn or *Cornu Ammonis* (CA1-CA3). The principle cell layer of Ammon's Horn is the *stratum pyramidale*, or the pyramidal cell layer. The other "C" is made up of the dentate gyrus, of which the *stratum granulosum*, or granule cell layer is the principle cell layer. Sometimes the hilus of the dentate gyrus, the area inside the 'C' created by the granule cells is referred to as CA4. Nowadays, the intrinsic connections between the

principle cell layers of the dentate gyrus and CA regions of the hippocampus are very well defined and often described as the hippocampal circuit.

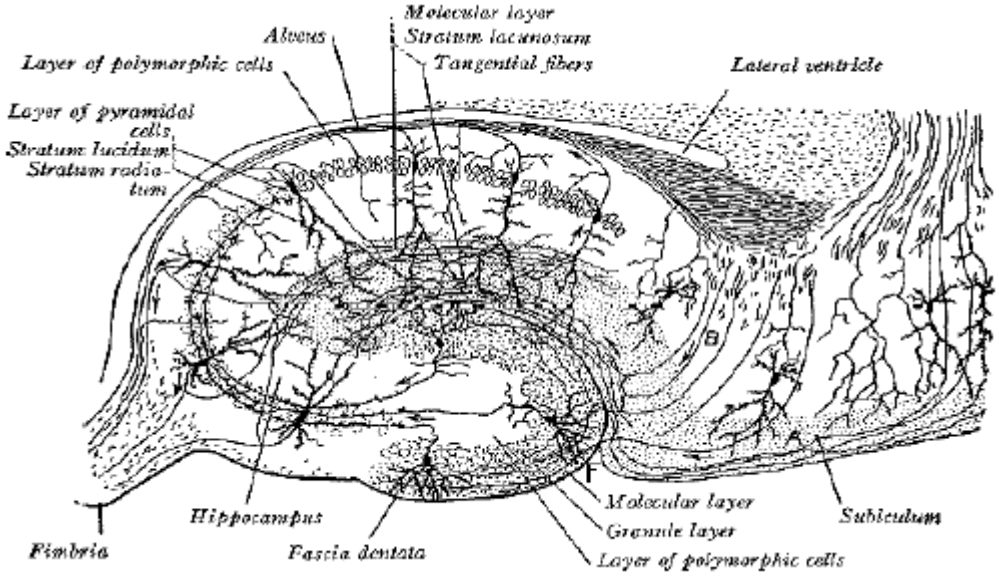

Figure 1**. The hippocampal formation.** The arrows show the direction of conduction. For a detailed description see text. Modified from Zador and Brown 1990 after an original drawing by Cajal

IV.1.2. Synaptic circuit in the hippocampus

A common characteristic of connections between regions of the neocortex is that they are largely reciprocal (Felleman and Van Essen, 1991). As first described by Ramon y Cajal (1893), this is clearly not the case for the hippocampal formation where the flow of information in this structure, is mainly unidirectional as presented below (see text below and Fig. 2).

a) Perforant Path

The entorhinal cortex (EC) is considered to be the starting point of this circuit because most of the sensory information that reaches the hippocampus enters through the entorhinal cortex. The neurons located in the layer II of the entorhinal cortex give rise to the perforant path (PP), that projects through (perforates) the subiculum and terminates both in the dentate gyrus and in the CA3 region of hippocampus. The perforant path splits into two anatomical distinct pathways, called the medial and lateral perforant path

(MPP and LPP). These fibers form excitatory synapses on the middle third (MPP) and outer third (LPP) regions of the dendritic tree of the granule cells.

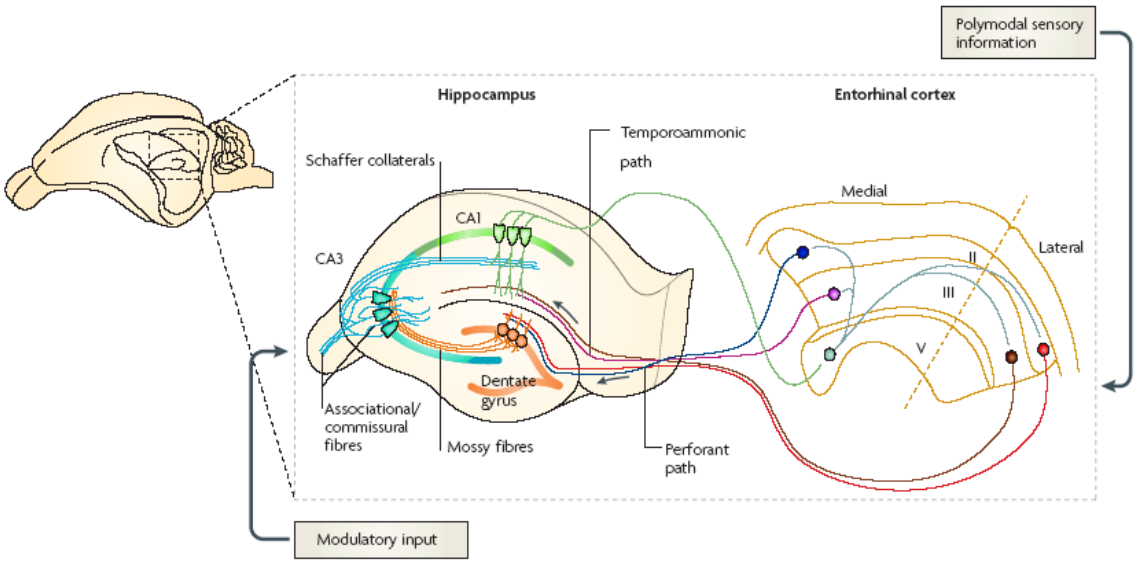

Figure 2. **Basic anatomy of the hippocampus**. The major input is carried by axons of the perforant path, which convey polymodal sensory information from neurons in layer II of the entorhinal cortex to the dentate gyrus. Perforant path axons make excitatory synaptic contact with the dendrites of granule cells: axons from the lateral and medial entorhinal cortices innervate the outer and middle third of the dendritic tree, respectively. Granule cells project, through their axons (the mossy fibers), to the proximal apical dendrites of CA3 pyramidal cells which, in turn, project to ipsilateral CA1 pyramidal cells through Schaffer collaterals and to contralateral CA3 and CA1 pyramidal cells through commissural connections. In addition to the sequential trisynaptic circuit, there is also a dense associative network interconnecting CA3 cells on the same side. CA3 pyramidal cells are also innervated by a direct input from layer II cells of the entorhinal cortex (not shown). The distal apical dendrites of CA1 pyramidal neurons receive a direct input from layer III cells of the entorhinal cortex. The three major subfields have an elegant laminar organization in which the cell bodies are tightly packed in an interlocking C-shaped arrangement, with afferent fibers terminating on selective regions of the dendritic tree. From Neves et al, 2008

This kind of anatomical organization gives the possibility to study two different populations of synapses on the same cell type by using extracellular stimulation. LTP was first observed in this pathway (Bliss and Lomo, 1973). Although the EC provides the major input to the dentate gyrus, the DG does not project back to the entorhinal cortex. The same is also true for the projections described below.

b) Mossy fibers

The dentate gyrus is the next step in the flow of information through the hippocampus and it gives rise to two types of excitatory projections that terminate on the proximal

dendrites of the CA3 pyramidal cells and on inhibitory interneurons. The former type of synapse, known also as the 'mossy fiber' synapse, has some unusual properties including large terminals, multiple sites of release and multiple postsynaptic densities. Besides glutamate, which is their primary neurotransmitter substance, mossy fibers (MF) also release other compounds like dynorphin (Weisskopf et al., 1993), zinc (Westbrook and Mayer, 1987), enkephalin (van Daal et al., 1989). It has also been shown recently that in neonatal rodents and in pathogenic condition, they can also release GABA (Safiulina et al., 2006; Trevino and Gutierrez, 2005).
The CA3 pyramidal cells give rise to highly collateralized axons that distribute fibers both within the ipsilateral hippocampus (ex., Schaffer collateral projections) and to the same fields in the contralateral hippocampus (commissural projections).

c) Associational\Commissural fibers

Associational (A) inputs are the projections from CA3 neurons to other CA3 cells on the same side of the brain. Commissural (C) inputs are CA3–CA3 connections between the two hemispheres. For simplicity reasons and since their properties can not really be distinguished between each other in term of physiological characteristics they are usually referred to as associational\commissural fibers (A\C). These projections are found throughout the stratum radiatum and oriens of the CA3 (Amaral and Witter, 1989).
Although CA3 pyramidal cells give rise to both the Schaffer collateral fiber synapses in CA1 and the A\C fiber synapses in CA3, the induction of LTP at these synapses may be regulated by different activity- and modulatory neurotransmitter-dependent processes (Moody et al., 1998).

d) Schaffer collaterals

CA3 pyramidal cells send projections to the CA1 region of hippocampus. Although it is common that Schaffer collaterals (SC) are illustrated as extending only through stratum radiatum, it is important to note that the stratum oriens is also highly innervated by CA3 axons. The Schaffer collaterals are probably the best-studied synaptic pathway in the hippocampus. Each CA3 axon synapses onto thousands of CA1 pyramidal neurons but usually with only one or two synaptic contacts per neuron (Sorra and Harris, 1993). This pathway has been extensively studied because of interest in various forms of synaptic

plasticity, which take place at this synapse. Stimulation of the SC activates both the excitatory and inhibitory cells present in this area.

CA1 pyramidal cells receive inputs also from other regions, for example the septal nuclei and 'nonspecific' regions of the thalamus (Dolleman-Van der Weel and Witter, 2000). Thus, once the information enters the entorhinal cortex, it follows the circuit described above, transverses the entire hippocampus and ultimately returns to the area from which it originated. The changes that take place during this signal propagation are most probably essential for enabling the information to be stored as long-term memories.

IV.1.3. Short- and long-term plasticity in the hippocampus

The excitatory synapses in the hippocampus exhibit different forms of activity-dependent synaptic plasticity. These are generally defined as changes in the amplitude of synaptic potentials that are dependent on the prior activity of the synapse. The different forms of plasticity are generally distinguished on the basis of their duration or time-course.

IV.1.3.1. Short-term plasticity

Activity-dependent changes of synaptic efficacy which range in duration from tens or hundreds of milliseconds to several seconds or minutes are commonly referred as shor-term plasticities. Paired-pulse facilitation and depression were first studied in the neuromuscular junction and appear to be a common characteristic of all chemical synapses.

Paired-pulse facilitation

The mechanism underlying this form of plasticity is believed to be presynaptic (Zucker and Regehr, 2002), thus the level of facilitation (second response is increased compared with the first one, see section VI.2.5) at a given synapse can be attributed to a transient increase in the concentration of presynaptic calcium produced by the two delivered stimuli. The concentration declines to basal values in a few hundred milliseconds, but the calcium influx at the time of the second stimulus adds to the residual calcium from the first stimuli, resulting in an enhanced calcium concentration and, in this way, to an increase in the probability of release (Wu and Saggau, 1994). The magnitude of facilitation is inverse proportionally to the probability of release.

Paired-pulse depression

A second phenomena can be observed when two consecutive stimuli are applied to a synapse, this is depression (second response is decreased compared with the first one, see section VI.2.5). This process is due to depletion of readily releasable transmitter from the presynaptic terminal or down-regulation of transmitter release via activation of presynaptic $GABA_B$ or mGlu autoreceptors in response to the first stimulus.

IV.1.3.2. Long-term plasticity

There are several forms of synaptic plasticity at glutamatergic, excitatory synapses in the hippocampus that can last from 30 minutes to hours, days or weeks. These forms usually take place after repetitive trains of synaptic stimulation or after specific pairings of presynaptic and postsynaptic firings. They are called long-term potentiation (LTP) and long-term depression (LTD) and are believed to contribute to the learning and memory functions of the hippocampus. Since this study is focusing exclusively on LTP at different hippocampal synapses and this form of plasticity is affected in some genetically modified mice, just LTP will be discussed further.

IV.1.3.2.1. Discovery of LTP

In 1973, Bliss and Lomo conducted a series of electrophysiological experiments on anesthetized rabbits, in order to explore the role of the hippocampus in short-term memory. They observed that stimulating the perforant path (see section IV.1.2, namely perforant path projections) with a series of electrical stimuli resulted in potentiation (Bliss and Lomo, 1973). Potentiation is an increase in synaptic efficacy, meaning how efficient a synapse transmits an action potential from the presynaptic axon to the postsynaptic dendrite. The resulting increase in the fEPSP is a measure of increased synaptic efficacy. This means that, given the same input, the synapses with higher efficacy are more likely to trigger an action potential than those with low efficacy.

Since its first discovery in anesthetized rabbits, hippocampal LTP has been described in numerous vertebrate species, including rodents, guinea pigs (Hanse and Gustafsson, 1992), macaque monkeys (Urban et al., 1996) and humans (Beck et al., 2000; Cooke and Bliss, 2006).

LTP has been studied more in the hippocampal formation than in any other brain region and in addition to the main hippocampal projections, it has been also observed at the PP

projections to CA3 (Do et al., 2002) and CA1 (Colbert and Levy, 1993), the projections from CA1 to subiculum (O'Mara et al., 2000) and even in synaptic connections made by CA1 neurons on a class of macroglia-like cells (Ge et al., 2006). During the years a series of studies showed that other regions of the nervous system like cerebellum (D'Angelo et al., 2005), amygdala (Sigurdsson et al., 2007), sensory and motor cortexies (Castro-Alamancos et al., 1995), prefrontal cortex (Blond et al., 2002) and nucleus accumbens (Schramm et al., 2002) are capable of plasticity.

IV.1.3.2.2. Protocols used for induction of LTP (*in vivo & in vitro*)

There are a large number of protocols for inducing LTP in different brain regions, for example high-frequency stimulation (HFS), theta-burst stimulation (TBS), pairing of presynaptic stimulation with a postsynaptic depolarization and sharp wave stimulation. Depending on the purpose of investigation, at the same synapse, more than one protocol can be used (Albensi et al., 2007). In this study (see Material and Methods section) we used TBS protocols for inducing LTP in the areas CA1 and DG (*in vitro* and *in vivo*), HFS protocols in area CA3 and SHFS in the DG (*in vitro*).

High-Frequency Stimulation

High-frequency tetanus is a train of 50-100 stimuli at frequency ≥ 100 Hz (Bliss and Collingridge, 1993). The efficacy of this protocol to induce potentiation depends on a series of factors, one of them being the number of trains applied. It was shown that three trains of HFS stimulation are effective in producing so-called late LTP that lasts 3 h or more and involves protein synthesis (Huang and Kandel, 1994; Krug et al., 1984) instead a single train at baseline stimulation intensity leads to early LTP (1–3 h) and is protein synthesis-independent (Huang and Kandel, 1994).
Since it is questionable if neurons naturally fire for one second at a frequency more than 100 Hz, more physiological protocols like theta-burst stimulation were developed to mimic physiological patterns of neuronal activity.

Theta-Burst Stimulation

As the name suggests, TBS protocols are based on the fact that during learning-related exploratory behavior, theta waves are presented in a hippocampal EEG. Comparative with the HFS protocols, this one can use a relatively small number of bursts and number of stimuli in the burst, if they are applied on the positive phase of the theta wave

(Holscher et al., 1997; Pavlides et al., 1988). Nowadays, common protocols for induction of LTP *in vivo* consist of several series of bursts at 400 Hz with an interburst interval of 200 ms (Namgung et al., 1995).

In vitro studies, showed that by using TBS protocols one can induce LTP which can be, depending on the frequency used, either NMDA dependent or/and VDCC dependent (Grover and Teyler, 1990) and, like HFS in the CA1 region, also involves transcription, translation, and protein kinase A (PKA) activation (Nguyen et al., 1994). The theta-burst protocols are so effective because of the interburst interval of 200ms. It seems that this interval is a time period when inhibitory post-synaptic potentials (IPSPs) are difficult to recruit. Therefore, repeated stimulation permits an effective temporal summation of EPSPs.

Pairing protocols

A standard protocol for inducing LTP using whole-cell recording is to pair low-frequency synaptic stimulation (100-200 pulses, 1-2 Hz) with a depolarizing voltage-clamp pulse (1-3 min duration). These stimuli applied alone to a presynaptic axon do not elicit any potentiation, but, together there is a persistent increase in the EPSP (Gustafsson et al., 1987; Wigstrom and Gustafsson, 1986). This protocol is extremely useful for investigating the properties of different types of cells and their behavior during LTP induction.

Spindle stimulation protocol

Spindles are non-REM sleep EEG rhythms of 7-14 Hz that take place independently or in association with slow oscillations. Using a spindle stimulation pattern protocol, derived from *in vivo* recordings in anesthetized cats, Rosanova and Ulrich (2005) managed to induce LTP in somatosensory cortex of rat slices. This protocol contained 22 spikes (mean firing rate c.a. 30Hz) that were group initially in a ~ 10 Hz rhythmic burst followed by other bursts at decreasing frequency. Its application induced L-type Ca^{2+} channels dependent LTP, whereas the short-term potentiation was NMDA dependent. Even though this pattern of stimulation has not produced LTP in other brain structures, up to now it remains the most natural protocol used to induce LTP (Rosanova and Ulrich, 2005).

IV.1.3.2.3. Neuronal plasticity in hippocampal synapses

Experimental analysis of the properties and the mechanism of LTP have mainly concentrated on the Hebbian form of synaptic plasticity exhibited by the Schaffer-collaterals input to CA1 and by the perforant path projection to the DGCs. While it is presumed that the LTP at the A\C synapses shares the same mechanisms with the above mentioned synapses, there are very few studies investigating synaptic plasticity at this synapse.

Forms and mechanisms of LTP at CA3-CA1 synapses

The most studied form of LTP at this particular synapse is NMDAR-depended potentiation. This form of plasticity proceeds in stages, beginning with a protein kinase independent phase (STP), lasting less than one hour followed by three stages of LTP (Reymann and Frey, 2007), requiring protein phosphorylation (LTP1), protein synthesis from existing mRNAs (LTP2) and gene transcription (LTP3). The very first step of this process involves a temporary co-activation of the NMDA and AMPA receptors and as a result, an increase of the intracellular Ca^{2+} concentration which is further augmented by Ca^{2+} release from intracellular stores (Harvey and Collingridge, 1992). Although activation of NMDAR is a necessary condition for the induction of a major component of LTP at SC-CA1 and PP-DG synapses, it is not always a sufficient condition. Just NMDA application can only induce STP, but neither NMDA nor glutamate when applied to hippocampal slices readily induces long-lasting potentiation. Other postsynaptic signaling pathways such as mGluRs, may be necessary co-triggers.
Several different Ca^{2+} sensitive enzymes have been proposed to play a role in converting the induction signal into persistent modifications of synaptic strength. Most interest has focused on the role of protein kinases (Bliss and Collingridge, 1993; Citri and Malenka, 2008; Malenka and Nicoll, 1999). Among these, Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) is a key component of the molecular machinery for LTP. Once activated, CaMKII can phosphorylate the GluR1 subunit of AMPAR and thus enhance the channel function, bind to the cytoplasmic domain of the NR1 and NR2B subunits of NMDAR and also take part in the trafficking of AMPAR, thus being involved in converting the silent synapses into functional contacts (Lisman et al., 2002). PKA activation has been shown to regulate AMPARs, both voltage-dependent K^+ and Ca^{2+} channels and furthermore, to be involved in gene transcription, through CREB

phosphorylation (Sweatt, 1999). The extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway has also been suggested to be important for LTP (Thomas and Huganir, 2004). In addition, Src kinase has been implicated in the enhancement of NMDAR function during LTP induction (Kalia et al., 2004). Finally, PKC and in particular the atypical PKC isozyme, PKM_{ζ}, has received attention because this isozyme is rapidly expressed upon induction of LTP and recent studies have implicated PKM_{ζ} in the maintenance of the late phase of LTP both in hippocampal slices and *in vivo* (Hrabetova and Sacktor, 1996; Kelly et al., 2007; Pastalkova et al., 2006; Serrano et al., 2005).

NMDAR-dependent LTP is not the only form of plasticity seen at SC-CA1 synapses. Stimulation at different frequencies generates different types of LTP at the same synapse: NMDAR-dependent (100 Hz) or independent (200 Hz). The latter form develops relative slowly, is of small amplitude but it can last a very long time and is triggered by Ca^{2+} entry through L-type VDCC (Grover and Teyler, 1990). A second form of NMDA independent plasticity can be achieved by a slight and transient increase in extracellular Ca^{2+} (Turner et al., 1982), whereas a third one can be induced by increasing extracellular K^{+} (Bernard et al., 1994) or by TEA application (Aniksztejn and Ben-Ari, 1991), which induce Ca^{2+} influx via L-type VDCCs.

Forms and mechanisms of LTP at PP-DG synapse

Unlike LTP at the Schaffer collaterals and at the mossy fibers, LTP at the perforant path has been studied mostly in anesthetized or freely moving animals. Relatively fewer studies have examined LTP in the PP in acute hippocampal slices. Most of these studies have been investigating just the early component of LTP and have been mainly focusing in dissociating between LTP at the medial and lateral perforant path (Colino and Malenka, 1993; Hanse and Gustafsson, 1992), initially thought to be mediated by different receptors (Bramham et al., 1991a; Bramham et al., 1991b). Since most of the *in vivo* studies do not differentiate between these two pathways, it is hard to say if the *in vitro* findings in either of the projections hold also true for the other path. Therefore here I will refer mostly to either the medial perforant path projection to the dentate gyrus or the perforant path as an assembly without distinction between the projections.

The mechanism underlying LTP at the PP-DG has not received the same amount of attention as the one at the SC-CA1. This is most probably due to the fact that *in vitro* LTP is possible to induce just in disinhibited slices (i.e., in presence of $GABA_A$

antagonists), although one study reported LTP induction in ACSF solution with a lower Mg^{2+} concentration (Nguyen and Kandel, 1996). Since it has been shown that LTP at this synapse also involves postsynaptic depolarization, NMDAR and mGluR activation and consequently an increase in the Ca^{2+} influx (Colino and Malenka, 1993; Hanse and Gustafsson, 1992; McHugh et al., 2007; Wu et al., 2001), the common belief is that it shares the same mechanism as NMDA dependent LTP at the SC-CA1 synapses. Several findings support this hypothesis. First, a recent study showed that LTP at the MPP-DG is mediated by either of two parallel cascades, one involving CaMKII and the other PKA or MAPK (Wu et al., 2006), both of these are also active during CA1 LTP. Secondly, a selective enhancement of the AMPAR but not of NMDAR labeling has been observed after *in vivo* LTP induction (Moga et al., 2006), reminiscent of the AMPAR trafficking described in the CA1 region. Furthermore, a late LTP requiring new RNA and protein synthesis has also been described both *in vitro* (Nguyen and Kandel, 1996) and *in vivo* (Krug et al., 1984; Otani and Abraham, 1989; Otani et al., 1989).

NMDAR-dependent LTP is not the only form of potentiation seen in the dentate gyrus. HFS applied at 200 Hz induced an APV and nifedipine insensitive, Ni^{2+} sensitive form of LTP suggesting that the Ca^{2+} influx occurred via low voltage dependent Ca^{2+} channels (Wang et al., 1997). The same study also showed that, although NMDAR-dependent and NMDAR-independent LTP use different Ca^{2+} channels, they share a common intracellular pathway.

Chemically induced LTP was also observed at this synapse. Acute intrahippocampal infusion of BDNF potentiated transmission at MPP in anesthetized adult rats (Messaoudi et al., 1998), whereas acute forskolin application to slices increased the phosphorylation levels of CREB and MAPK. Surprisingly, while TEA application induces potentiation in the CA1 region, it has been shown that in the dentate gyrus it induces an NMDAR-dependent depression (Song et al., 2001).

Forms and mechanisms of LTP at CA3-CA3 synapses

A/C synapses are a dense associative network of ipsilateral and contralateral excitatory connections between CA3 pyramidal cells that are found throughout the strata radiatum and oriens of the CA3 (Amaral and Witter, 1989). LTP at these synapses has been shown to be associative (Chattarji et al., 1989; Martinez et al., 2002), to require postsynaptic depolarization (Zalutsky and Nicoll, 1990) and to be NMDAR dependent

(Gibson et al., 2005; Ito et al., 1997). Thus, although the mechanisms have not been studied, A/C and SC-CA1 synapses have been reported to share some pharmacological properties (Zalutsky and Nicoll, 1990). In contrast to the CA1 region, where LTP can be induced by 5-Hz stimulation in presence of β-adrenergic receptor agonist, at the A/C synapse the same protocol failed to induce potentiation (Moody et al., 1998). This finding suggests that different activity dependent processes may regulate induction of LTP at these synapses.

Most of the studies investigating LTP at this synapse have been mainly focused on different factors, which modulate the synaptic plasticity. Serotonin, glucocorticoid receptor activation and nitric oxide synthase inhibitors have been shown to inhibit LTP (Nicolarakis et al., 1994; Pavlides and McEwen, 1999; Villani and Johnston, 1993), whereas mineralocorticoid receptor activation enhanced it (Pavlides and McEwen, 1999). mGluR1 has been also shown to play a role, being implicated in generation of the spike train timing dependence of A/C LTP (Kobayashi and Poo, 2004).

IV.1.3.3. LTP as a possible substrate for learning and memory

The interest in studying the hippocampus formation started in the early 1950s, when it was recognized that this structure plays a fundamental role in learning and memory. During this time, it was shown that bilateral hippocampal removal generates a permanent loss of the ability to encode new information into the long-term memory, although the retrieval of old information and short-term memory were not affected (Scoville and Milner, 1957), a phenomenon know as anterograde amnesia. The patient's ability to form long-term procedural memories was still intact, thus he could, for example, learn new motor skills, despite not being able to remember learning them.
Two decades after the initial publication of this observation, it was discovered that hippocampal synapses are capable of undergoing stable and long-lasting changes in synaptic strength referred to as LTP (see section IV.1.3.2.1). The discovery of LTP has attracted a great deal of attention since, it was initially observed in a 'memory structure' and because some of its properties like persistence, input specificity, associativity, cooperativity (Bliss and Collingridge, 1993; Malenka, 1994) serve as arguments for supporting the hypothesis that LTP may be a biological substrate for at least some forms of memory.

The question of whether these changes have to last as long as memory is still debatable. The fact that hippocampal LTP has a decremental time-course (Abraham et

al., 1995) is some time presented as an argument against the possibility that LTP is a substrate for memory formation. Since it is speculated that actually this structure plays a time-limited role in the information storage, with the cortex being the ultimate station for different kinds of memory, the same feature can serve as a solid argument for the hypothesis that LTP is a substrate for memory formation. Several other pieces of evidence have consolidated this view:

1) A series of pharmacological and molecular genetic manipulations enhance both LTP and memory. One of the best studied examples are the 'ampakines', which were shown to facilitate the induction of hippocampal LTP (Staubli et al., 1994a) and enhance the encoding of memory in a variety of tasks (Lynch, 2006; Staubli et al., 1994b).

2) Pharmacological or genetic manipulation impairs both LTP and learning. NMDARs are one of the key players in synaptic plasticity (Collingridge et al., 1983) and learning (Castellano et al., 2001). The injection of AP5, a specific NMDAR antagonist blocks spatial learning (Butcher et al., 1990; Morris, 1989) and hippocampal LTP *in vivo* and *vitro* (Davis et al., 1992). Another important player is the AMPA receptor. Mice lacking the GluR1 subunit, have impaired LTP in area CA1 and severe deficits in spatial working memory (Schmitt et al., 2005).

3) A critical feature of both memory consolidation and formation of long-lasting synaptic plasticity is the requirement for new proteins. A number of interventions can interfere with both memory consolidation and stabilization of LTP, like for example : a) disrupting the activity of CaMKII (Lisman et al., 2002) or MAPK/ERK (Sweatt, 2004; Thomas and Huganir, 2004); b) disrupting the expression of immediate early genes such as *Zif268* (Jones et al., 2001) or *arc* (Guzowski et al., 2000); c) inhibition of macromolecular synthesis (Frey and Morris, 1998).

4) Aged rats have deficits in both the induction and maintenance of LTP (Burke and Barnes, 2006). For example, in old rats LTP at PP - DG synapses has an increased rate of decay that correlates with the rate of forgetting (Barnes, 1979). A similar deficit was also observed at PP - CA3 synapses (Dieguez and Barea-Rodriguez, 2004), whereas at SC - CA1 synapses, the primary deficits with aging seem to be a reduction in the magnitude of LTP (Tombaugh et al., 2002). Actually, LTD and depotentiation appear to be much easier to induce in aged animals (Norris et al., 1996).

Another important aspect that sustains the hypothesis that LTP is connected with learning and memory is the fact that synaptic plasticity has distinct functions in different parts of the nervous system. In the amygdala, it has been implicated in conditioned fear (LeDoux, 2000), in the spinal cord, LTP-like changes have been implicated in hyperalgesia to painful stimuli.

As regard to hippocampus, no clear mechanism has been revealed regarding what exactly happens to information, when it passes through this structure or what is the precise role of its different areas so, one cannot really say for sure that LTP at different synapses of the hippocampus play certain roles. Nowadays, the current state of the literature suggests that the areas of hippocampal formation are involved in different types of learning and memory as following : a) DG is involved in pattern separation (O'Reilly and McClelland, 1994); b) a series of studies point out that CA3 is important for associative memory recall (Nakazawa et al., 2002) and its recurrent network serve as a temporary storage site for short-term, episodic or working memories (Kesner and Rolls, 2001); c) CA1 area acts as a comparator: detecting novelty or mismatches between actual sensory information from the EC and the expectation from memory in CA3 (Moser and Paulsen, 2001; Vinogradova, 2001); d) The direct entorhinal-CA1 path was shown to be sufficient for recollection-based recognition memory whereas recall depends on intact CA3-CA1 connectivity (Brun et al., 2002).

IV.2. Neural cell adhesion molecule NCAM

Cell adhesion molecules (CAMs) are cell surface molecules that control cell-cell, cell-matrix and cell-substratum interactions, thus mediating processes like neuronal adhesion, migration, neurite outgrowth, fasciculation and synaptogenesis. Subfamilies of immunoglobulin (Ig) CAMs in the nervous system have been categorized according to the number of Ig-like domains, the presence and number of fibronectin type III repeats, the mode of attachment to the cell membrane and the presence of a catalytic cytoplasmic domain (Fig. 3). Neural cell adhesion molecule, NCAM, was the first Ig-like CAM to be isolated (Jorgensen and Bock, 1974) and is the CAM that has been characterized in the greatest detail. It is the foundation for the large family of neural adhesion molecules of the Ig superfamily and it is a very attractive molecule for medical research since it has been implicated in schizophrenia pathology. In the cerebrospinal fluid (CSF) of patients with schizophrenia and mood disorders, NCAM concentration was elevated (Poltorak et al., 1997; van Kammen et al., 1998), the same being true also

when the CSF of schizophrenic patients was compared with their normal monozygotic twin siblings (Poltorak et al., 1997).

IV.2.1. NCAM structure

Alternative splicing of a primary transcript encoded by a single NCAM gene gives rise to three major isoforms: NCAM - 180, -140 and -120. The numbers following their names represent their approximate molecular weights. The NCAM gene is located on chromosome 9 in chicken (Owens et al., 1987) and mice (D'Eustachio et al., 1985) and on chromosome 11 in humans (Nguyen et al., 1986). The extracellular part of NCAM contains five immunoglobulin like domains (Ig I-V) and two fibronectin type III domains (FN III). The Ig IV domain may contain additionally the ten aminoacids long sequence VASE (Variable Alternatively Spliced Exon) or π-exon, which may be possibly related to psychiatric disorders. NCAM-180 and NCAM-140 are transmembrane isoforms with different intracellular extensions, thus able to interact with the cytoskeleton and to be more rigidly anchored in the membrane, whereas NCAM-120 is attached to the lipid bilayer via a glycosylphosphatidylinositol (GPI) anchor. Additionally, several forms of soluble NCAM exist which are generated by truncation or proteolysis (Olsen et al., 1993). These forms of soluble NCAM were proposed to be developmentally toxic, since a gene targeting study showed that removing all cytoplasmic domains of NCAM was lethal in mice (Rabinowitz et al., 1996).

Posttranslational modifications of NCAM include structural changes like palmitoylation and polysialylation. The palmitoylation process take place at four cystein residues of the intracellular part of NCAM, and thus helps NCAM to be anchored to lipid rafts. Interference with the palmitoylation has been shown to affect the distribution of NCAM within the plasma membrane and to disrupt NCAM-mediated signalling and neurite outgrowth (Niethammer et al., 2002). Attachment of PSA results in a large hydration sphere and modulates the function of NCAM molecule. The amount of PSA residues on NCAM is regulated developmentally, changing from 30% of mass in embryonic development to only 10% in the adult (Edelman and Crossin, 1991). In contrast with vertebrate NCAM, NCAM homologues in Aplysia and Drosophila, apCAM and Fas II respectively, are not polysialylated.

PSA is not the only carbohydrate carried by NCAM. HNK-1, also found on TN-C and TN-R molecules can be also attached to NCAM (Schachner and Martini, 1995).

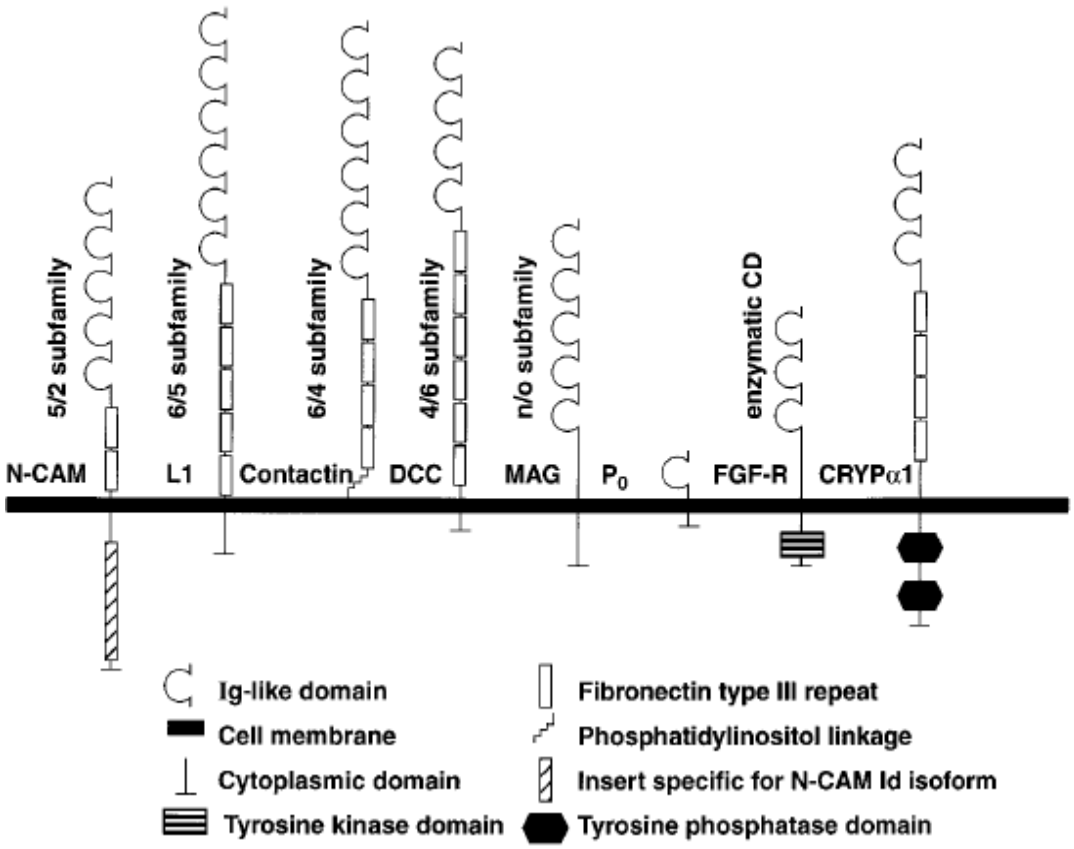

Figure 3. **Diagram of neural cell adhesion molecule subfamilies of the immunoglobulin superfamily.** The majority of molecules of the immunoglobulin superfamily that are found in the nervous system belong to different classes depending on the number of Ig-like domains and fibronectin repeats, which are denoted with numbers for each. For example, the 5/2 family is exemplified by N-CAM and contains five Ig domains and two fibronectin type III-like repeats. The N/0 subfamily molecules contain varying numbers of Ig-like domains but no fibronectin repeats. The enzymatic cytoplasmic domain (CD) category exhibit protein tyrosine kinase or phosphatase activities in their cytoplasmic domain. Adapted from (Crossin and Krushel, 2000).

IV.2.2. NCAM expression

NCAM is expressed on the surface of most cells in the central and peripheral nervous system and despite its name, NCAM expression is not restricted just to neural tissue, but it can be also expressed by several cell types in many tissues, for example in muscle (Andersson et al., 1993), heart (Chuck and Watanabe, 1997) and gonads (Moller et al., 1991).

Regarding its expression in the CNS, it was shown that the main isoforms of NCAM have distinct expression profiles. NCAM-120 is predominantly expressed on glia (Kiss and Muller, 2001), NCAM-180 seems to be expressed exclusively on

neurons, particularly at the postsynaptic side of synapses (Persohn and Schachner, 1990), whereas NCAM-140 can be found on both glia cells and neurons. In neurons NCAM-140 is expressed on both pre-and postsynaptic membranes.

In the hippocampus, NCAM is expressed in the hilus, in the inner molecular layer of dentate gyrus, in the mossy fiber tract and in the stratum radiatum and oriens of the CA1 region. Stimulation of AMPARs has been reported to activate an NCAM promoter in slices, this effect is reduced by CNQX, an AMPAR antagonist (Holst et al., 1998). This observation indicates that AMPARs mediated synaptic transmission may regulate NCAM expression. Further evidence for the theory that NCAM expression is upregulated upon increased synaptic activity came from a study of synaptic plasticity, which showed that NCAM-180 expression is increased in the molecular layer of DG after induction of LTP in adult rats (Schuster et al., 1998).

As mentioned above, one of the crucial posttranslational modifications of NCAM is the attachment of PSA to NCAM, which is developmentally regulated. PSA expression in the adult brain is considerably lower than at younger stages of development but it still persists in some brain regions, which are known to exhibit neurogenesis, cell migration axonal outgrowth and synaptic plasticity. Thus, PSA was found to be present in the rostral migratory stream (Lois et al., 1996), in the hippocampal formation (Seki and Arai, 1993b), in the suprachiasmatic nucleus (Glass et al., 1994), in amygdala (Nacher et al., 2002b) and hypothalamus (Theodosis et al., 1991), in prefrontal (Varea et al., 2005) and piriform cortex (Nacher et al., 2002a) and in some nuclei of the spinal cord (Seki and Arai, 1993a). In the hippocampus, PSA expression can be seen on a number of cells in the dentate gyrus (Seki and Arai, 1993b), particularly in the deepest part of the granular layer, coincident with the localization of neural stem cells.

IV.2.3. Biosynthesis of PSA by STX & PST enzymes

As a linear homopolymer (n can be > 100) of α2, 8-linked sialic acid, it is a remarkably simple macromolecule. Unlike most carbohydrates found on the cell surface, PSA in the vertebrates appears to be expressed just on NCAM. In invertebrates, the polysialylation seems to be absent. Addition of PSA to NCAM takes place in the trans Golgi compartment as a regular step in the biosynthetic pathway of protein glycosylation in eukaryotes (Scheidegger et al., 1994). Initially, it was thought that NCAM polysialylation represented a complex process involving several enzymes. Although the exact number of enzymes involved in PSA biosynthesis is still under discussion, *in vitro*

experiments suggest that ST8Sia II / STX and ST8Sia IV / PST polysialyltransferases are able to catalyze the entire reaction. Certain differences exist in the level of polysialylation catalyzed by PST or STX. Both of them can polysialylate all NCAM isoforms (Franceschini et al., 2001) but ST8Sia IV is more efficient in NCAM polysialylation and forms a larger polysialylation on NCAM than does ST8Sia II (Angata et al., 1997).

The expression patterns of these two enzymes are always correlated with the expression of PSA. Both ST8Sia II and IV transcripts are barely detectable at E8, but increase after E9. ST8Sia II is prominent in embryonic tissue, but its levels decrease substantially after birth and it is almost undetectable in the adult animal. In contrast, the decline of ST8Sia IV is moderate and the enzyme persists in the mature brain (Hildebrandt et al., 1998; Ong et al., 1998).

By using an *in vitro* assay system, both enzymes were shown to directly add PSA to fetuin and NCAM (Angata et al., 1997; Nakayama and Fukuda, 1996). This demonstrates that either PST or STX alone can form PSA by adding the first α2, 8-linked sialic acid to α2, 3 and α2, 6 sialic acid in a glycoprotein acceptor, followed by the multiple addition of α2, 8 linked sialic acid residues. Since the two enzymes are often expressed in the same region of the brain, it is likely that they work cooperatively on polysialylation rather than competing. In accordance with this assumption, NCAM polysialylation by a mixture of ST8Sia II and IV was more extensive than the combined amount of NCAM polysialylation by each enzyme in separate reactions (Angata et al., 1998; Angata et al., 2002).

Although there is no direct evidence that mutations in the polysialyltransferases cause certain diseases, decreased PSA expression is reported in schizophrenia and disorganized expression of PSA increases in Alzheimer disease (Barbeau et al., 1995; Mikkonen et al., 2001).

IV.2.4. Roles of NCAM and PSA in synaptic plasticity

The presence of adhesion molecules in or near the synaptic cleft raises the possibility that, in addition to serving as recognition molecules for synaptogenesis, they may also participate in initiating and maintaining synaptic changes. In this respect, NCAM is clearly well placed to have an active role in synaptic restructuring following LTP or learning and memory. Indeed, interference with NCAM or PSA-NCAM was found in several studies to prevent synaptic plasticity, namely LTP induction. This result has

been obtained using different approaches. Initial *in vitro* studies showed that application of antibodies directed against NCAM before LTP induction reduces levels of LTP in the CA1 region of the adult hippocampus (Luthi et al., 1994; Ronn et al., 1995) and that NCAM is important for the stabilization of the early phase of LTP rather than for its maintenance. Later studies performed in constitutively NCAM deficient mice (Muller et al., 2000) and in conditionally NCAM deficient mice (Bukalo et al., 2004) confirm the initial observations made by Luthi in the CA1 region of the hippocampus. Interestingly, impaired LTP in hippocampal slices or organotypic cultures prepared from the constitutively NCAM knockout mice could be recovered by addition of the neurotophic factor BDNF (Muller et al., 2000). Also the impairment in CA1 LTP in conditionally NCAM deficient mice could be rescued by increasing the extracellular concentration of Ca^{2+} (Bukalo et al., 2004).

In the area CA3, LTP is impaired just in the constitutively NCAM deficient mice (Cremer et al., 1998), but not of conditionally NCAM deficient mice (Bukalo et al., 2004) pointing thus to a role of NCAM in the normal development of mossy fibre–CA3 synapses (Cremer et al., 1998).

Up to now, it is not known what role, if any, NCAM plays at the perforant path-dentate gyrus synapses but it was reported that at these synapses, LTP is associated with an increase in the extracellular concentration of NCAM in a NMDAR-dependent manner (Fazeli et al., 1994). Also, tetanic stimulation of the perforant path increases the percentage of axospinous synapses expressing NCAM-180, the largest splice variant of NCAM with the longest intracellular domain of all NCAM isoforms, in the dentate gyrus of adult mice (Schuster et al., 1998).

Since NCAM is a carrier of PSA in the mammalian brain, in the experiments mentioned above, one can not dissect which effects are attributable to NCAM and which to PSA. Initially studies investigating this issue made use of the endo-N enzyme which cleaves specifically PSA from NCAM, making possible a clear functional separation. Thus, a similar reduction (see above) of LTP in the CA1 region has been demonstrated *in vitro* (Becker et al., 1996; Muller et al., 1996) when slices from wild-type mice were treated with this enzyme, indicating that the PSA expression on NCAM is necessary for normal LTP to occur, since the basal synaptic transmission was unaffected. It was also reported that enzymatic removal of sialic acid from cortical or hippocampal membranes modulates AMPA-binding to AMPARs (Hoffman et al.,

1997), suggesting that the expression of PSA-NCAM may influence the activity of synaptic transmission mediated by glutamate receptors.

The endo-N treatment partially recapitulates the phenotype observed in the NCAM deficient mice, suggesting that some roles of NCAM are determined by the attached PSA. Furthermore, since the enzymes catalyzing this process were well characterised, recently mice deficient in these enzymes became available, thus allowing the distinction between NCAM and PSA deficiency. Both PST (Eckhardt et al., 2000) and STX deficient mice (Angata et al., 2004) have impaired LTP in area CA1 of hippocampus, whereas in the area CA3 LTP is normal despite the fact that STX mutants have abnormal lamination of mossy fiber projections (Angata et al., 2004).

The current knowledge regarding the synaptic plasticity in these three mutant mice is presented in table 1. The data summarized in the table were obtained using *in vitro* approaches in the CA1 and CA3 areas of the hippocampus. To date, data regarding the dentate gyrus have not been obtained.

The studies mentioned above show a role for NCAM and PSA in synaptic plasticity, most of the physiology results are complemented by studies which show the involvement of NCAM and PSA in learning and memory. Thus, perturbation of NCAM

LTP	**STX -/-**	**PST -/-**	**NCAM -/-**
LTP in CA1	+	-*	-
LTP in CA3	+	+	-
LTP in DG	?	?	?

Table 1. **The role of NCAM and PSA in synaptic plasticity.** LTP is impaired in the KO mice compared with the WT mice; +, LTP in the KO is normal comparative with the WT; *, just in the adult PST deficient mice.

function with NCAM antibodies caused amnesia in a passive avoidance task (Doyle et al., 1992) and defects in spatial learning in rats (Arami et al., 1996). Both constitutively and conditionally NCAM deficient mice are impaired in spatial learning in the Morris water maze paradigm (Bukalo et al., 2004; Cremer et al., 1994). Regarding the involvement of PSA, it was also shown that PSA-NCAM expression is upregulated 10-24 hours posttraining in the water maze (Sandi et al., 2003; Venero et al., 2006), while intra-hippocampal enzymatic removal of PSA results in spatial learning and memory deficits (Becker et al., 1996; Venero et al., 2006). These kind of deficits were also observed in mice deficient in the STX enzyme (Angata et al., 2004) but surprisingly the mice lacking the PST enzyme show normal spatial learning (Markram et al., 2007)

despite the fact that CA1 LTP was impaired. Furthermore, mutant mice lacking NCAM (Stork et al., 2000) or mice deficient in the polysialyltransferase ST8SiaII (Angata et al., 2004) show impaired contextual and cued fear memories, the contrary being true for mice deficient in ST8SiaIV which have intact fear conditioned tone memory (Markram et al., 2007). It is thus obvious that the role of PSA in synaptic plasticity and in learning and memory is far from being fully understood, but all the results point to a rather selective involvement of this molecule in the processes mentioned above.

IV.3. Extracellular matrix molecules

IV.3.1. General properties of the tenascin family

The extracellular matrix (ECM) is a complex network consisting of different types of molecules such as collagens, proteoglycans and glycoproteins. These molecules are, on one side, able to interact with each other and on the other side capable of activating signal transduction pathways via diverse cell-surface receptors, being able, in the end to coordinate cell functions such as proliferation, differentiation, migration and survival.

A particular class of glycoproteins is the tenascin family which is present throughout our body (Chiquet-Ehrismann and Chiquet, 2003; Jones and Jones, 2000). They are considered to be unique to vertebrates since this gene family has not been found outside the phylum *Chordata* and are not present in *C. elegans* or *Drosophila* (Erickson, 1993; Rubin et al., 1999). The tenascin family is composed of the following members: TN-C, TN-R, TN-X, TN-Y and the most recently characterised member TN-W described in zebrafish (Weber et al., 1998) and mouse (Scherberich et al., 2004), also referred to as TN-N in (Neidhardt et al., 2003).

All tenascins are built from a common set of structural motifs as represented in Fig. 4. The protein modules consist of four domains: a cysteine-rich amino-terminal or tenascin assembly domain (TA), epidermal growth factor-like repeats (EGFL), fibronectin type III domains (FN-III) and a C-terminal globular domain shared with fibrinogens (fibrinogen globe-FC). These proteins module are lined up like beads on a string and give rise to long and extended molecules.

The presence of tenascins during developmental and pathological states is now well recognized but still not well understood. All members of the family share the characteristic of having tightly regulated expression during development and throughout an organism's life. During adulthood, various tenascins have been implicated in pathological states such as tissue injury and tumorigenesis when remodeling processes

are prominent. Since the tenascins-C and -R molecules will be discussed later in greater detail, a short characterisation of the other members of the family is given below.

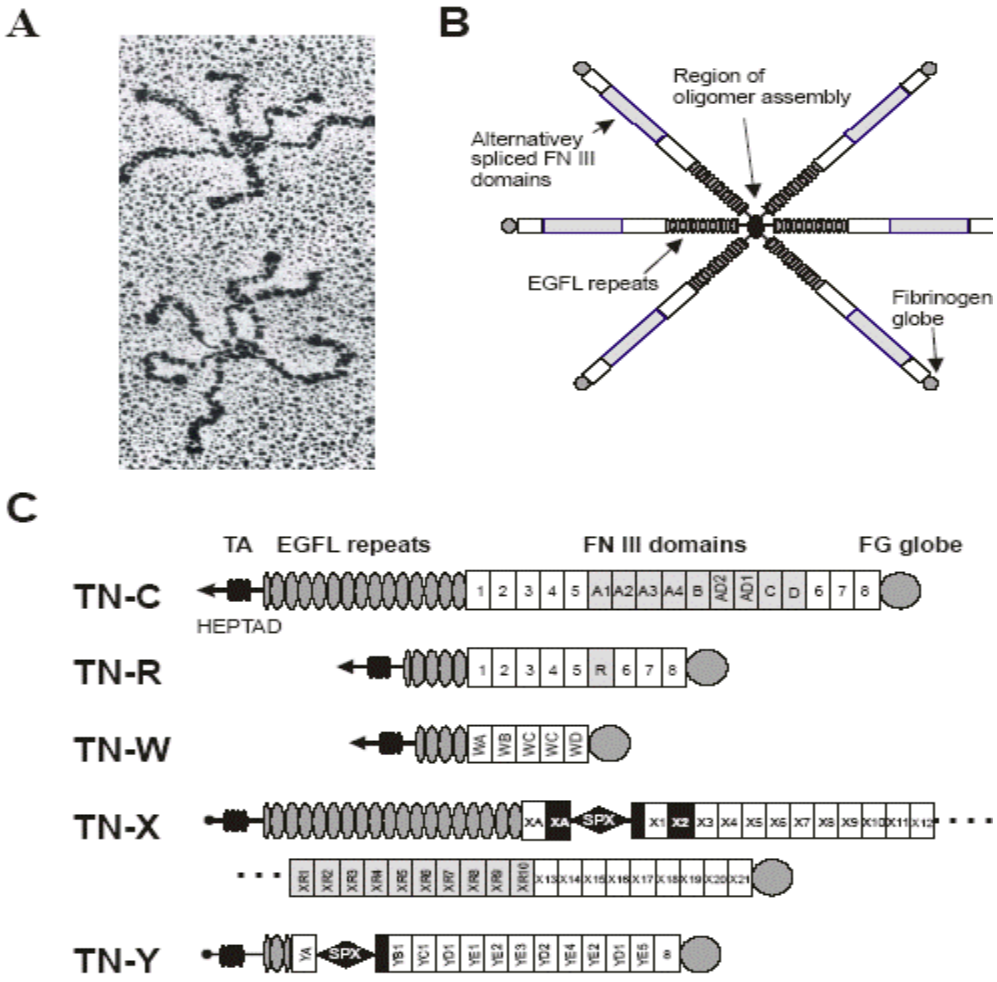

Figure 4. **Tenascin family structure.** A. Rotary shadowing images of two mouse TN-C hexabrachions. Each arm is circa 100 nm in length. B. Model of the TN-C hexabrachion. The tenascin assembly domain links six TN-C chains via heptad repeats. C. Schematic diagrams known tenascins. Proceeding in a carboxiterminal direction, the domains are: TA domain, an array of EGFL repeats (ovals), two types of FN-III domains: those conserved in all variants of TN-C (white rectangles) and those that are alternatively spliced (gray rectangles), and the terminal fibrinogen globe (circle). The figure has been adapted from Jones and Jones (2000).

TN-X is the largest known member of the family and widely expressed during development. Adult expression however is mostly limited to musculoskeletal, cardiac and dermis tissue. It is the first tenascin whose deficiency has been clearly associated with a pathological disorder on humans, a connective tissue disorder known as Ehler-Danlos Syndrome, which is associated with fibrillar collagen defects. Patients suffering

from this disease, show clinical symptoms consistent with ECM structural defects including skin and joint laxity, vascular fragility and poor wound healing.

TN-Y is expressed in connective tissues but also occurs in the brain.

TN-W has been identified in the zebrafish, where it is predominately expressed in neural crest pathways and colocalizes with TN-C in several different tissues during development (Weber et al., 1998). A recent study showed that TN-W is widely expressed in the mammary tumors of mice known to develop metastases but not in non-metastatic tumors (Scherberich et al., 2005).

IV.3.2. Tenascin-C

The first member of the tenascin family which is now known as tenascin-cytotactin (TN-C) was discovered more then 20 years ago (Bourdon et al., 1983). Several groups simultaneously identified the protein and different names were proposed, of which tenascin, proposed by Chiquet and Fambrough (Chiquet and Fambrough, 1984), finally survived. As the first tenascin to be identified, TN-C remains the best characterised member of the family, accounting for most reports examining the component domains shared by tenascin family proteins.

IV.3.2.1. Structure of tenascin-C

All members of the tenascin family except the TN-W have splice variants which alter the number of FN-III domains in the molecule (Jones and Jones, 2000). For example, all the TN-C molecules have eight FN-III domains (FN-III 1-8), whereas additional nine distinctive repeats can be independently included or excluded depending on the splice variant. Thus, the TN-C molecule shows the greatest number and diversity of isoforms with as many as 27 different mRNA variants having been identified in the developing mouse brain (Joester and Faissner, 1999).

Until recently TN-C was considered to be a unique member of the family because it seemed that it was the only one able to form hexamers. A recent study published in 2004 using rotary shadowing followed by electron microscopy showed that TN-W, like TN-C is able to form hexabranchions (Scherberich et al., 2004). In the latter case, this structure is the result of the interactions between TA domains, which in a first step will form trimers and in a second step, these trimers are assembled into the so called hexabranchion which is stabilized by disulfide bonds between cysteine-residues in the subunits (Kammerer et al., 1998). The TN-C hexamer consists of six arms which

are elongating from a central core. The proximal parts of these arms are thin and rigid being made of EGFL repeats while the distal parts are thick and flexible consisting of FN-III domains. The end of the arms contains an electron dense globular particle domain that resembles the carboxyl terminal portion of the β and γ chains of fibrinogen (Fig. 4).

It was reported that TN-C can also form nanomers (Schenk and Chiquet-Ehrismann, 1994), thus one can assume that native TN-C molecules exist predominately as multiples of three. The hexabranchion structure is mainly made of one type of monomer, but heterotypic hexamers have been also observed. In these structures, a trimer containing a particular variant of TN-C is linked to a trimer containing a different variant (Fischer et al., 1995).

IV.3.2.2. Tenascin-C expression

TN-C has been detected as early as embryonic day 10 in the developing mouse nervous system (Kawano et al., 1995). It is transiently expressed during organogenesis and is absent or much reduced in the developed organs (Chiquet-Ehrismann and Chiquet, 2003). The molecule is mainly secreted by immature and reactive astrocytes and by subsets of radial glia cells. In addition, a restricted number of immature neurons including granule cells in the hippocampus and some motoneurons of the spinal cord as well as a subclass of neurons of the developing retina produce TN-C (Bartsch, 1996).

While TN-C is absent from most regions of the normal adult brain, the protein persists in areas known to retain a high degree of plasticity, in particular in the nuclei of the hypothalamus which are implicated in endocrine regulation (Theodosis et al., 1997). It was also detected in the olfactory system which permits continuous neuronal regeneration and axonal growth throughout life (Gonzalez and Silver, 1994).

Upregulation of human TN-C has been described in hippocampal sclerosis of children and young adults (Scheffler et al., 1997). Several studies have found a strong association of TN-C expression with gliomas and astrocytomas of the CNS and as well with cancers of other organs (Gladson, 1999; Scherberich et al., 2005).

IV.3.2.3. Tenascin-C and synaptic plasticity

The connection between TN-C expression and synaptic plasticity was first recognized when TN-C was found to be upregulated after chemical (Nakic et al., 1996) and electrical (Nakic et al., 1998) stimulation of the rat hippocampus. Despite the fact that

gross hippocampal architecture (Evers et al., 2002) and a series of behavioral tests (Morellini and Schachner, 2006) showed no striking abnormalities in the TN-C deficient mice, selective impairments of several forms of synaptic plasticity have been reported.

Short-term plasticity measured in the cerebellum showed that the level of inhibition at the climbing fibers-Purkinje cells synapses is not as strong as in the wild-type, while the level of facilitation measured at the parallel fibers-Purkinje cells synapses has a more complex behavior: some cells present higher levels of facilitation than others which actually show depression (Andjus et al., 2005). Long-term synaptic plasticity studies performed in the hippocampus showed that TN-C deficient mice have normal basal synaptic plasticity, reduced LTP and completely abolished LTD at the Schaffer collaterals-CA1 synapse, whereas LTPs at the mossy fibers-CA3 and lateral/medial perforant path-DG synapses are normal (Evers et al., 2002). The impaired synaptic plasticity observed at the SC-CA1 synapse was proposed to be due to VDCCs (Evers et al., 2002). It was also shown that intrahippocampal injection of the FN 6-8 but not of the FN 3-5 repeats impairs retention of memory in a step-down paradigm and reduce levels of LTP in area CA1 (Strekalova et al., 2002).

IV.3.3. Tenascin-R

IV.3.3.1. Structure of tenascin-R

As mentioned earlier the structure of TN-R is similar to that of TN-C, having the same structural motifs (Fig. 4). The homology between FN-III repeats of TN-R and TN-C is about 70%, emphasizing the homogenity of both glycoproteins (Pesheva et al., 1989). TN-R is expressed in the CNS of different vertebrates as two major molecular forms of 160 kD and 180 kD (Pesheva et al., 1989). The former isoform tends to form dimers while the larger one forms trimers (Pesheva and Probstmeier, 2000).

IV.3.3.2. Tenascin-R expression

TN-R is synthesized and secreted by oligodendrocytes during development and adulthood (Bartsch et al., 1993; Wintergerst et al., 1993) and its expression seems to be limited exclusively to the CNS, although it was reported to be also present in a cell line originating from the PNS (Probstmeier et al., 2001). In CNS it is expressed on the one hand by oligodendrocytes with highest expression during the period of active myelination and on the other hand by subpopulations of neurons such as horizontal cells

in the retina, Purkinje and basket cells in the cerebellum, motoneurons in the spinal cord and some subtypes of interneurons in the hippocampus (Wintergerst et al., 1993). It was also found to be localized in distinct zones of the olfactory bulb (Saghatelyan et al., 2004). It was reported that, in the hippocampus, TN-R is located between fasciculating non-myelinated axon bundles of mossy fibers and axons of the perforant path, between cell somata of CA3 pyramidal neurons and at the gap junction between astrocytic processes (Schuster et al., 2001).

In contrast to oligodendrocytes, protein expression in neurons does not decrease after the postnatal period and the secreted protein is concentrated in perineuronal nets that surround different types of neurons in many brain regions (Bruckner et al., 2000). Perineuronal nets appear during the first postnatal weeks, possibly playing a role in synaptic stabilization (Celio and Blumcke, 1994; Celio et al., 1998). TN-R immunoreactivity in perineuronal nets increases to the adult level between postnatal days 21 and 40 (Bruckner et al., 2000) and the lack of TN-R leads to abnormal distribution and shape of these particular nets (Weber et al., 1999).

IV.3.3.3. Tenascin-R and synaptic plasticity

In vitro electrophysiological analysis has shown that TN-R deficiency causes impairment of synaptic transmission and synaptic plasticity in the CA1 region of the hippocampus, increase in basal excitatory synaptic transmission in CA3-CA1 synapses and reduction of perisomatic inhibition of pyramidal cells (Bukalo et al., 2001; Saghatelyan et al., 2001; Saghatelyan et al., 2000). The reduction of perisomatic inhibition seen initially in the hippocampus was also found in the cortex of the TN-R deficient animals, which show enhanced cortical EEG and cortical auditory-evoked potentials (Gurevicius et al., 2004). A series of studies suggest that the reported hippocampal physiological abnormalities seen in these mutants are due to a deficiency in the HNK-1 carbohydrate which is carried by the TN-R. In normal conditions, TN-R via HNK-1 inhibits postsynaptic $GABA_B$ receptors, thus GABA released from the presynaptic terminals activates just the postsynaptic $GABA_A$ receptors (Saghatelyan et al., 2001). TN-R deficiency causes an activation of the $GABA_B$ receptors that will eventually result in an increase in the K^+ conductance, which inhibits evoked GABA release via retrograde signalling (Saghatelyan et al., 2001). This model is additionally supported by the fact that in the TN-R mutant mice, the postsynaptic $GABA_B$ receptor mediated currents are increased (Saghatelyan et al., 2003). Thus, TN-R/HNK-1 ablation

causes a reduction in GABAergic inhibition and impaired LTP, which was recently shown to be due to a 10mV shift in the threshold for LTP induction (Bukalo et al., 2007).

IV.4. Neurogenesis in the adult brain

IV.4.1. General features of neurogenesis

Prenatal development of the mammalian brain involves a complex series of precisely timed events, which can be divided into different phases: 1) generation of new cells from stem cells; 2) migration of new neurons to the target brain regions; 3) differentiation of new neurons into mature synaptically active cells and 4) death of neurons via apoptosis. For more than a century, neuroanatomists have speculated about the capacity of the adult brain to change its structure (Stahnisch and Nitsch, 2002), but it was not until about forty years ago when Joseph Altman and colleagues demonstrated the possibility of a persistent neurogenesis in the brain of adult rodents (Altman and Das, 1965). Initially this observation passed unnoticed until 1977 when, using electron microscopy, Kaplan and Hinds confirmed the fact that the brain is capable of adult neurogenesis in at least some areas of the adult rodent brain, such as the dentate gyrus of the hippocampal formation and the olfactory bulb (Kaplan and Hinds, 1977). The new neurons derive from multipotent neural progenitor cells (NPCs) (Gage et al., 1998) and although the NPCs are ubiquitous in the adult brain, strikingly, as mentioned above, neurogenesis is very much restricted to the subgranular zone (SGZ) of the DG and the subventricular zone (SVZ) of the lateral ventricles. The SGZ and SVZ give rise primarily to the dentate gyrus granule cells and interneurons of the olfactory bulb.

Over the following years, a series of papers show evidence of this phenomenon in the adult brain but only during the last decade has neurogenesis received intense attention by the neuroscience community. Until now, adult neurogenesis has been observed in the hippocampus of virtually every mammalian species examined, including mice, rats, cats, rabbits, guinea pigs, macaques and humans (Altman and Das, 1965; Eriksson et al., 1998; Gould et al., 1999b; Kaplan and Hinds, 1977). For most of these species the same holds true also for the SVZ but in the case of humans, adult neurogenesis in the olfactory bulb remained in question until recently. Two studies published in 2004 on this subject reached different conclusions: one reported that, as in rodents, new neurons are generated in the SVZ and migrate, via the rostral migratory stream, to the olfactory bulb (Bedard and Parent, 2004), whereas the other did not find

such evidence (Sanai et al., 2004). However, a recent paper convincingly demonstrated that adult neurogenesis in the olfactory bulb not only occurs in humans but is a robust phenomenon, comparable in magnitude to that of rodents (Curtis et al., 2007). Although adult neurogenesis has been reported to take place also in other regions of the adult brain like neocortex (Gould et al., 2001), striatum (Dayer et al., 2005), amygdala (Bernier et al., 2002), hypothalamus (Kokoeva et al., 2005), as well as in the brain stem (Bauer et al., 2005), the existent data are conflicting. Even if these regions are capable of adult neurogenesis, the number of generated new neurons is invariably fewer than those reported for the hippocampus and olfactory bulb which probably will explain the contradictory findings. Since the topic of this thesis is dealing with studying the role of NCAM in neurogenesis in the dentate gyrus area using an electrophysiological approach, I will focus mainly on the present state of literature regarding hippocampal neurogenesis.

In the hippocampus, the proliferative cells are located in a germinal zone along the border between the granule layer and the hilus of the DG (Altman and Bayer, 1990) and give rise to new neurons and glial cells. It is important to note that neurogenesis is not taking place throughout the germinal zone, since recently it was discovered in rats the existence of anatomically identifiable 'nerogenically quiescent zone' that consistently lacks neurogenesis under basal conditions, but when stimulated can undergo proliferation (Melvin et al., 2007).

The first step of adult neurogenesis, termed *proliferation*, is the unidirectional division of neural stem cells, to generate another stem cell as well as a neural progenitor cell. The latter starts migrating through the granule cell layer while simultaneously differentiating from an undifferentiated neuroblast to a mature neuron. The second step is the *survival* step, as only about 10% of newborn cells undergo terminal differentiation to functional neurons.

Newborn neurons strongly but transiently express, until three weeks of age, (Seki, 2002a; Seki, 2002b) PSA-NCAM along with a series of other biochemical markers like NeuN and calbidin. PSA-NCAM has a particular importance since it helps cell movement by inhibiting cell-to-cell adhesion. Within four to ten days, these new neurons project their axons toward the CA3 region where they contact pyramidal cells and hilar interneurons and their dendrites extend toward the molecular layer and continue to grow for several weeks.

The first synaptic input received is excitatory GABAergic and precedes that of other neurotransmitters (Tozuka et al., 2005; Wang et al., 2005), thus from a morphological and electrophysiological point of view, all these integration processes are a complete recapitulation of neuronal development (Ben-Ari, 2002; Ming and Song, 2005).

GABA inputs are thought to initiate and promote differentiation of new born neurons in the adult brain with glutamatergic inputs shortly following. Glutamate signaling has been proposed to promote neurogenesis via group I mGluR (Baskys et al., 2005), while the stimulation of NMDA and AMPA receptors by glutamate appears to decrease neurogenesis (Kitayama et al., 2003; Poulsen et al., 2005).

Most of these newly generated neurons in DG will differentiate into granule cells, but a small proportion (about 14%) will become GABAergic basket cells (Liu et al., 2003). Although these findings suggest that two subpopulations of new neurons exist in this brain region (one with excitatory and one with inhibitory properties), an alternative possibility is that new neurons temporarily exhibit characteristics of inhibitory neurons as part of a natural process of maturation (see above).

Several factors and conditions have been shown to affect the number of new neurons in the DG of adult vertebrates. Well-studied negative regulators include drugs, alcohol, stress and aging. The last two factors affect this process via the action of glucocorticoids. Similarly, there are a series of factors which increase neurogenesis like, physical exercise (van Praag et al., 1999), enriched environment, antidepressants (Malberg et al., 2000) and mood stabilizers (Duman, 2002). In several species, including rat and vole, the proliferation of new neurons is influenced both by gender and endogenous levels of the gonadotropic steroid hormone estradiol (Tanapat et al., 2005), whereas no such differences are present in adult mice (Lagace et al., 2007). There are individual differences in the reaction to the same condition, for example, the reaction to novelty. Rats that are highly reactive to novelty exhibit lower levels of cell proliferation and therefore of neurogenesis or survival than their less reactive counterparts (Lemaire et al., 1999). The same has also been shown for aggressive behavior. Dominant animals have substantially more new neurons than subordinate ones (Kozorovitskiy and Gould, 2004).

Alongside neurogenesis, there is substantial death of granule cells, especially of newborn neurons in the adult rodents and primates under control conditions. Neuron number in the DG appears to be tightly regulated. In addition to massive cell

production, neurite extension and synaptogenesis, there is massive cell death, neurite retraction and synapse elimination.

IV.4.2. Neurogenesis and synaptic plasticity

The granule cell layer consists of neurons ranging in age from hours to years. In young adult rats, the number of newly generated cells makes up about 6% of the total size of the GC layer within one month (Cameron and McKay, 2001). Thus, even though many thousands of new neurons are added to the DG every day; this is a relatively small proportion of the large number of mature cells produced during development and that survive through the adulthood. For the newly cells to have an impact on adult hippocampal function, they must have unique properties that empower them. Growing evidence suggests that immature neurons may be functionally stronger, having a series of physiological properties that differ from those of mature neurons, thus they might serve as the major mediators for structural plasticity. For example, it was shown that PSA-NCAM^{+} newborn DGCs have distinct membrane properties: a) exhibit an enhanced excitability due to their very high input resistance (Schmidt-Hieber et al., 2004; Wang et al., 2000) and to the expression of low-threshold T-type Ca^{2+} channels (Schmidt-Hieber et al, 2004) and b) have slow membrane time constant (~120ms) and therefore, a slow decay time course of the EPSPs, leading to the effective temporal summation of the excitatory synaptic inputs. These cells are at the same functional stage as the neonatal-generated granule cells, since when compared, their physiological properties are indistinguishable (Overstreet-Wadiche et al., 2006) and the expression of the Ca^{2+} binding protein calbindin is lower then in the mature ones, thus having a low Ca^{2+} buffering capacity. Of particular interest is the finding that new neurons have a lower threshold for LTP induction and exhibit a more robust LTP then the adult ones (Schmidt-Hieber et al., 2004; Wang et al., 2000) and this type of plasticity cannot be inhibited by $GABA_A$ agonists, unlike LTP elicited from mature granule neurons (Snyder et al., 2001).

Considering that long-term potentiation is a prime candidate to explain the mechanisms underlying hippocampal learning and memory, the correlation between animal learning and enhanced neurogenesis suggests a close relationship between LTP and neurogenesis. In support, physical exercise enhanced not only neurogenesis and spatial memory, but also LTP induction in the DG of mice (van Praag et al., 1999) and rats (Farmer et al., 2004). Also, weak gamma radiation impaired both neurogenesis and

the induction of AP5-insensitive, NR2B antagonist-sensitive LTP but had no effect on the induction of AP5-sensitive LTP in rat DG (Snyder et al., 2001), suggesting that the newly generated DG cells contribute to the induction of NR2B mediated LTP. LTP induction in mossy fiber-CA3 synapses resulted in enhanced neurogenesis in the rat DG. These results suggest bi-directional interactions between LTP and neurogenesis: newly generated granule cells contribute to LTP induction (Saxe et al., 2006) and, conversely LTP induction affects both proliferation of progenitor cells and survival of recently born GCs (Bruel-Jungerman et al., 2006).

IV.4.3. Role of neurogenesis in the adult brain

The daily production of thousands of neurons and their incorporation into the existing circuitry are costly in terms of energy consumption. Thus, they have to be play an important role since so many are generated. There are a series of findings, which propose different roles for this phenomenon. An intuitive explanation would be that the continual influx of new cells provides an important mechanism for certain functions of DG that can not be obtained with a structure comprising mature neurons exclusively. Adding new neurons to a already existing network has been suggested to be important for generation of temporal associations in memory (Aimone et al., 2006), for avoiding catastrophic interference when adapting to new environments (Wiskott et al., 2006) or/and for enabling the brain to accommodate continued bouts of novelty, thus preparing the hippocampus for processing greater levels of complexity (Kempermann, 2002).

A widely held view is that the hippocampal formation plays a temporary role in storing new memories. This theory is sustained by a series of studies which show that memories for certain information are eliminated by lesioning the hippocampal formation. Such a rapid change in the pool of granule cells may serve as a substrate for this temporary role of hippocampus in information storage like it was suggested in canaries, where the seasonal changes in song correlate with the transient recruitment of more new neurons into the relevant circuitry (Nottebohm, 2002).

A series of studies indicate a connection between neurogenesis and learning and memory. Activities known to enhance learning and memory, such as exercise or living in an enriched environment, promote neurogenesis in the adult hippocampus (Kempermann et al., 1997; Nilsson et al., 1999; van Praag et al., 1999). Learning itself has been shown to enhance the survival of the newly generated neurons in the dentate

gyrus (Gould et al., 1999a). The most direct evidence so far was demonstrated in an experiment in which inhibition of the neurogenesis using a mitotic inhibitor impaired hippocampal-dependent, but not hippocampal-independent memory (Shors et al., 2001). Inhibition of cell neurogenesis via focal irradiation of the hippocampus impairs performance of a place recognition task in a T maze but not of an object recognition task (Madsen et al., 2003; Rola et al., 2004).

New theories regarding the role of adult neurogenesis came from the endocrinological field. Investigation of the effects of early stressors on the development of the HPA axis and on adult neurogenesis revealed potential link between adult generated cells and hippocampal neuroendocrine function. For example, early stressors have been show to generate persistent changes in the HPA axis and to decrease the production of new neurons during adulthood (Coe et al., 2003; Lemaire et al., 2000).

A growing body of evidence points to a role of new granule cells in the etiology of a series of diseases like schizophrenia and epilepsy. Two molecules have been recently implicated both in adult neurogenesis and in pathology of schizophrenia: reelin and the transcription factor neuronal PAS domain protein 3 (NPAS3). Reelin deficiency reduces and disorganizes (newborn cells do not align along the SGZ, but scattered all over the DG) adult neurogenesis in reeler mice (Won et al., 2006). NPAS3 knockout mice show about 84% decrease the hippocampal neurogenesis (Pieper et al., 2005) and display several schizophrenia-like behavior abnormalities. Epileptic seizures can increase the rate of neurogenesis in the adult DG, and newly generated neurons can migrate to abnormal locations (hilus), become engaged in synchronous neuronal activity and develop aberrant connections (Scharfman et al., 2000).

Even though this field of research has develop incredibly fast during the last decade and the different approaches of the experiments clarified some of the important features of these newborn neurons, the question of the fundamental biological significance of adult neurogenesis still remains open.

V. AIMS of the STUDY

During the last twelve years, a series of studies showed the importance of NCAM and its polysialylated form, PSA-NCAM, in hippocampal synaptic plasticity and hippocampus-dependent forms of learning and memory. To relate behavioral and synaptic abnormalities in mice deficient in NCAM or in one of two major enzymes necessary for polysialylation of NCAM, it is important to have a complete picture of synaptic physiology in all major excitatory synapses in the hippocampi of these mutants. The roles of NCAM and PSA in synaptic plasticity have been analyzed *in vitro* in the CA1 or CA3 regions of hippocampus, while the dentate gyrus was completely unexplored. In order to fill this gap in our knowledge and to extend the analysis of synaptic plasticity to *in vivo* situation, the first aim of this study was to investigate the role of NCAM and PSA-NCAM in synaptic plasticity at the perforant path-dentate gyrus synapse in anesthetized mice deficient in either of these molecules. Since the dentate gyrus is one of the two main areas capable of neurogenesis in the adult brain and immature neurons in the dentate gyrus strongly express PSA-NCAM, my second aim was to find out whether NCAM is required for plasticity of synapses formed on these immature neurons.

Not only cell adhesion molecules are important players in synaptic plasticity, but also tenascin-C and tenascin-R, two members of the tenascin family of extracellular matrix molecules have been shown to modulate long-term potentiation in the CA1 subfield of hippocampus. As for NCAM, there were no data regarding the role of these molecules in synaptic plasticity *in vivo*. Therefore, the third aim of the present study was to verify whether any of abnormalities observed in mice deficient in tenascins in the CA1 subfield are also present in the dentate gyrus. In order to reach this aim, we performed electrophysiological recordings at the perforant path-dentate gyrus synapse in anesthetized mice deficient in tenascin-C or tenascin-R. Furthermore, we extended analysis of tenascin-R deficient mice by performing *in vitro* analysis of synaptic plasticity at the associational/commissural synapse in the CA3 region.

VI. MATERIALS and METHODS

VI.1. Mice used in this study

VI.1.1. Constitutive NCAM deficient mice

NCAM deficient mice used in this study were generated by Cremer and co-workers (Cremer et al., 1994) and were backcrossed for more than 8 generations on the C57BL6J background.

These mice have been reported to show a reduced size of the olfactory bulb and deficits in cell migration, lamination of the CA3 mossy fiber projections and spatial learning (Bruses and Rutishauser, 2001; Cremer et al., 1998; Cremer et al., 2000; Cremer et al., 1994; Muller et al., 1996; Tomasiewicz et al., 1993). Also LTP in CA1 and CA3 was abnormal (Bruses and Rutishauser, 2001; Cremer et al., 1998; Muller et al., 1996; Tomasiewicz et al., 1993) and these mice showed increased aggression and anxiety (Stork et al., 1997; Stork et al., 2000).

VI.1.2. PSA deficient mice

Mice deficient in polysialyltransferases PST (ST8SiaIV) or STX (ST8SiaII) which are the two major enzymes necessary for synthesis of PSA in the brain, were obtained by introducing SV129Ola embryonic stem cells carrying a mutation either in the PST or STX gene into C57BL/6J mice (Angata et al., 2004; Eckhardt et al., 2000). These mice were back-crossed for six generations onto the C57BL/6J background. Heterozygous mice were then intercrossed to obtain homozygous mice and strains were maintained as homozygous lines for several generations (Weinhold et al., 2005). Both strains, STX and PST, have the same genetic background, as they bear the same percentage of C57BL/6J and SV129Ola genome, and therefore only one group of wild-type mice was used for comparison with STX -/- and PST -/- mice.

VI.1.2.1. PST deficient mice

These mice have normal development and normal morphological characteristics like migration of neural precursors and fasciculation of mossy fibers but they show a

specific loss of PSA in mossy fibers and in the CA1 region in adults. This loss leads to an impaired synaptic plasticity at the adult CA3-CA1 synapse, whereas at the DG-CA3 synapse LTP is normal (Eckhardt et al., 2000). In young animals both LTP and LTD are normal in CA1 region of the hippocampus. In terms of behavior, PST deficient mice are impaired in spatial as well as in reversal learning in the water maze. PST -/- mice exhibited no impairments in the acquisition or retention of cued fear memories (Markram et al., 2007), whereas contextual fear memory was impaired (Senkov et al., 2006).

VI.1.2.2. STX deficient mice

In spite the fact, that in embryos at early developmental stages there is a high expression of ST8SiaII enzyme, STX mutants do not have any severe abnormality, developing normally and being fertile. In contrast to the NCAM -/- and PST -/- mice, STX -/- mice have normal synaptic plasticity both in the CA1 and CA3 areas of the hippocampus. The major abnormalities in these mice were observed at the morphological level, namely, the suprapyramidal mossy fiber projections were thinner and the infrapyramidal projections were thicker and much more extended then normal, than in wild-type mice, until CA3a, where they formed ectopic synapses. This abnormality correlated with an increase in exploratory behavior and reduced responses to fear conditioning (Angata et all, 2004).

VI.1.3. Mice deficient in extracellular matrix molecules

VI.1.3.1. Tenascin - C deficient mice

TN-C constitutive deficient and wild-type mice with a mixed C57Bl/6J x 129O/Sv genetic background have been backcrossed for six generations into C57Bl/6J background (Evers et al., 2002). The TN-C -/- animals have reduced long-term potentiation and long-term depression in the CA1 region of the hippocampus but normal LTP at the medial and lateral perforant path - dentate gyrus synapses (Evers et al., 2002). They show subtle but significant morphological abnormalities in the cerebral cortex (Irintchev et al., 2005). TN-C deficiency caused on one hand weaker muscle strength, but normal coordination and motor learning (Morellini and Schachner, 2006) and on the other hand impaired memory in the step-down passive avoidance task (Strekalova et al., 2002).

VI.1.3.2. Tenascin - R deficient mice

TN-R deficient and wild-type mice were obtained from heterozygous breeding animals with a C57BL/6J genetic background after 5 backcrosses of mice from the original C57Bl/6J x 129/Ola colony (Weber et al., 1999). The TN-R -/- mice have increased basal synaptic transmission and impaired LTP in the CA1 region of the hippocampus (Saghatelyan et al., 2001) but normal LTD at the same synapses (Bukalo et al., 2001). The observed impairment is due to a metaplastic increase in the threshold for induction of LTP (Bukalo et al., 2007). TN-R deficient mice show decreased motivation to explore and increased anxiety in the free choice open field, open field and elevated plus maze tests (Freitag et al., 2003). The mutant mice also show motor coordination impairments in the wire hanging, rotarod and pole test (Freitag et al., 2003).

Treatment of animals

All mice were singly housed in pathogen-free, climate-controlled conditions under a 12h light/dark cycle, with free access to standard mouse diet and water. The mice were housed in these quarters for at least 1 week prior to testing. All efforts were made to minimize both the suffering and the number of animals used. All surgical procedures had been approved by the Committee on Animal Health and Care of the local governmental body.

VI.2. *In vivo* experiments (Perforant Path – Dentate Gyrus synapse)

VI.2.1. Pharmacological treatment

In one series of experiments (see chapter VII.2.3.1.), mice were injected with the $GABA_A$ receptor agonist muscimol (1 mg/kg, i.p., in ACSF) or with ACSF 24 h before the beginning of the experiment. At this concentration it was shown that muscimol inhibits morphine-induced hyperactivity of animals (Yoon et al., 2002).

VI.2.2. Anesthesia

Three- to five-month-old male mice were anesthetized with urethane with an initial dose corresponding to 1.5 g/kg of body weight. The mice that were heavier than 30g were injected with an initial dose of 0.045g urethane (1.5 g urethane * 0.03 kg). Approximately 20 minutes following initial anesthesia, all the mice were tested for any

sign of arousal and additional doses of urethane were administrated when needed in order to abolish the hind pinch reflex. In most of the cases, mice needed more than one additional injection. The dosage of supplementary injections was 1/6 of the initial one administrated every 15 - 20 minutes until the animals did not respond to the hind pinch. During the time of recording, the mice were checked visually for any sign of recovering from anesthesia and if needed an additional dose were injected intramuscularly.

VI.2.3. Tracheotomy

A tracheotomy was performed in order to avoid breathing problems. After the beginning of anesthesia, the mice were lying on their back on the plane surface with the frontal legs fixed in order to allow easy access to the ventral neck. A median cervical skin incision (from the upper thorax to the lower jaw) was performed and layerwise skin, fascia, fat and connective tissue covering the thyroid gland were removed. The thyroid lobes were pulled aside and kept retracted in order to have a full access to larynx and trachea. Tracheotomy was performed by a transversal cut between two tracheal rings (using a normal needle) in the middle part of the trachea. A 1 cm-long plastic tube of approximately 1mm outer diameter was inserted into the incised trachea. This procedure did not take longer than 5-6 minutes and every effort was made to prevent bleeding. After the insertion of the tube the thyroidal lobes and the skin were pulled back to cover the trachea.

VI.2.4. Fixation in the stereotaxic frame

Following the tracheotomy, mice were fixed in a stereotaxic frame (Narishige, Tokyo, Japan). The temperature of the mice was then constantly monitored via a rectal probe and was kept at approx. 36^{o}C using a heating pad (HSE-Harvard, March-Hugstetten, Germany). Before fixing the mouse head, mice were visually inspected for 10 min for any sign of breathing problems or discomfort.
The head was positioned by means of a mouse nose clamp adaptor supplemented by ear bars. The position of the head was adjusted so that the height of the skull surface at bregma and lamda (the intersection of sagittal suture with lambdoid suture) was the same. The dorsal skull was exposed by a midsagittal incision through the scalp. The soft tissue was removed from the skull using cotton applicators soaked with ethanol. Then the surface was dried in order to have a better visibility. Pencil marks were made on the

skull centered at the intended penetration sites. Two holes were drilled ipsilaterally at the following coordinates:

a) AP: 2.0 mm; Lat: 1.5 mm (for the recording electrode)
b) 0.5 mm anterior to lambda, Lat: 3.5-4.00 mm (for the stimulating electrode).

An additional hole was drilled on the surface of the contralateral frontal cortex for the ground electrode.

Sometimes a bleeding occurred during the drilling procedure. In this case a cotton tip was applied to the bleeding site till it stopped. After all holes were drilled, the skull was washed with the normal saline solution (NaCl 0.9%) and then dried. The holes were then inspected using a magnifying glass to ensure that the dura was properly removed and the penetration sites are not occluded by thin layer of residual bone.

Insertion of the electrodes

After the holes were drilled, the homemade electrodes were placed in the proper positions:

a) a bipolar stimulating electrode (made from 75 μm Teflon-insulated stainless steel wires) was placed in the angular bundle at the coordinates mentioned in section VI.2.4 under an angle of 70 degrees between the skull and sagittal plane.
b) The recording electrode was placed perpendicularly to the skull near the granule cell layer at the coordinates mentioned in section VI.2.4. Through the study, the recording electrode was made of metal; the only exception was in the case of the pharmacological experiments where glass pipettes were used (see chapter VII.2.3.2.).

The electrodes were positioned stereotaxically as precisely as possible using a piezo-driven micromanipulator in the case of the recording electrode and a manipulation tower for the stimulating one. The electrodes were advanced until the waveforms with a large positive field EPSP and a superimposed negative population spike were evoked. Electrodes depth was adjusted in order to maximize the field responses to a biphasic pulse of 100μs and 100-500μA. The recorded signals were amplified x 1000 and filtered (0.1-5 kHz bandpass) using an AC amplifier (A-M Systems) and digitized using a Pico ADC board. The signals were acquired and measured using the LTP Program (Anderson and Collingridge, 2001).

VI.2.5. Stimulation Protocols

After the identification of characteristic responses at the perforant path-dentate gyrus synapses, two different parameters were measured: slope of the fEPSP and the so-called population spike (pop-spike or PS) (Fig.5) and a series of protocols were applied (see below). The slope of fEPSP represents a measure of the synaptic strength, whereas the amplitude of population-spike is a measure of synchronous excitatory activity describing the firing of the cells in the recording area.

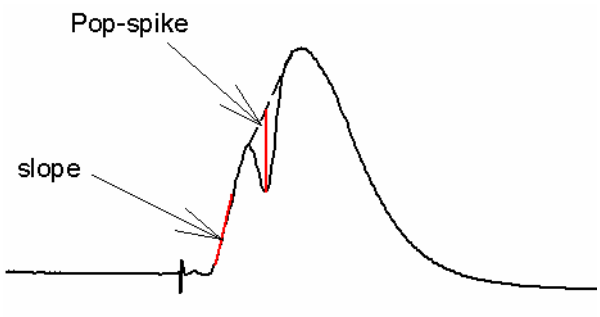

Figure 5. **Responses elicited by stimulation of PP in the DG are characterized by two main parameters.** Slope of fEPSP at the initial positive phase of the fEPSP offers information about the synaptic strength, whereas the amplitude of pop-spike, the negative inflection, describes the sum of action potentials recorded from granule cells extracellularly.

1) Stimulus-response curves. At the beginning of the experiment, the relationship between the stimulation current and evoked response was determined in order to estimate basal synaptic transmission (data not shown).

2) Analysis of paired-pulse facilitation at different interpulse intervals (10, 25, 50, 100 ms).

This protocol was applied in order to estimate the short-term plasticity in the dentate gyrus. The intensity of stimulation used was rather small (25% of supramaximal level) to avoid firing of neurons. The slopes of each pair of responses were computed, and the slope of the second fEPSP was expressed as a percentage of the first one. Five sweeps were averaged for each inter-pulse interval and the averaged fEPSPs were used for measurements.

3) Analysis of paired-pulse depression at different interpulse intervals (10, 25, 50, 100, 200, 500, 1000 ms). This protocol was applied in order to estimate the levels of feed-back inhibition. The stimulation currents were set to evoke a maximal population spike. Population spike amplitudes for each of the pair of responses were compared and the

amplitude of second population spike was expressed as the percentage of the first one. Five consecutive responses were averaged for each inter-pulse interval and the averaged response was used for measurements.

4) Pre-tetanus baseline. The intensity of stimulation was adjusted to evoke a population spike of 2-3 mV. The baselines for slope and population spike for each animal were calculated over 20 minutes period prior to the tetanization. All the values shown represent the averages of 5 consecutive sweeps. If the responses were not stable during the baseline recording, the intensity was readjusted and the baseline recording was repeated.

5) LTP induction. Following a stable baseline, LTP was induced by theta-burst stimulation (TBS) of the perforant path. TBS consisted of 6 series of 6 trains of 6 stimuli applied at 400 Hz, 200 ms between trains and 20 s between series (Davis et al., 1997). The intensity of stimulation used during TBS was twice higher that compared with the intensity used for the baseline recording.

6) Long-term potentiation was recorded for 3 hours at the same intensity as used for the pre-TBS baseline. Again, 5 consecutive responses were averaged and the slope and population spike amplitude at all time points were expressed as the percentage of the mean value of the baseline.

During my master diploma, I established this technique, and at that time we confirmed that the coordinates mentioned above for electrode positioning were the right ones, as verified by using a morphological analysis.

VI.3. *In vitro* experiments

VI.3.1. Preparation of hippocampal slices for field recordings

Mice were anesthetized with CO_2 and the brain was quickly removed in the dissection artificial cerebrospinal fluid (dACSF). The cerebellum was removed and the two hemispheres were separated. Depending of the type of synapse studied different kind of slices with a 350 µm thickness were prepared: a) transversal slices for recordings in CA3 - CA1 and MPP - DG synapses and b) horizontal slices for recordings at the A/C synapse. After preparation, the slices were kept at room temperature for two hours, in an approximately 500 ml incubation chamber filled with ACSF (table 2 and table 3). During the incubation and recording time, slices were kept in ACSF and continuously perfused with 95% CO_2 and 5% O_2) at a rate of 3 ml/min (Bukalo and Dityatev, 2006).

Compound	NaCl	KCl	NaH_2PO_4	$NaHCO_3$	$MgCl_2$	$CaCl_2$	Glucose
Molecular Weight	58.44	74.55	138.0	84.01	203.3	147.0	180.2
Molarity (mM)	120	2.5	1.25	24	1.5	2.0	27.5

Table 2. **The composition of ACSF.** The final pH was adjusted to 7.35 – 7.4.

Compound	KCl	NaH_2PO_4	$NaHCO_3$	MgSO4	$CaCl_2$	Glucose	Sucrose
Molecular Weight	74.55	138.0	84.01	246.5	147.0	180.2	342.3
Molarity (mM)	2.5	1.25	24	1.5	2.0	2.5	250

Table 3**. The composition of dACSF.** The final pH was adjusted to 7.35 – 7.4.

VI.3.2. Recordings of extracellular field excitatory responses

Excitatory postsynaptic potentials (EPSP) were recorded by glass pipettes filled with ASCF and having a resistance of 2-3 MΩ. Stimulation pulses of 0.2 ms were applied via monopolar stimulating glass electrodes with resistance of < 1 MΩ. In order to activate a higher number of fibers, the tip of the stimulating electrode was gently broken. Data acquisition and analysis were performed using the Pulse program (Heka Electronik, Lambrecht/Pfalz, Germany).

For all the *in vitro* experiments, short-term plasticity (paired-pulse facilitation or depression) was studied at an intensity of 50 % of the maximum response.

VI.3.2.1. Recordings at CA3-CA1 synapse

The stimulating electrode and the recording electrode were placed in *str. radiatum* (as shown in the Fig. 6). Stimulation is applied to the CA3 axons every 20 seconds and both the electrodes were slowly advanced until they evoke maximal amplitude of EPSP. LTP was induced using a TBS protocol which consists of 10 burst delivered at 5 Hz. Each burst consisted of four pulses delivered at 100 Hz. The inter-TBS interval was 20 seconds and in total 4 TBSs were applied at the intensity of stimulation being 50% of the supramaximal one (Bukalo and Dityatev, 2006).

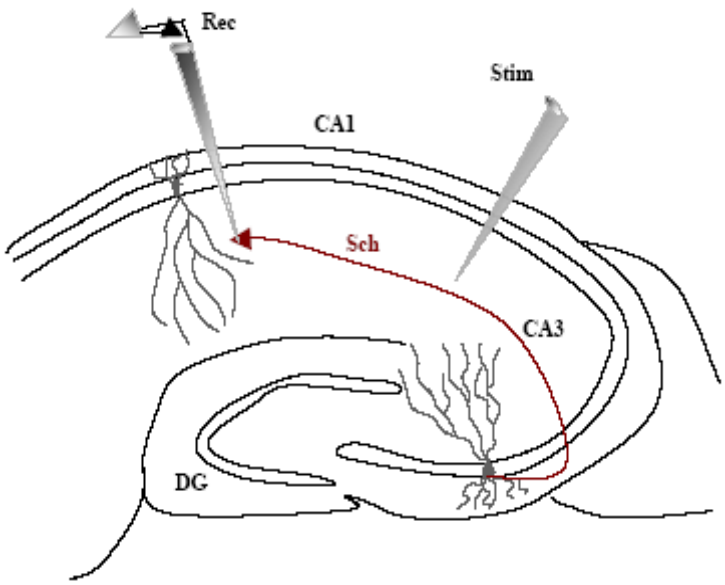

Figure 6. **Positions of the recording (Rec) and stimulating (Stim) electrodes used to evoke responses at the CA3-CA1 synapse.** From Eka Lepsveridze's PhD thesis.

VI.3.2.2. Recording at the A/C synapse

The stimulating electrode was positioned in the stratum radiatum of the CA1 field and activated associational projections antidromically (Fig. 7). We used this approach to avoid direct stimulation of local CA3 interneurons. The recording electrode was placed in the stratum radiatum of the CA3 field, where CA3-CA3 synapses are located. LTP was induced by two 1 second trains of HFS delivered at 100 Hz with 20s inter-train interval with a stimulation strength being 50% of the supramaximal one (Ito et al., 1997).

VI.3.2.3. Recording at the MPP - DG synapse

The field potential recordings were performed in the medial molecular layer of the dentate gyrus in response to stimulation of the medial perforant pathway (as shown in the Fig. 8). At this synapse, stimulation with an interpulse interval of 50 ms gives rise to depression of the second response, in contrast to the stimulation of lateral perforant path (LPP) in which it elicits facilitation. The baseline frequency of stimulation was 0.05Hz. At this synapse, two different forms of synaptic plasticity were recorded: LTP of the adult granule cells and LTP of the newly generated neurons in dentate gyrus.

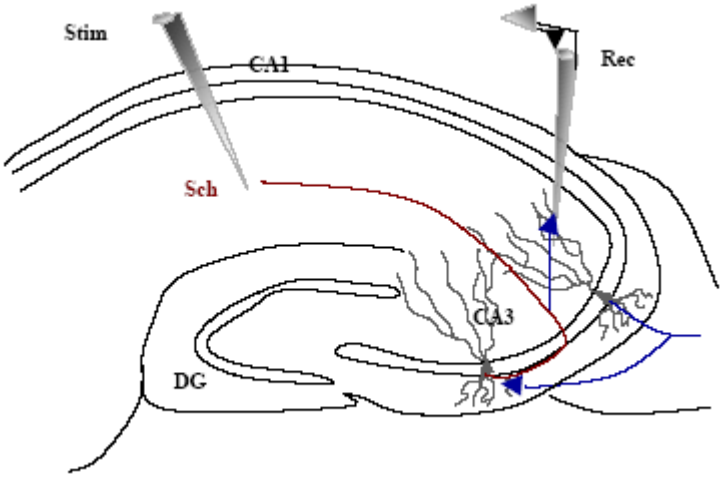

Figure 7. **Scheme of the hippocampal slice showing location of stimulating (Stim) and recording (Rec) electrodes for measuring of associational/commissural-CA3 responses.** From Eka Lepsveridze's PhD thesis.

LTP recordings of adult neurons can be obtained just in disinhibited slices (Hanse and Gustafsson, 1992), thus throughout the recording, 100 μM picrotoxine was present in the bath. For LTP induction, a short high-frequency stimulation (SHFS) protocol was used. Five trains of stimulation, spaced 20s apart from each other, were applied. Every train consisted of 10 pulses at 100 Hz delivered at double intensity used for baseline recordings (see also VI.2.5 subsection 'LTP induction').

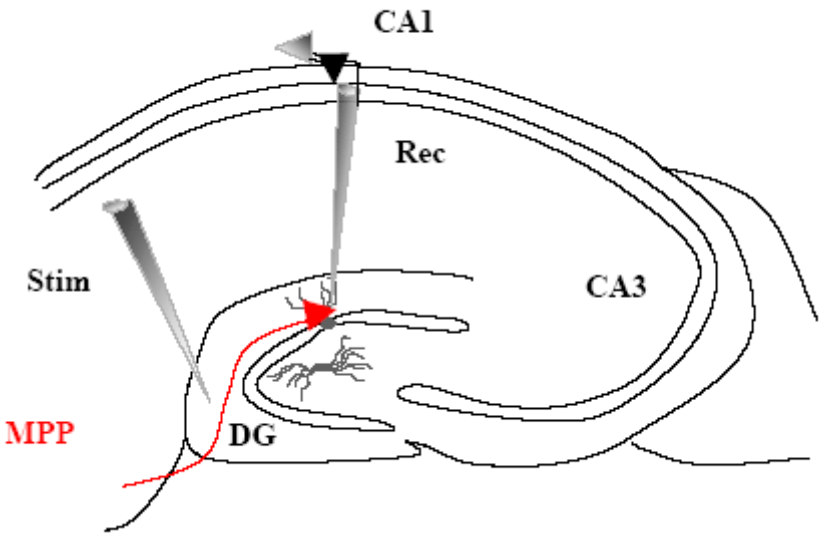

Figure 8. **Recordings at the MPP-DG synapse. Positioning of the recording (Rec) and stimulating (Stim) electrodes.** From Eka Lepsveridze's PhD thesis.

In order to induce LTP of the newly generated neurons we used a high-frequency protocol designed by Wang and Wojtowicz (Wang and Wojtowicz, 1997) which is now widely accepted of being able to induce synaptic plasticity in these cells. The protocol consisted of 4 trains, 500 ms each, 100 Hz within the train, repeated every 20 seconds. The stimulation intensity was the same as the one used for baseline recordings. It was shown that this intensity is sufficient (Snyder et al., 2001) to activate just the newly generated neurons in dentate gyrus (see chapter IV.4.2), which are not affected by the GABAergic inhibition activated during tetanization.

VII. RESULTS

PROJECT 1. Electrophysiological analysis of NCAM and PSA deficient mice

VII.1.1. Short-term plasticity in the dentate gyrus

To determine weather NCAM or PSA plays a role in short-term plasticity at the perforant path-dentate gyrus synapse two different paired-pulse stimulation protocols were used: paired-pulse facilitation of synaptic responses and paired-pulse modulation of population spikes.

VII.1.1.1. Paired-pulse facilitation

The intensity of stimulation used for this protocol was adjusted so that it evokes pure fEPSPs without population spike "contamination". Paired-pulse stimulation with inter-pulse intervals of 10, 25, 50 and 100 ms was applied at a 30 second intervals and produced a small paired-pulse facilitation of fEPSPs in wild-type and mutant mice (Fig. 9), as previously reported for C57BL/6J mice (Bampton et al., 1999). For each interval, five responses to paired-pulse stimulation were collected and the initial slopes of responses were used to calculate the ratio.

When paired-pulse stimulation was applied with an interval of 10 ms, most often the second response appeared on the decaying phase of the first one. To correct for this "contamination", the slope of decay of the first response (determined from responses elicited by a single stimulus) was subtracted from the slope of the composite response following the second stimulus. These values are given in table 4.

Analysis of paired-pulse facilitation of fEPSPs using two-way analysis of variance (ANOVA) with Genotype as a between-subjects factor and Inter-pulse interval as within subject factor revealed a significant interaction ($P < 0.05$) between these two factors for PST -/-, but not for NCAM -/- and STX -/- mice. In other words, the two curves shown in figure 9B are significantly different from each other but those in figures 9A and C are not.

However, no significant difference in paired-pulse facilitation was detected for PST -/- mice for any particular inter-pulse interval using t-test with Bonferroni correction for

multiple comparisons, although mutants showed a tendency for larger paired-pulse facilitation at 10 and 25 ms (Fig. 9B).

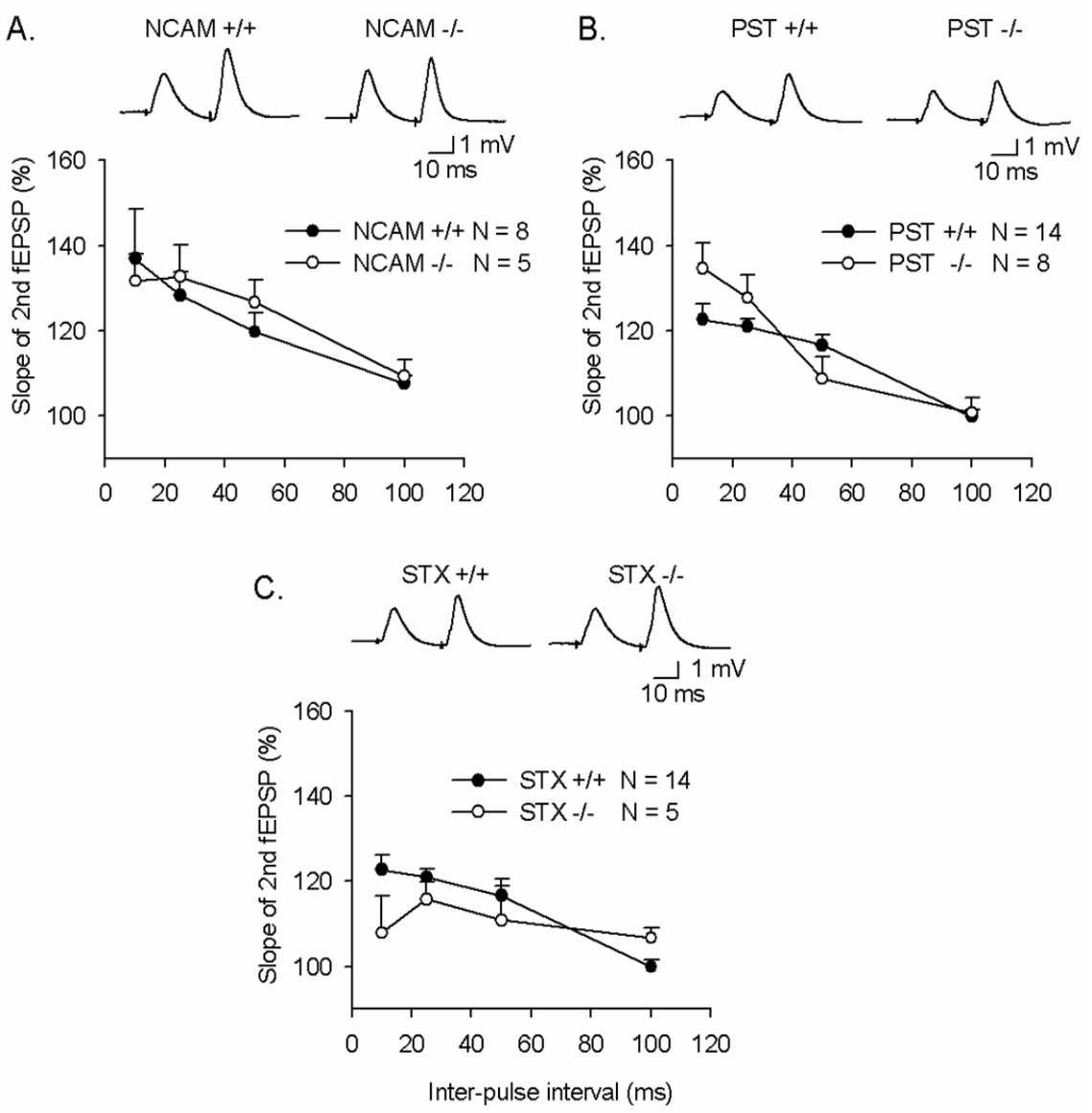

Figure 9. **Paired-pulse facilitation in mice deficient in NCAM (A), PST (B) or STX (C) vs. wild-type mice.** Paired-pulse facilitation of fEPSPs was elicited by subthreshold double-pulse stimulation of the angular bundle with inter-pulse intervals of 10, 25, 50 and 100 ms (the x axis is the same in all figures). The data shown in A, B and C are mean values + SEM. The second slope is expressed as a percentage of the first slope. Analysis of paired-pulse facilitation using two-way ANOVA detects a significant interaction between Genotype and Inter-pulse interval for PST deficient mice but not for other tested genotypes. N indicates the number of mice analyzed.

Inter-pulse interval	10 ms (%)	25 ms (%)	50 ms (%)	100 ms (%)
NCAM +/+	137 ± 11.7	128.3 ± 5.3	119.8 ± 4.5	107.7 ± 1.8
NCAM -/-	131.7 ± 6.3	132.7 ± 7.5	126.7 ± 5.4	109.3 ± 3.7
PST/STX +/+	122.7 ± 3.5	121 ± 1.8	116.6 ± 2.4	100 ± 1.5
PST -/-	134.7 ± 6	127.7 ± 5.5	108.7 ± 5.2	100.8 ± 3.6
STX -/-	107.8 ± 8.7	115.7 ± 4.1	110.8± 9.7	106.7 ± 2.4

Table 4. **Analysis of paired-pulse facilitation at the perforant path-dentate gyrus synapse in mice deficient in NCAM, PST or STX**. For each genotype, the mean values of second slope at different inter-pulse intervals are presented a percentage of the first slope ± SEM. Number of tested animals is the same as shown in figure 9.

VII.1.1.2. Paired-pulse modulation of population spikes

Stronger paired-pulse stimulation, eliciting a supramaximal PS in response to the first stimulus, resulted in a block or strong inhibition of the PS in response to the second stimulus when the inter-pulse interval was 10 or 25 ms (Fig. 10A, B and C). This inhibition is believed to be mediated by feedback inhibition in the dentate gyrus (Bampton et al., 1999). Paired-pulse stimulation with intervals longer than 50 ms produced an increase of the PS amplitude, as also reported previously (Bampton et al., 1999). This increase most likely reflects enhanced synchrony in firing of neurons in response to the second pulse.

Population spike amplitudes for the first and second responses were calculated and the second amplitude was expressed as a percentage of the first. Five determinations were made at each inter-pulse interval and a mean value was calculated. The final values are shown in table 5.

Analysis of paired-pulse modulation of PSs using two-way ANOVA revealed a weak but significant interaction ($P < 0.05$) between Genotype and Inter-pulse interval for NCAM and STX deficient mice but not for PST -/- mice. No significant difference in paired-pulse modulation was detected for any particular inter-pulse interval using the t-test with Bonferroni correction for multiple comparisons, although there was a tendency for all mutant mice to have smaller depressions at 10 ms and 25 ms intervals and smaller facilitation at intervals > 25 ms (Fig. 10B and C).

In summary, we can say that there were rather subtle abnormalities in paired-pulse modulation of either fEPSPs or population spikes in all genotypes studied.

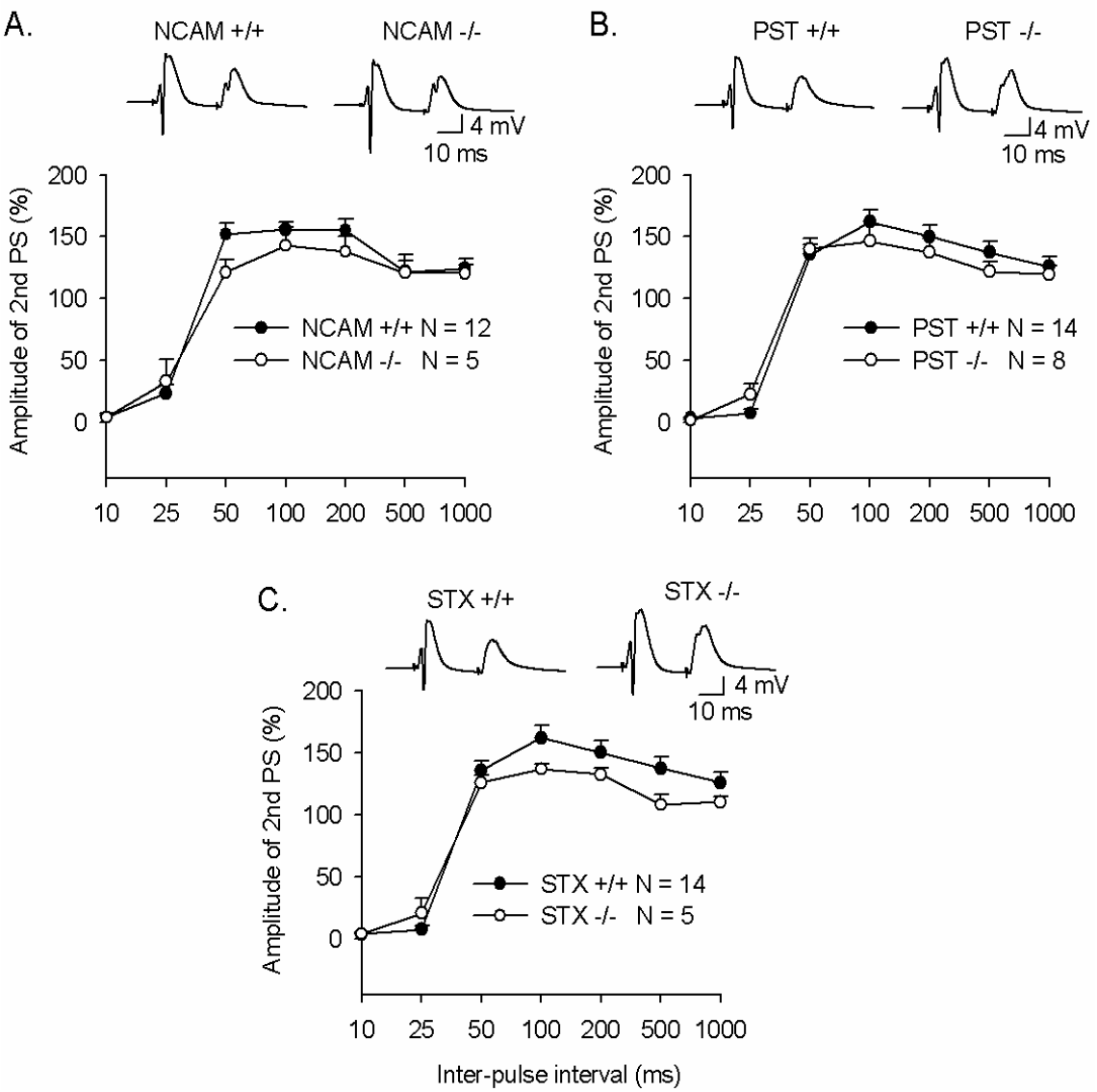

Figure 10. **Paired-pulse modulation of the population spike** (PS; negative-going fast component) was elicited by supramaximal stimulation of the angular bundle, resulting in a strong suppression of spikes at short intervals (10 and 25 ms) and facilitation of PS amplitude at longer intervals (50, 100, 200, 500 and 1000 ms-the same in all Figures). The data shown in A, B and C are the mean + SEM. The second PS is expressed as percentage of the first PS. Two-way ANOVA detected a significant interaction between genotype and inter-pulse interval for NCAM and STX deficient mice. N indicates the number of mice analyzed.

Interpulse interval	NCAM +/+	NCAM -/-	PST/STX +/+	PST -/-	STX -/-
10 ms	3.5 ± 1	5 ± 4.4	3.6 ± 1	2 ± 0.5	4 ±1.4
25 ms	19.2 ± 5.5	38 ± 22.3	7.6 ± 3	22.8 ± 8.3	20.5 ± 12.3
50 ms	157 ± 10.4	120 ± 12.6	135.8 ± 8	140 ± 8.7	125.8 ± 6.2
100 ms	160 ± 5	147.3 ± 18.7	162 ± 10	146.4 ± 13	136.8 ± 4.3
200 ms	168 ± 14.7	141.4 ± 15.4	150 ± 9.6	137.6 ± 11.5	132.5 ± 5.3
500 ms	132.5 ± 11	122.8 ± 18.5	137.5 ± 9	122 ± 7.7	108 ± 8
1000 ms	135.2 ± 9.6	123.2 ± 145	126 ± 8.2	119.7 ± 7	110.3 ± 4.5

Table 5. **Analysis of paired-pulse modulation at the perforant path-dentate gyrus synapse in mice deficient in NCAM, PST or STX.** For each genotype, the mean amplitudes of second spikes at different inter-pulse intervals are presented as a percentage of the first ± SEM. Numbers of tested animals are the same as shown in Fig. 10.

VII.1.2. Analysis of basal synaptic transmission in the dentate gyrus

Due to the fact that in our recordings intensities of stimulation vary between subjects, we made a rule to select the intensity for baseline recording that elicits population spike with amplitudes of 2-3 mV. Indeed, the mean amplitude of population spikes was the same for mice deficient in NCAM or PST or STX and their corresponding wild-type mice (Fig. 11-13A and table 6), the same holds true also for the stimulation intensities necessary to evoke such population spikes (Fig. 11-13B).

This intensity was kept constant through the experiment (except for the moment when LTP is induced at a double stimulation strength) and allowed further characterization of the basal synaptic transmission. Thus, in NCAM -/- and PST -/- mice, the slopes of fEPSPs recorded at the selected stimulation intensity matched those in wild-type mice (Fig. 11C and 12C) however, in STX -/- mice there was a strong reduction in the slope of fEPSPs (Fig. 13C and table 6).

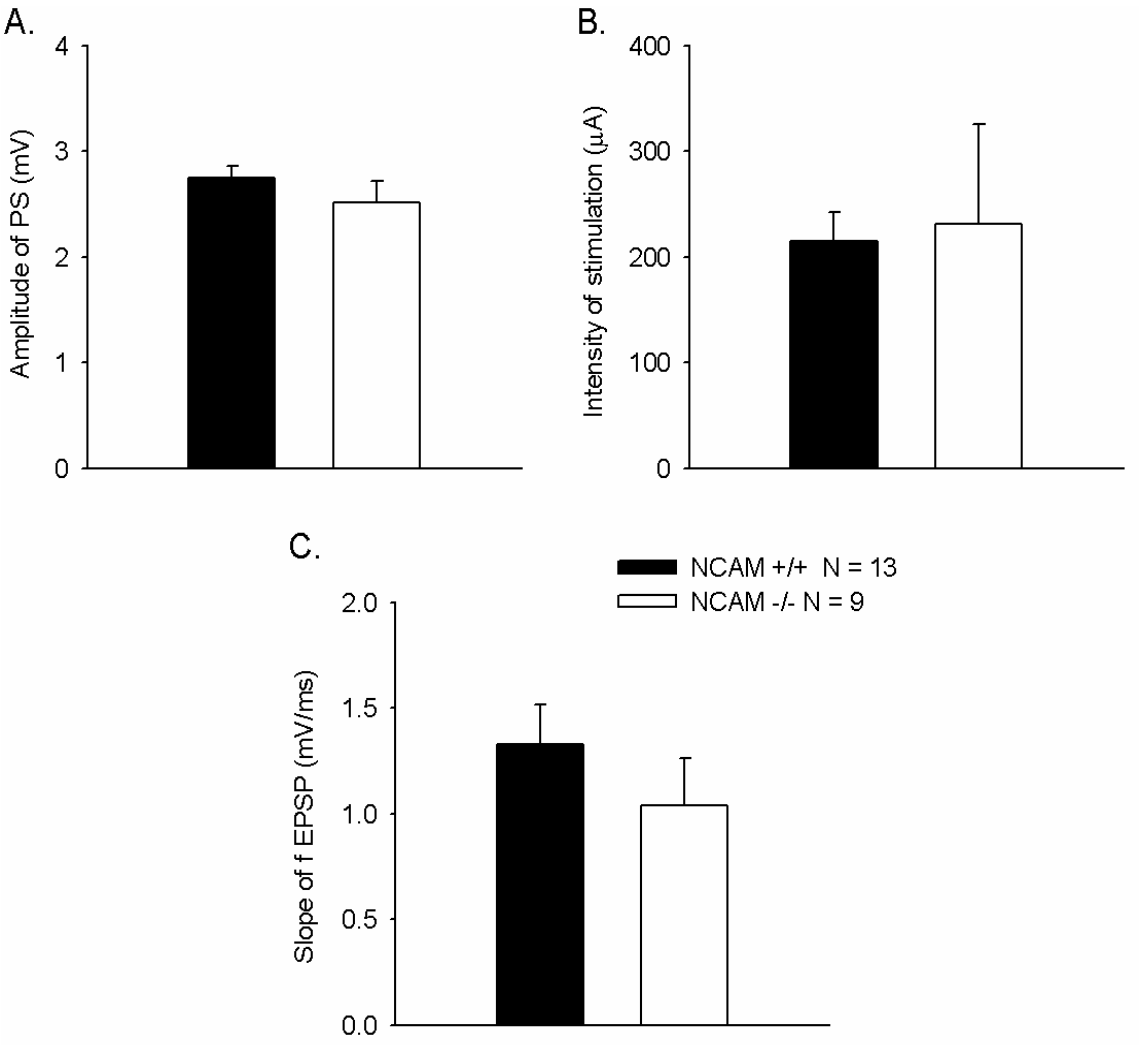

Figure 11. **Normal basal synaptic transmission in NCAM -/- mice**. Data are mean + SEM of three parameters characterizing basal synaptic transmission: amplitude of PSs (A); intensity of stimulation used for baseline & LTP recordings (B); and slope of fEPSPs (C). All values are collected at the same stimulation intensity. N indicates the number of mice analyzed.

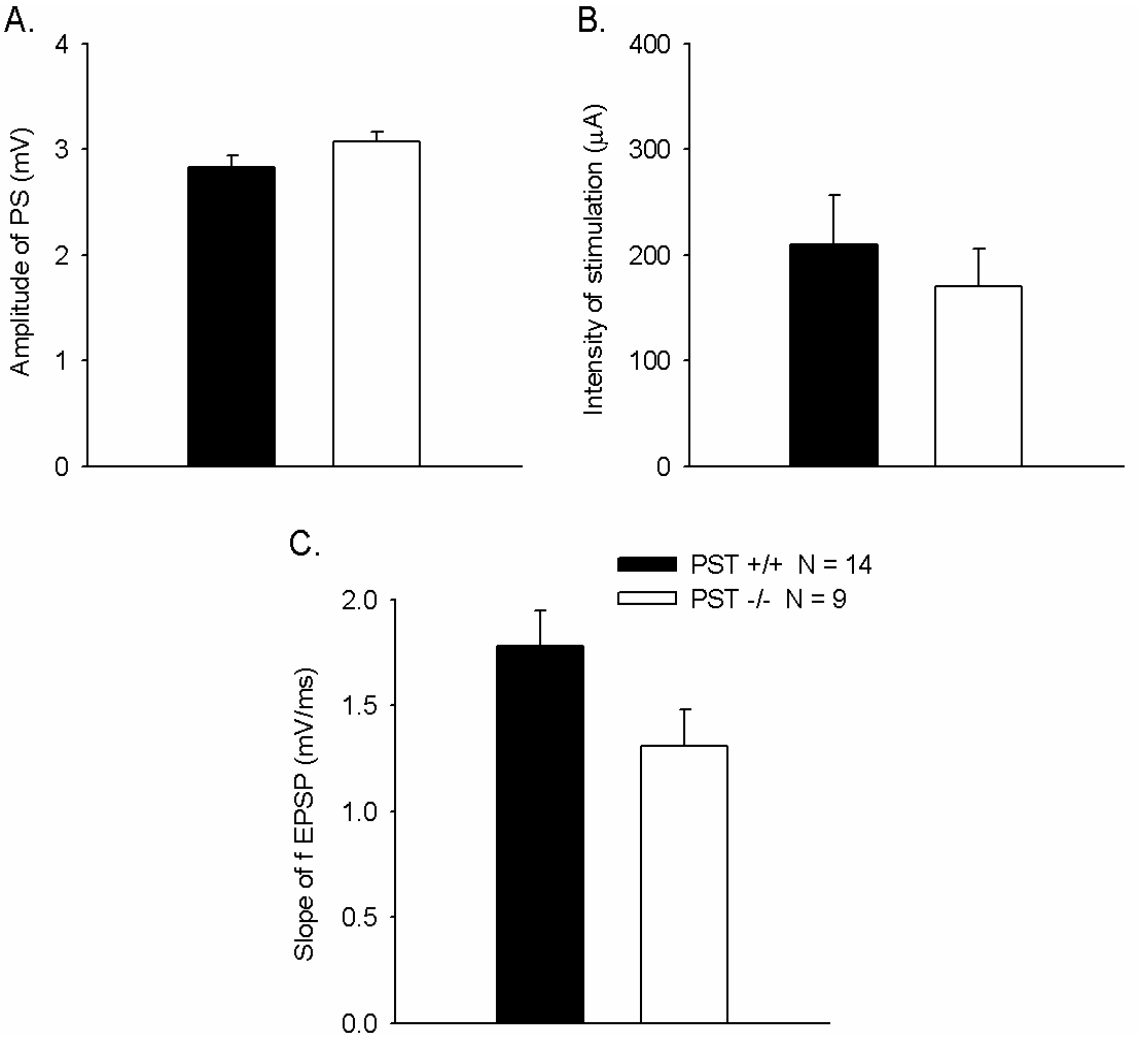

Figure 12. **Normal basal synaptic transmission in PST -/- mice.** Data are mean + SEM of three parameters characterizing basal synaptic transmission: amplitude of PSs (A); intensity of stimulation used for baseline & LTP recordings (B); and slope of fEPSPs (C). All values are collected at the same stimulation intensity. N indicates the number of mice analyzed.

Animals	Amplitude of PS (mV)	Intensity of stimulation (µA)	Slope of fEPSP (mV/ms)
NCAM +/+	2.75 ± 0.10	215.38 ± 27.13	1.32 ± 0.19
NCAM -/-	2.51 ± 0.20	231.11 ± 95.04	1.03 ± 0.22
PST/STX +/+	2.82 ± 0.11	209.64 ± 46.62	1.77 ± 0.17
PST -/-	3.07 ± 0.10	170.55 ± 35.46	1.30 ± 0.17
STX -/-	2.55 ± 0.16	336.87 ± 107.20	0.76 ± 0.12 **

Table 6. **Analysis of basal synaptic transmission in the dentate gyrus of NCAM or PST or STX deficient mice.** **P < 0.005, unpaired two-sided t-test

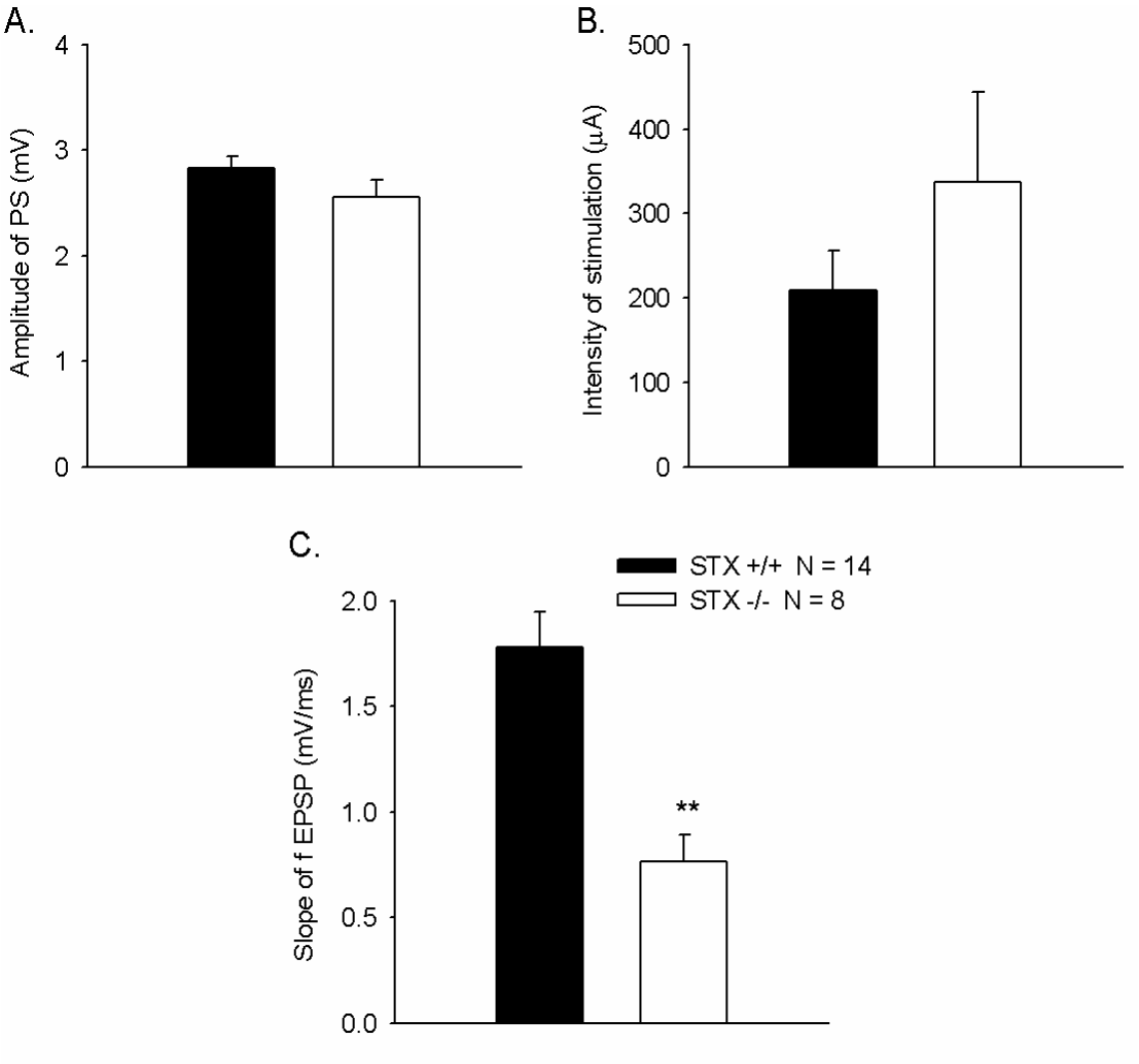

Figure 13. **Impaired basal synaptic transmission in STX -/- mice.** Data are mean + SEM of three parameters characterizing basal synaptic transmission: amplitude of PSs (A); intensity of stimulation used for baseline & LTP recordings (B); and slope of fEPSPs (C). All values are collected at the same stimulation intensity. N indicates the number of mice analyzed. **P < 0.005, unpaired two-sided t-test

The impairment observed in the STX mutants in the slope of fEPSP, stimulated us to perform further analysis of basal synaptic transmission in these mutants. Thus, stimulus-response curves in STX -/- mice were determined to investigate the relationships between the intensity of stimulation and the amplitude of population spikes or slopes of fEPSPs. The stimulus-response curves for population spikes were similar in STX -/- and their corresponding wild-type mice (Fig. 14A), whereas the slope of fEPSPs was significantly lower in mutant mice as compared to wild-types for all stimulation intensities tested (Fig. 14B). Analysis of relationships between the slope of fEPSPs and the amplitude of PSs, i.e. so-called E-S curves, revealed a significant shift of the curve

to the left (Fig. 14C) in STX -/- mice, indicating a reduction in the threshold of action potential generation.

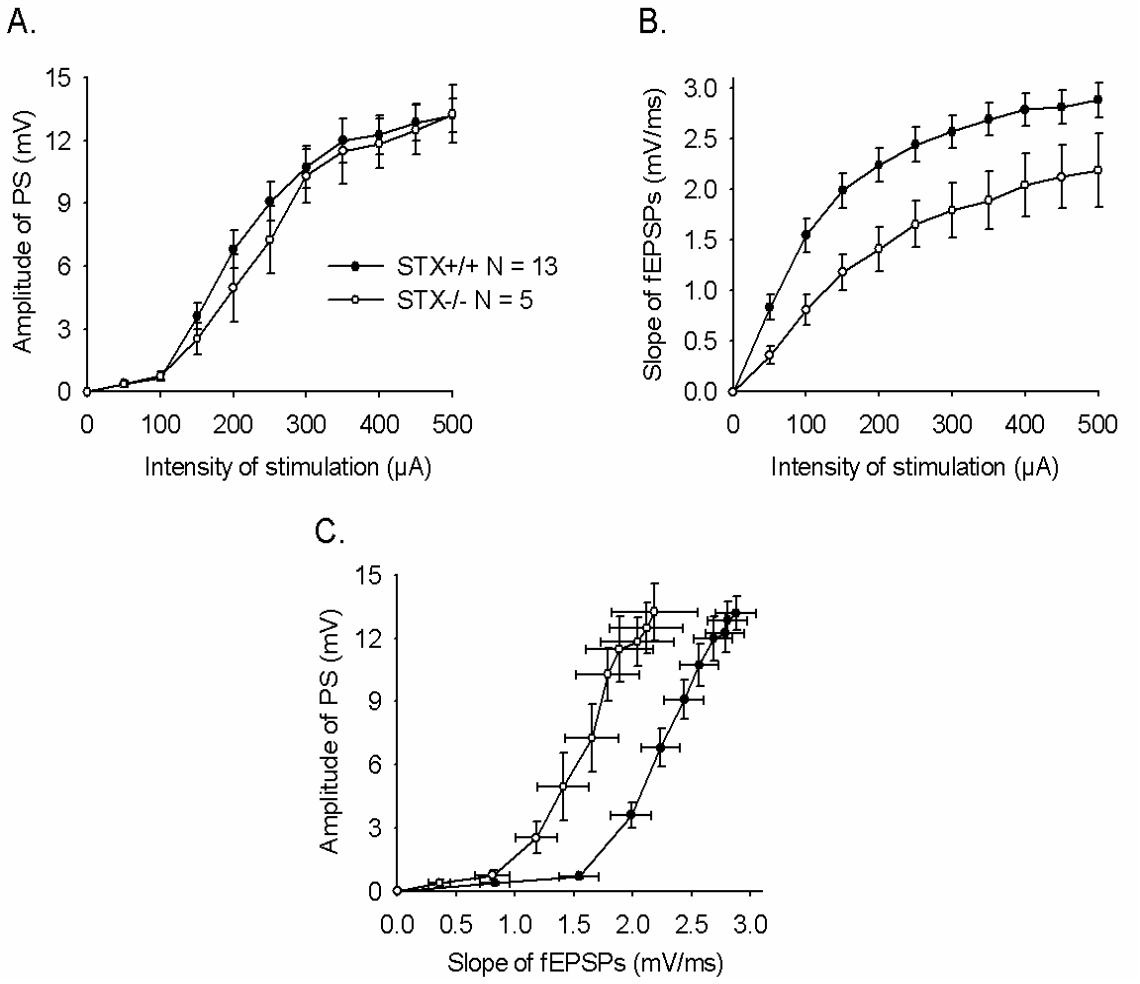

Figure 14. **Changes in stimulus-response and E-S curves in STX -/- versus wild-type mice.** Data demonstrate relationships between the intensity of stimulation and the amplitude of PSs (A), the intensity of stimulation and the slope of fEPSPs (B) and the slope of fEPSPs versus the amplitude of PSs (E-S curves in C). Means ± SEM are shown; N indicates the number of mice analyzed.

VII.1.3. LTP recording in the dentate gyrus

The next step after recording of short-term plasticity and basal synaptic transmission was to further investigate the role of NCAM or PSA in synaptic plasticity. Thus, as described above (see methods), after the identification of the proper intensity of stimulation (i.e. intensity which elicits PS of 2-3 mV) and the recording of a 20 min baseline, LTP was induced by theta-burst stimulation using a doubled stimulation intensity as compared to the one used for baseline recordings. TBS protocol elicited a robust LTP both for the population spike and the slope of fEPSP in urethane-

anaesthetized wild-type mice, as it was previously reported (Bampton et al., 1999; Bliss et al., 2000; Errington et al., 1997). Once induced, LTP was maintained throughout the entire duration of the recording. If during the three hours of recording, the potentiated slope decreased below 90% of the baseline, the recordings were excluded from analysis. The post-tetanic potentiation (PTP), i.e. increase of fEPSP and PS immediately after induction of LTP was greatly impaired in NCAM -/- mice (Fig. 15 and table 7) when compared with their corresponding NCAM +/+ mice.

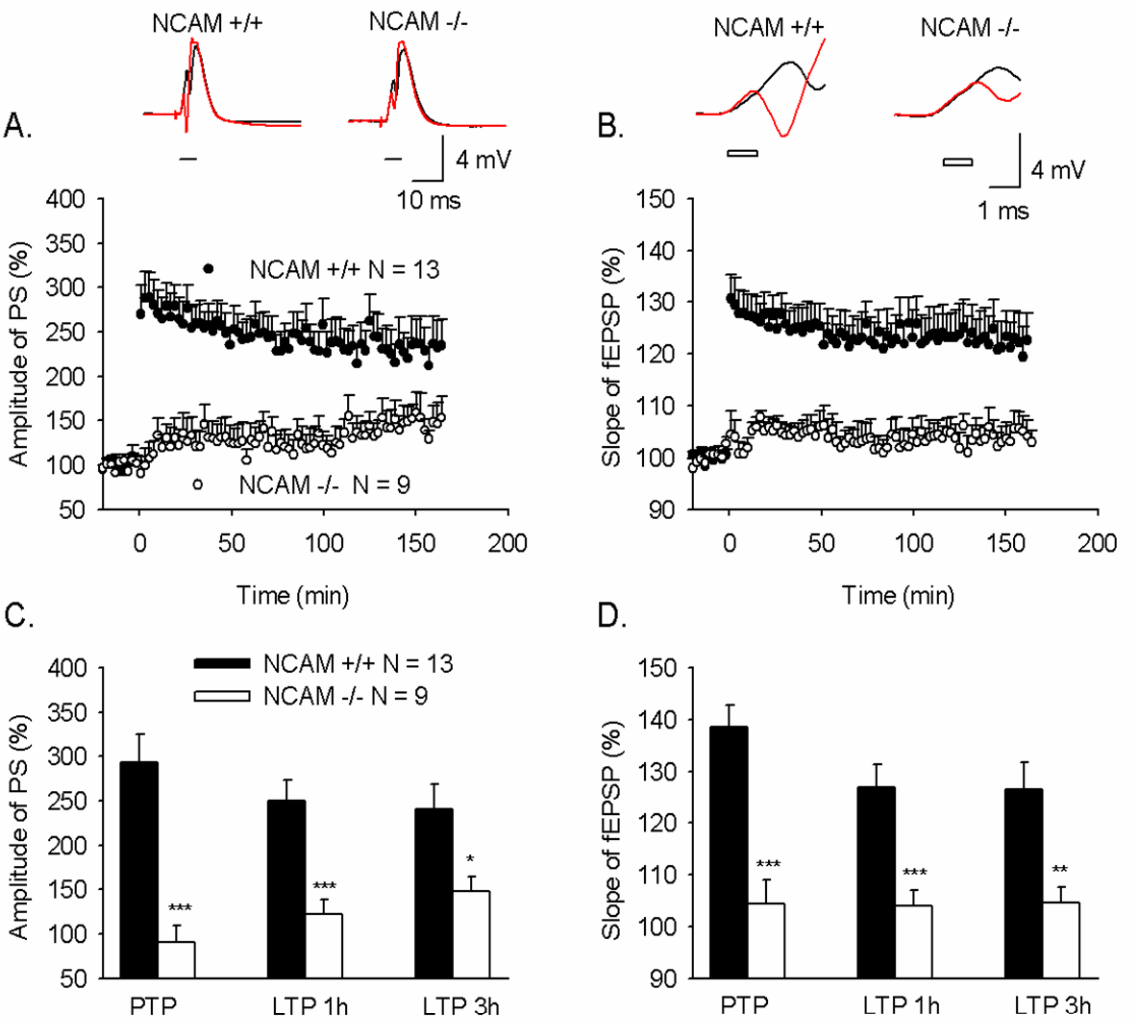

Figure 15. **Impaired long-term plasticity in NCAM -/- mice.** Data show mean + SEM of population spike (PS) amplitudes (A, C) and the slope of fEPSPs (B, D) expressed as a percentage of the baseline (time from -20 to 0 min). Theta-burst stimulation (applied at time '0') elicited a robust LTP in wild-type mice but not in de NCAM deficient mice (A-D). Examples of responses collected before and immediately after LTP induction are shown above the LTP profiles. Horizontal bars in A indicate the time intervals shown with a higher resolution in B. Horizontal boxes in B indicate the time intervals used for slope measurements. C, D) Cumulative data showing levels of PTP and LTP for all recorded mice. N indicates the number of mice analyzed (the same number from A to D). *** $P < 0.001$, ** $P < 0.005$, * $P < 0.05$ t-test.

This impairment was observed until the end of the three hours of recordings, when the NCAM +/+ mice had a significantly higher LTP than NCAM -/- mice (table 7).

The finding that LTP is impaired in mice which have ablation of the NCAM led us to the question if this is due to the absence of PSA. Since it has been shown that removal of PSA with endo-N impairs CA1 LTP and learning and memory (Becker et al, 1996) and since PSA expression is rather high in the dentate gyrus of adult mice, we expected that PSA mutants may exhibit some abnormalities in the perforant path-dentate gyrus synapse.

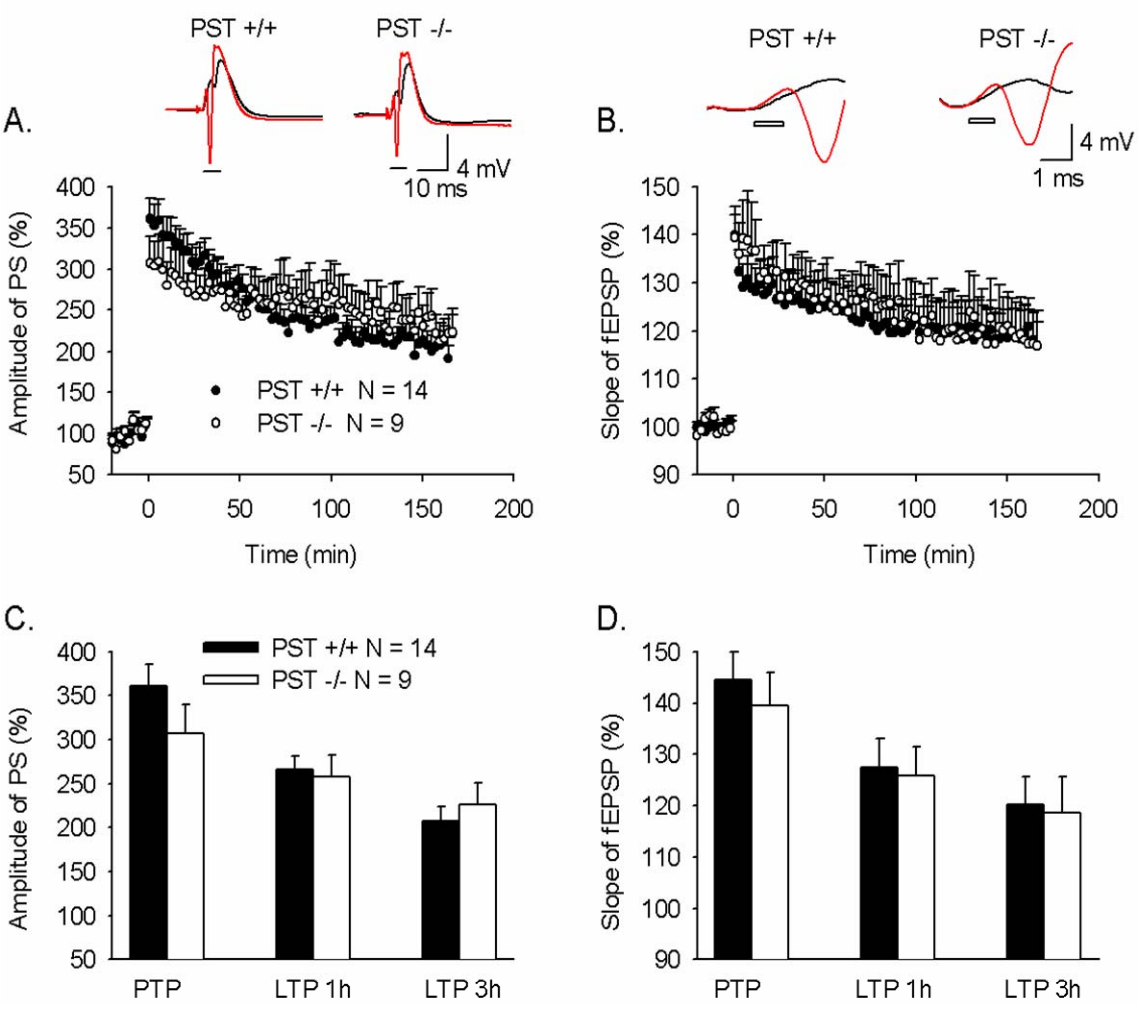

Figure 16. **Normal long-term plasticity in PST -/- mice.** Data show mean + SEM of population spike (PS) amplitudes (A, C) and the slope of fEPSPs (B, D) expressed as a percentage of the baseline. TBS induced a robust LTP both in the wild-type and PST deficient mice. Examples of responses collected before and immediately after LTP induction are shown above the LTP profiles. Horizontal bars in A indicate the time intervals shown with a higher resolution in B. Horizontal boxes in B indicate the time intervals used for slope measurements. C, D) Cumulative data showing levels of PTP and LTP for PS and slope of fEPSP from all recorded mice. N indicates the number of mice analyzed (the same number from A to D).

When the same kind of experiments as the ones described above were performed in PST or STX deficient mice, surprisingly both post-tetanic potentiation and long-term synaptic plasticity were normal in these mutants, which lack either one of the two enzymes necessary for the polysialylation process (Fig. 16, 17 and table 7).

A more detailed analysis revealed the fact that there was a strong correlation between PTP and LTP levels (Fig. 18A and B) for all the mice studied (NCAM +/+, NCAM -/-, PST/STX +/+, PST -/- and STX -/-), except for NCAM -/- animals. The fact that the post-tetanic potentiation is impaired in NCAM -/- mice suggests that in these mutants, the abnormalities observed in the synaptic plasticity are due to abnormal induction of LTP. In order to verify this hypothesis, we measured the population spikes and slopes of fEPSPs during theta-burst stimulation (Fig. 18C) and, indeed, observed a strong difference only in the NCAM -/- mice. Thus in NCAM wild-type mice, the slope of fEPSPs gradually increased after each theta-burst stimulation, reaching the level of PTP recorded during the first minute after induction of LTP, whereas in the NCAM deficient animals, such facilitation was not observed (Fig. 18D). The amplitudes of PSs elicited during induction of LTP were also significantly smaller in NCAM -/- as compared with NCAM +/+ mice (Fig 18E). Thus, there were significant differences between NCAM -/- and NCAM +/+ mice in the pattern of synaptic activity during and immediately after induction of LTP, suggesting that the induction phase is impaired in NCAM -/- mice. This is in agreement with previous *in vitro* data in the CA1region that indicated that NCAM is involved in early stages of CA1 LTP (Staubli et al., 1998; Bukalo et al., 2004).

Mice	PTP (%)		LTP 1h (%)		LTP 3h (%)	
	Slope	Pop-spike	Slope	Pop-spike	Slope	Pop-spike
NCAM +/+	138.6± 4.2	293.3 ± 32	127 ± 4.4	250.3 ± 23	126.6 ± 5.2	240.33 ±29
NCAM -/-	104.4 ±4.6	90.7 ± 18.2	104 ± 3	122.5 ± 16	104.6 ± 3	148 ± 17
PST/STX +/+	144.5 ±5.4	360.5±25.2	127.4 ± 5.6	266 ± 15.8	120.1 ± 5.6	207.2±16.8
PST -/-	139.4±6.5	306.8 ±32.5	125.76± 6	257 ± 24	118.53 ± 7	226 ± 24.8
STX -/-	139 ± 8.7	289.4 ±53.3	129.6 ± 6	263.6±45.3	125 ± 9.6	250 ± 38

Table 7**. Analysis of long-term potentiation in the dentate gyrus of PST or STX deficient mice and their corresponding wild-type controls.** Data show mean (presented in % of the baseline) ± SEM.

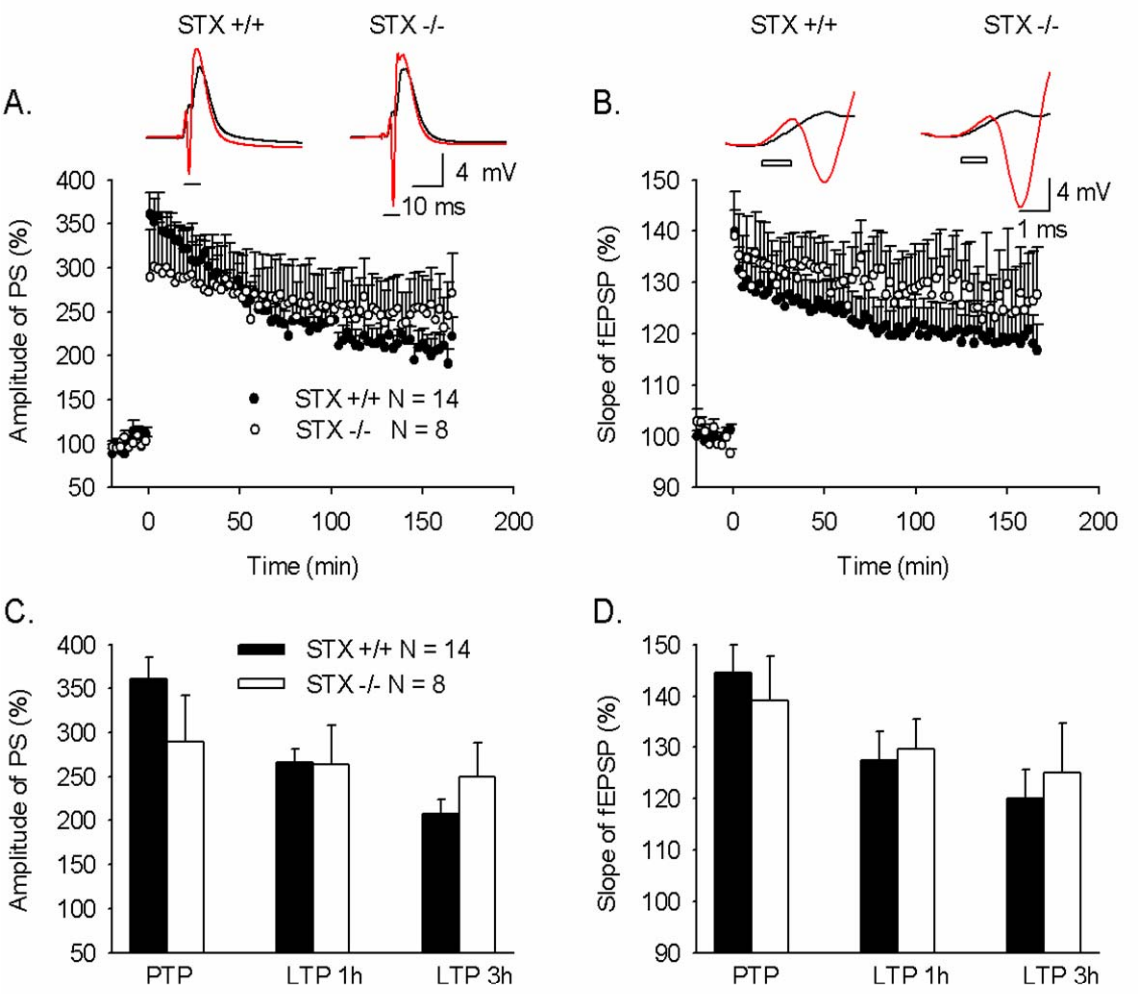

Figure 17. **Normal long-term plasticity in STX -/- mice.** Data show mean + SEM of population spike (PS) amplitudes (A, C) and the slope of fEPSPs (B, D) expressed as a percentage of the baseline. TBS induced a robust LTP both in the wild type and STX -/- mice. Examples of responses collected before and immediately after LTP induction are shown above the LTP profiles. Horizontal bars in A indicate the time intervals shown with a higher resolution in B. Horizontal boxes in B indicate the time intervals used for slope measurements. C, D) Cumulative data showing levels of PTP and LTP for all recorded mice. N indicates the number of mice analyzed (the same number from A to D). Please note that the wild-type mice are the same for PST and STX deficient mice (see also Figure 16).

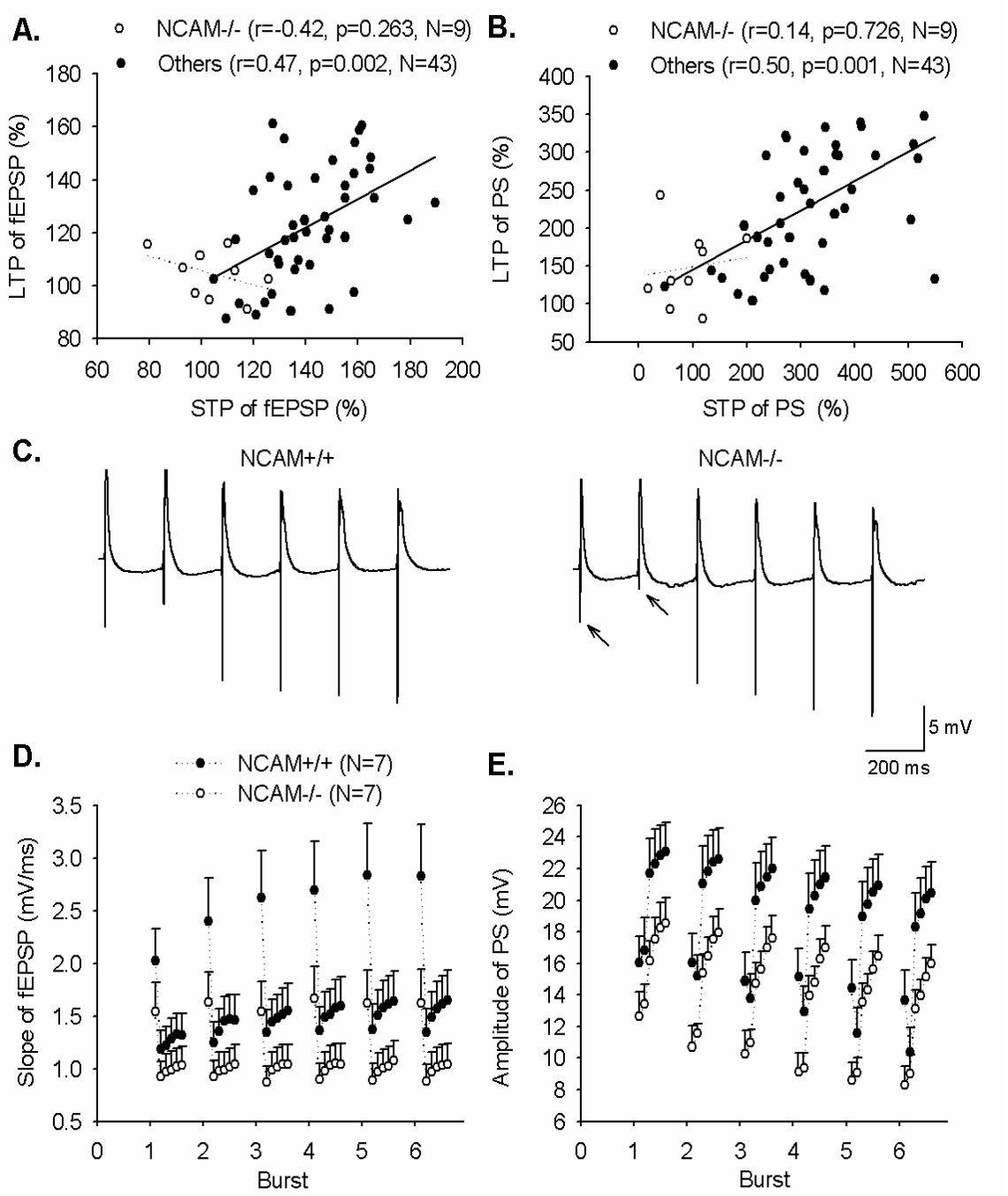

Figure 18. **Abnormal patterns of activity in NCAM-/- mice during and immediately after induction of LTP** (A and B). Analysis of the relationship between levels of short-term potentiation (first minute after TBS) and long-term potentiation (3 h after TBS) of fEPSPs (A) and population spikes (B). N is the number of mice analyzed, r, the coefficient of correlation, P, the significance of correlation. 'Others' include NCAM +/+, PST -/-, STX -/- and their wild-type controls. Solid and dashed lines are linear regression lines drawn through data from NCAM -/- mice and other mice ('others'), respectively (C). Examples of fEPSPs elicited by the sixth train of six bursts for NCAM +/+ and NCAM –/– mice. Negative-going components are PSs. Arrows point to reduced PSs in the NCAM -/- mice (D and E). Data are mean + SEM of slope of the first fEPSPs (D) and of PS amplitude (E) measured for each of 6 x 6 bursts applied for induction of LTP. N indicates the number of mice analyzed.

Earlier *in vitro* work showed the importance of both NCAM and PSA at the Schaffer collaterals – CA1 synapse, the absence of NCAM or just PSA alone resulting in impaired synaptic plasticity at this specific synapse of hippocampus. Our *in vivo* results, clearly show that at the perforant path – dentate gyrus synapse, the neural cell adhesion molecule is of particular importance for the synaptic plasticity whereas, the loss of PSA synthesized by either of two major polysialyltransferases seems not to affect the long-term potentiation. The summarized findings of all parameters recorded in the three knock-outs analyzed are presented in table 8.

Parameter	NCAM -/-	PST -/-	STX -/-
Basal synaptic transmission, slope of fEPSP	=	=	↓
Basal excitability, amplitude of population spike	=	=	=
Paired-pulse facilitation of fEPSPs	=	≠	=
Paired-pulse modulation of population spikes	≠	=	≠
LTP of fEPSPs	↓	=	=
LTP of population spikes	↓	=	=

Table 8. **Summary of electrophysiological findings in the dentate gyrus of mice deficient in NCAM or in one of the two polysialyltransferases PST/STX.** All the findings are relative to the wild-type mice. Abbreviations: =, not different from the wild type mice; ≠ different from the wild type mice; ↓ significantly reduced compared with the wild type mice.

VII.1.4. LTP recording in newly generated granule cells in the dentate gyrus

Neurogenesis in the context of active cell replacement program occurs in only two restricted areas of the adult brain, namely the subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus in the hippocampus formation. Although the exact function of adult neurogenesis still remains to be clarified, it has been recently proposed that neurogenesis in the SVZ and recruitment of new neurons into olfactory circuits might be important for olfactory discrimination (Lledo et al., 2006), whereas the

new born granule neurons have been recently shown to play a role in the synaptic plasticity in the hippocampal dentate gyrus (Snyder et al., 2001).

Nowadays, it is well accepted that NCAM and mostly PSA-NCAM expression is a characteristic feature of the postnatal neurogenic sites. Furthermore, since expression of PSA is conspicuously upregulated during the first three weeks after the generation of these cells, it can be used as a marker for the identification of newly generated neurons. A recent study addressed the role of PSA-NCAM in newly generated neurons in an *in vitro* model of neurogenesis (Vutskits et al., 2006) and it demonstrated that removal of the polysialic tail of NCAM by endo-N dramatically decreases the number of newly generated neurons. Similar results have been obtained when PSA was blocked by a specific antibody and in cultures prepared from the NCAM deficient mice (Vutskits et al., 2006).

The above mentioned data, suggest the fact that NCAM might be an active player in the neurogenesis process. Since, i) we showed that it plays an important role in the *in vivo* synaptic plasticity at the perforant path-dentate gyrus synapse and ii) in our *in vivo* situation it is impossible to distinguish between changes in synapses on immature versus mature granule cells, we decided to find an approach to investigate the role of NCAM specifically in the synaptic plasticity in the newly generated neurons.

Thus, we switched to an *in vitro* preparation, where without additional pharmacological manipulation (recording were performed only in ACSF) one can record long-term potentiation induced by a high-frequency stimulation protocol. Our next step was to establish this form of LTP in our laboratory in accordance with the protocol published by the Wojtowicz group (Snyder et al 2001).

According to this protocol, the medial perforant path was stimulated with a brief electrical pulse to evoke a response with amplitude of 50% from the maximum one. Stimulation was applied every 20 seconds in order to avoid the frequency depression characteristic for this pathway. LTP was induced by four trains of 100 Hz, each of them lasting 500ms, applied with an interval of 20s, at the same intensity as one used for baseline recordings. This stimulation produced in average a small potentiation of approximately 10%, with a commonly observed tendency to decrease after approximately 30 minutes (Fig. 19A and 20A, black circles). In order to be sure that the recorded LTP is generated by the newly neurons in dentate gyrus, we performed recordings in presence of Ro 25-6981 (Ro), a specific blocker of the NR2B subunit

containing NMDA receptor subtype, which has been reported to play a critical role for induction of LTP in immature granule cells.

The presence of Ro in the bath did not affect the basal synaptic properties since the intensities used for LTP recordings (59.5 ± 2.5 µA) did not differ compared with the ones used in the normal ACSF solution (63.46 ± 3.53 µA). Furthermore, analysis of the relationship between the amplitude of responses versus intensity of stimulation (Fig. 19B), did not reveal any abnormalities. The maximum response in the ACSF normal solution had an amplitude of 1.42 ± 0.16 mV and the one recorded in the presence of Ro had a value of 1.47 ± 0.12 mV.

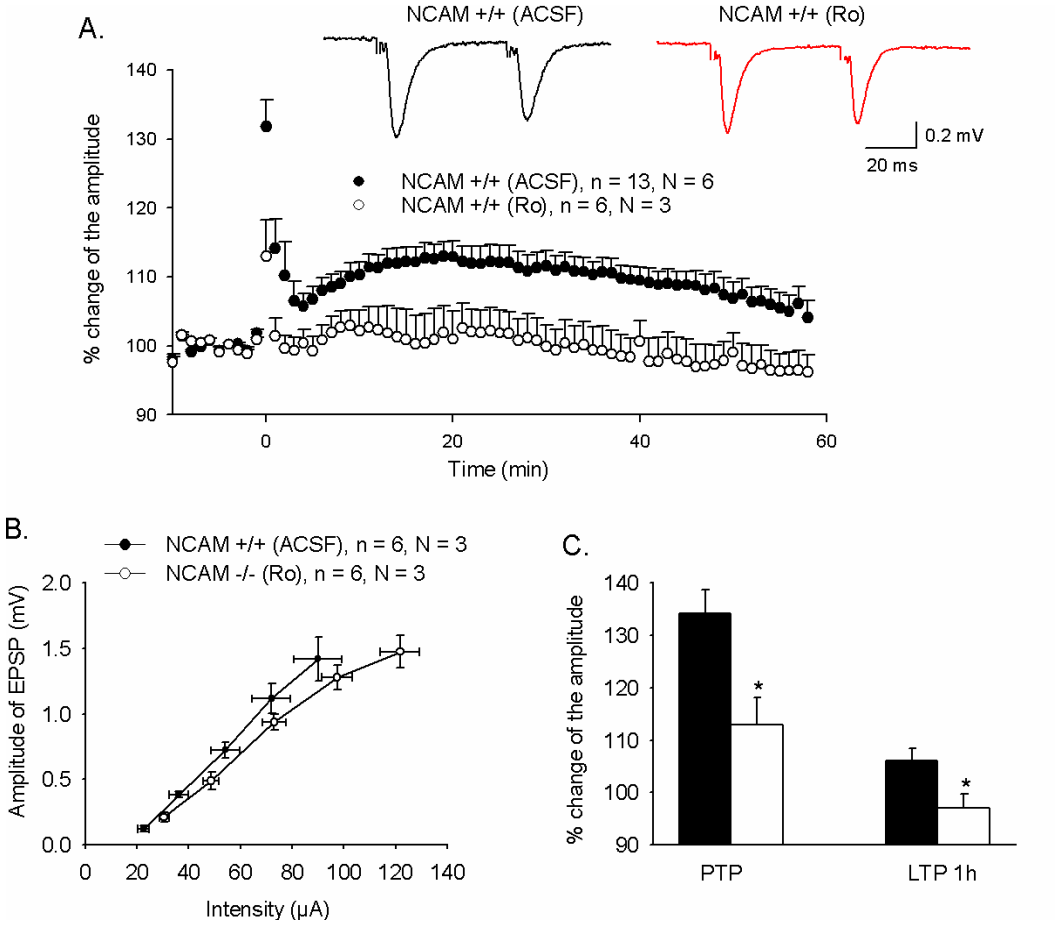

Figure 19. **Long-term plasticity of the new neurons in the dentate gyrus is NR2B dependent.** (A) 1 µM Ro impairs LTP in the immature neurons. Upper are shown example traces recorded at MPP-DG synapse in ACSF (black) and in presence of Ro (red). Stimulus response curves recorded in ACSF and in Ro (B). C) Levels of post-tetanic potentiation and of the last ten minutes of LTP. N indicates the number of mice and n represents number of slices analyzed; $P < 0.05$, t-test.

As described above, using an intensity of stimulation evoking 50% of the maximum amplitude, short- and long-term potentiation could be induce in the NCAM +/+ mice in ACSF presence (PTP: 134 ± 4.6 %; LTP: 106 ± 2.3%) but both PTP (113 ± 5.2%) and LTP (97 ± 2.7%) were significantly impaired ($p<0.05$) when synaptic plasticity was induced in the presence or Ro (Figure 19A and C).

Thus, following the protocol developed by Snyder and colleagues (2001), we could induce NR2B dependent long-term potentiation in immature neurons of the dentate gyrus.

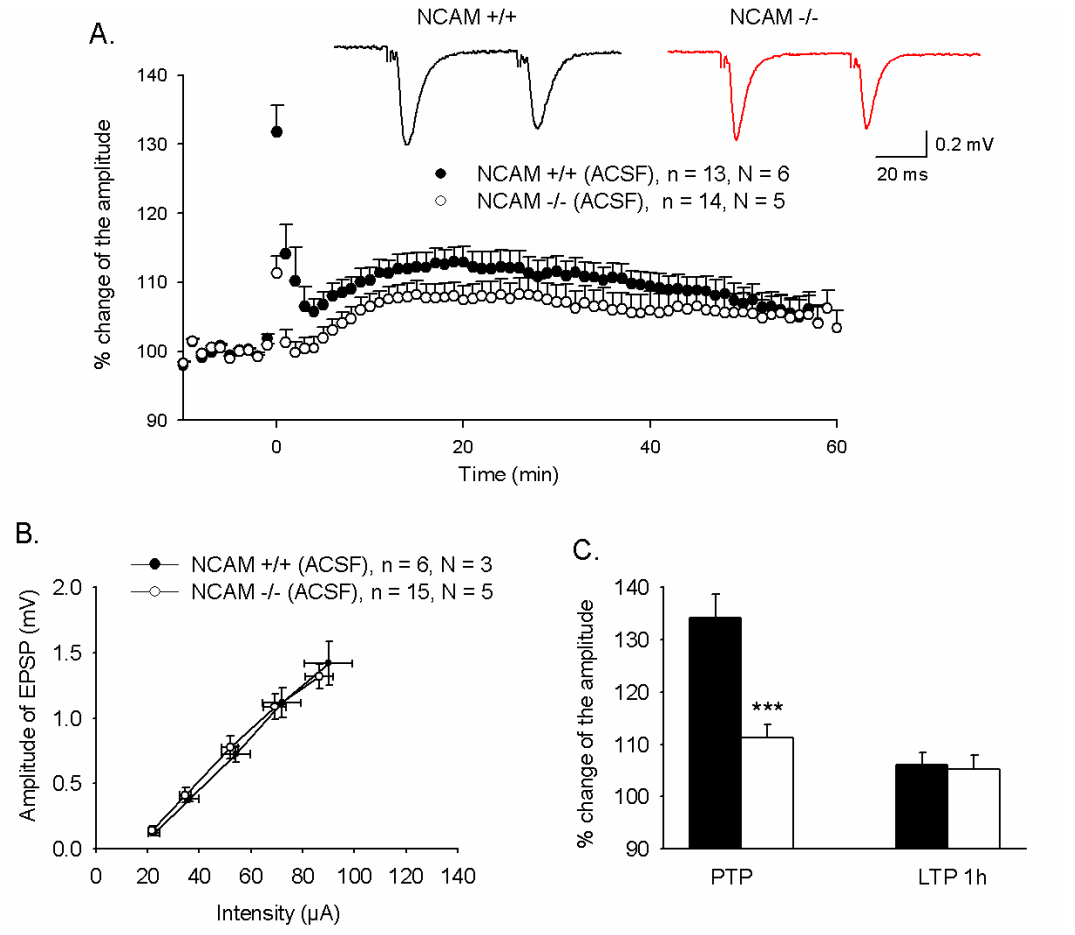

Figure 20. **Normal long-term potentiation in immature neurons in the dentate gyrus of the NCAM -/- mice.** A) LTP profiles recorded in wild-type and NCAM deficient mice. Upper are shown responses recorded in NCAM +/+ and NCAM -/- mice. B) Input-output curves for amplitudes of EPSPs evoked by stimulation of MPP projections to the DG at different stimulation strengths. C) Impaired post-tetanic potentiation but normal LTP in the NCAM deficient mice. N indicates the number of mice and 'n' represents number of slices analyzed; $P < 0.001$, t-test

After establishing this protocol, we further proceeded to investigate the role of NCAM in the synaptic plasticity of immature neurons, by performing analysis in the NCAM -/- and NCAM +/+ mice. Analysis of the input-output curves showed no abnormality in the NCAM -/- mice, maximum amplitude being 1.42 ± 0.16 mV in the control animals and 1.31 ± 0.09 mV in NCAM -/- mice (Figure 20B).

As stated above, taking into consideration the current state of literature, which points to an active role of NCAM/PSA-NCAM in neurogenesis we were expecting to see differences also in this form of synaptic plasticity. Much to our surprise this was not the case, except for a clear impairment in the levels of short-term potentiation (134.11 ± 4.63 % for NCAM +/+ and 111.30 ± 2.44 % for NCAM -/-), LTP values measured 50-60 min after high-frequency stimulation showed no difference between genotypes (Fig. 20A and C; $LTP_{NCAM\ +/+}$ 106.11 ± 2.27 % and $LTP_{NCAM\ -/-}$ 105.17 ± 2.65 %). Absence of NCAM effect on the synaptic plasticity in immature neurons in the dentate gyrus motivated us to perform further analysis of the synaptic plasticity at the medial perforant path-dentate gyrus synapse in order to check if the effect was specific for immature neurons.

This time we used the conventional approach for *in vitro* recording of LTP in the dentate gyrus, using disinhibited slices. Blocking the GABAergic inhibition is known to be a precondition for successful induction of large LTP in the dentate gyrus *in vitro* (Hanse and Gustafsson, 1992), thus throughout the recording 100μM picrotoxin was present in the bath.

Analysis of the basal synaptic transmission revealed no abnormalities between genotypes; maximum response recorded was 0.96 ± 0.19 mV in the wild-type and 0.97 ± 0.20 in the deficient animals (Fig. 21B). LTP was induced using four short trains of high-frequency stimulation at the doubled intensity as compared to one used for baseline recordings (82.66 ± 4.96 μA in the case of NCAM -/- mice and 80.36 ± 3.82 μA for their controls). In this case, with improved conditions for induction of LTP (larger depolarization during high-frequency stimulation due to disinhibition of neurons) both post-tetanic and long-term potentiation in the NCAM -/- mice (PTP: 163.37 ± 5.11%; LTP: 128.72 ± 6.70%) did not differ compared to NCAM +/+ controls (PTP: 161.25 ± 7.66; LTP: 129.20 ± 5.92%). These surprising findings might be due to our stimulation protocol, namely the short high-frequency stimulation, which does not generate a long-lasting depolarization in contrast to the protocol used in our *in vivo* situation. As shown in figure 18D, during the first burst of tetanization protocol, no real

difference was observed between slopes recorded in NCAM +/+ and NCAM -/- mice. The significant difference appeared rather after the 3rd burst.

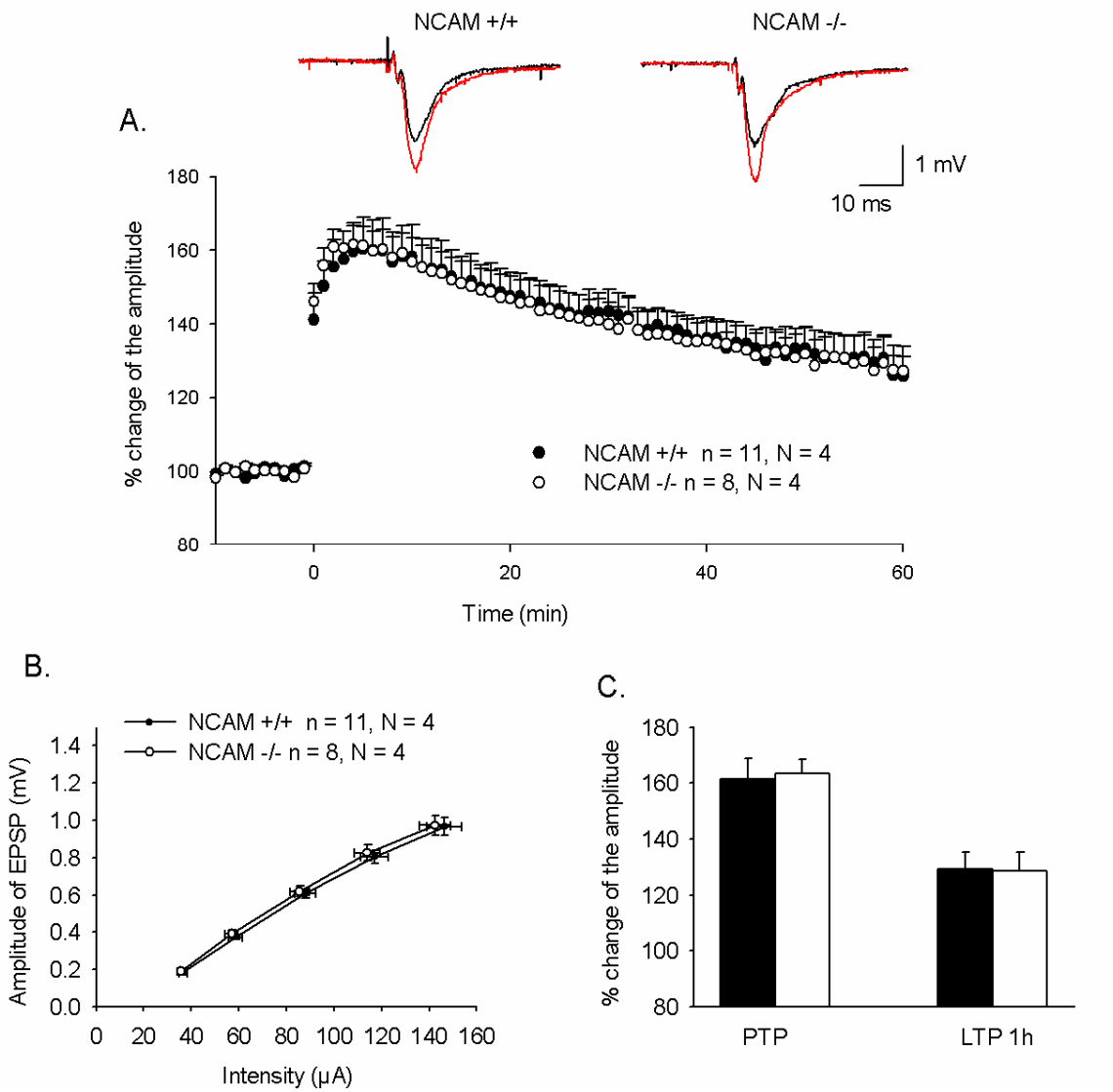

Figure 21. **Normal LTP in the dentate gyrus of NCAM deficient mice.** A) Short-high frequency stimulation of the medial perforant path (applied at the time 0) evoked similar levels of potentiation in slices from NCAM +/+ and NCAM -/- in the presence of 100 µM picrotoxin. Mean amplitude of EPSP recorded 10 minutes before LTP induction was normalized at 100%. Data represent mean +SEM; n indicates the number of tested slices and N, the number of tested animals. B) Input-output curves for amplitudes of EPSPs evoked by stimulation of medial perforant path at different stimulation strengths. No difference between genotypes was found. C) Cumulative data showing levels of PTP and LTP from all the recorded slices. Number of tested slices is the same as in figure A and B.

PROJECT 2. Electrophysiological analysis of mice deficient in extracellular matrix molecules tenascin-C and tenascin-R

VII.2.1. Short-term plasticity in the dentate gyrus of mice deficient in ECM molecules

The first step in identifying the role of TN-C,-R in synaptic plasticity at the perforant path-dentate gyrus synapse was to study their involvement in the paired-pulse facilitation and paired-pulse modulation at this synapse.

VII.2.1.1. Paired-pulse facilitation of fEPSPs

Analysis of short-term synaptic plasticity in TN-C -/- and TN-R -/- mice was performed using paired-pulse stimulation of the angular bundle. Subthreshold double-pulse stimulation with inter-pulse intervals of 10, 25, 50 and 100 ms produced small paired-pulse facilitation of the fEPSPs in both mutants studied and their corresponding wild-type mice (Fig. 22). For each interval, five pairs of stimuli were collected at a 30 second interval. The second slope was expressed as a percentage of the first one. The way the responses were measured is presented in section VI.2.5 and the data are presented in table 9.

Inter-pulse interval	10 ms (%)	25 ms (%)	50 ms (%)	100 ms (%)
TN-C +/+	111.5 ± 6.8	117.8 ± 3.7	116.6 ± 4.5	103.2 ± 2
TN-C -/-	107 ± 6.6	123.9 ± 2.5	122 ± 5.2	109 ± 1.7
TN-R +/+	117.2 ± 3.5	125.5 ± 2	116.2 ± 2.4	108 ± 1.5
TN-R -/-	112 ± 3.4	118 ± 2.7	114 ± 1.5	101 ±1.8

Table 9. **Analysis of paired-pulse facilitation at the perforant path-dentate gyrus synapse in mice deficient in either TN-C or TN-R.** The values represent the levels of facilitation of the second slope of fEPSP and they are expressed as percentage of the first slope ± SEM. Number of tested animals is the same as shown in figure 22.

In the case of TN-C -/- (Fig. 22A), analysis of paired-pulse facilitation of fEPSPs using two-way ANOVA with Genotype as a between-subjects factor and Inter-pulse interval as within-subjects factor did not revealed any significant effect of Genotype or

interaction between Genotype and Inter-pulse interval. The same type of analysis was also performed for the TN-R deficient mice. Using two-way ANOVA with Genotype as between-subjects factor and Inter-pulse interval as within-subjects factor revealed a significant effect of Genotype ($p = 0.04$). However, no significant difference in paired-pulse facilitation was observed for any of the inter-pulse intervals ($p > 0.05$, *t* tests with Bonferroni corrections for multiple comparisons, Fig. 22B).

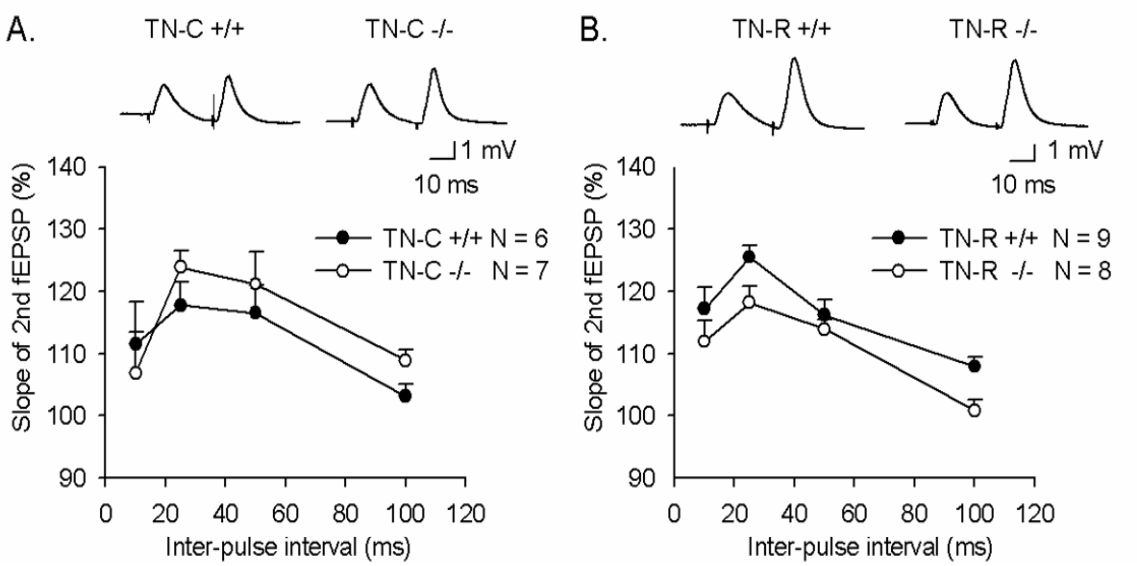

Figure 22. **Paired-pulse facilitation in mice deficient in extracellular matrix molecules.** (A) TN-C -/- mice show normal paired-pulse facilitation while TN-R -/- mice present an impairment in this form of synaptic plasticity (B). Paired-pulse facilitation of fEPSPs was elicited by subthreshold double pulse stimulation of the angular bundle with inter-pulse intervals of 10, 25, 50 and 100 ms. The data represent mean+SEM. The second slope is expressed as a percentage of the first slope. Upper graphs represent example traces recorded in TN-C -/- and TN-R -/- mice and their correspondent wild-types at 25 ms interval duration between the two pulses. N indicates the number of mice analyzed.

VII.2.1.2. Paired-pulse modulation of population spikes

The second protocol applied was to test activity-dependent modulation of population spikes. It also consisted of a pair of stimuli applied at different inter-pulse intervals ranging from 10 to 1000 ms. The intensity used for stimulation was the one eliciting a supramaximal population spike in response to the first stimulus. The second pulse resulted in a complete block or strong inhibition of population spikes when the inter-pulse interval was 10 or 25 ms (Fig. 23). Paired-pulse stimulation with intervals longer

than 50 ms produced an increase of the population spike amplitude. This increase most likely reflects reduced inhibition or enhanced synchrony in firing of neurons in response to the second pulse. Analysis of paired-pulse modulation of population spikes in both TN-C -/- (Fig. 23A) and TN-R-/- (Fig. 23B) mice using two-way ANOVA revealed no effect of Genotype and no significant interaction between Genotype and Inter-pulse interval, showing thus, that none of the molecules play a role in this type of plasticity. Data are shown in table 10.

VII.2.2. Analysis of basal synaptic transmission in the dentate gyrus

In order to analyze the basal synaptic transmission in the dentate gyrus of mice deficient in the extracellular matrix molecules TN-C or TN-R, the same criteria as described in the section VII.1.2 were also applied here. Thus, a stimulation intensity providing a population spike with amplitude of 2-3 mV was first determined and this intensity was used throughout the whole experiment.

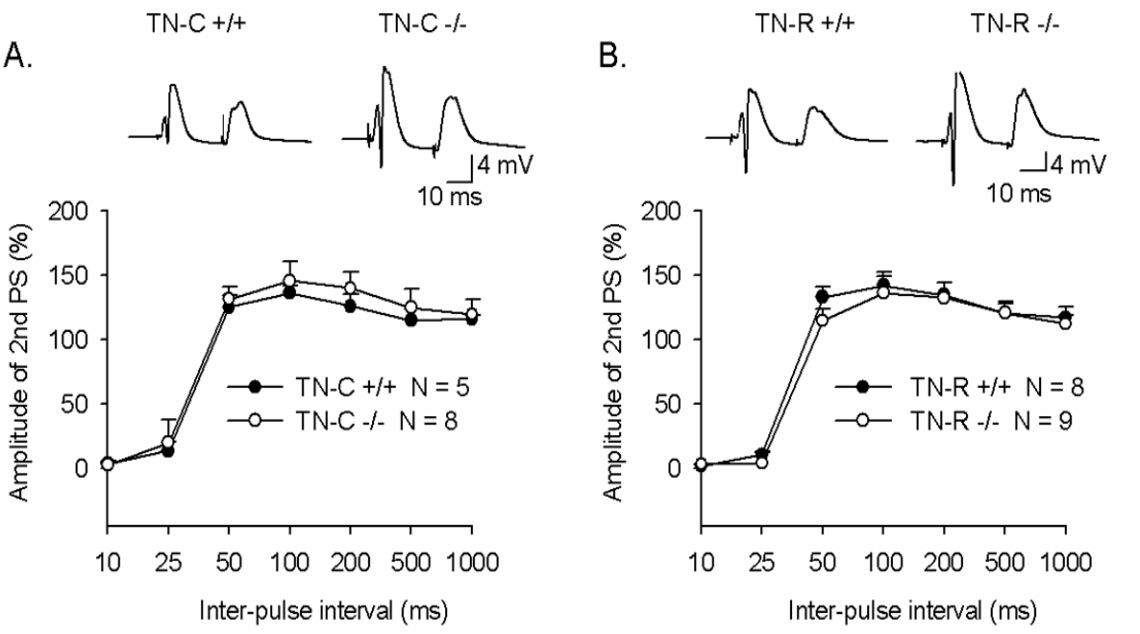

Figure 23. **Paired-pulse modulation in mice deficient in extracellular matrix molecules.** Both TN-C -/- (A) and TN-R-/- (B) mice have normal paired pulse modulation. This form of plasticity was induced by supramaximal stimulation of the angular bundle. It resulted in a strong suppression of spikes at short intervals (10 and 25 ms) and facilitation of PS amplitude at longer intervals (50, 100, 200, 500 and 1000 ms). The data shown represent mean + SEM. The second PS is expressed as a percentage of the first PS. The upper graphs represent example traces recorded at 25 ms interval between pulses in TN-C -/- and TN-R -/- and their correspondent wild-types. N indicates the number of mice analyzed.

Interpulse interval	TN-C +/+	TN-C -/-	TN-R +/+	TN-R -/-
10 ms	3.4 ± 1.4	2.6 ± 3.5	2 ± 1	3 ± 0.5
25 ms	13.7 ± 6.8	20 ± 17.8	10.5 ± 3	4 ± 8.3
50 ms	125.3 ± 9.3	131.6 ± 10	133 ± 8	115 ± 8.7
100 ms	136.5 ± 5.7	146 ±15	142.4± 10	136.6 ± 13.2
200 ms	126 ± 9.3	140 ± 12.4	135 ± 9.5	132.7 ± 11.5
500 ms	115 ± 9	125 ± 14.4	120.6 ± 9	120.7 ± 7.7
1000 ms	116.3 ± 3	119.7 ± 11.5	117.3 ± 8.2	112.3 ± 7

Table 10. **Analysis of paired-pulse modulation at the perforant path-dentate gyrus synapse in mice deficient in TN-C or TN-R.** For each genotype the mean values at different type point interval is presented in percents ± sem. Five different sweeps are averaged for each interval of inter-pulse stimulation Number of tested animals is the same as shown in figure 23.

The PSs values were in the predicted range of 2-3 mV for both mutants and their corresponding wild-type mice as shown in the figure 24A (TN-C -/-) and in figure 25A (TN-R -/-). There was no difference between genotypes in stimulation intensity necessary to evoke such a PS (Fig. 24B and 25B). Although there was a tendency in the TN-R -/- to have a higher slope of the fEPSP than the wild-type mice (Fig. 25C), it did not reach a significant level. In the TN-C -/- mice, the latter parameter was also normal when compared with the wild-type mice, suggesting that, basal excitatory synaptic transmission is normal in the dentate gyrus of TN-C -/- and TN-R -/- mice. Summarized data are presented in table 11.

Animals	Amplitude of PS (mV)	Intensity of stimulation (μA)	Slope of fEPSP (mV/ms)
TN-C +/+	2.53 ± 0.06	182.14 ± 38.20	1.11 ± 0.19
TN-C -/-	2.49 ± 0.12	231.25 ± 65.54	1.35 ± 0.09
TN-R +/+	2.97 ± 0.22	131.66 ± 18.48	1.48 ± 0.13
TN-R -/-	2.95 ± 0.09	134.77 ± 30.13	2.07 ± 0.31

Table 11. **Analysis of basal synaptic transmission in the dentate gyrus of TN-C -/- and TN-R -/- mice.** Data are collected at the same intensity as the one used for baseline recordings. For the TN-C mice, the number of averaged mice is the same as in figure 24, whereas the one for TN-R mice is the same as in figure 25.

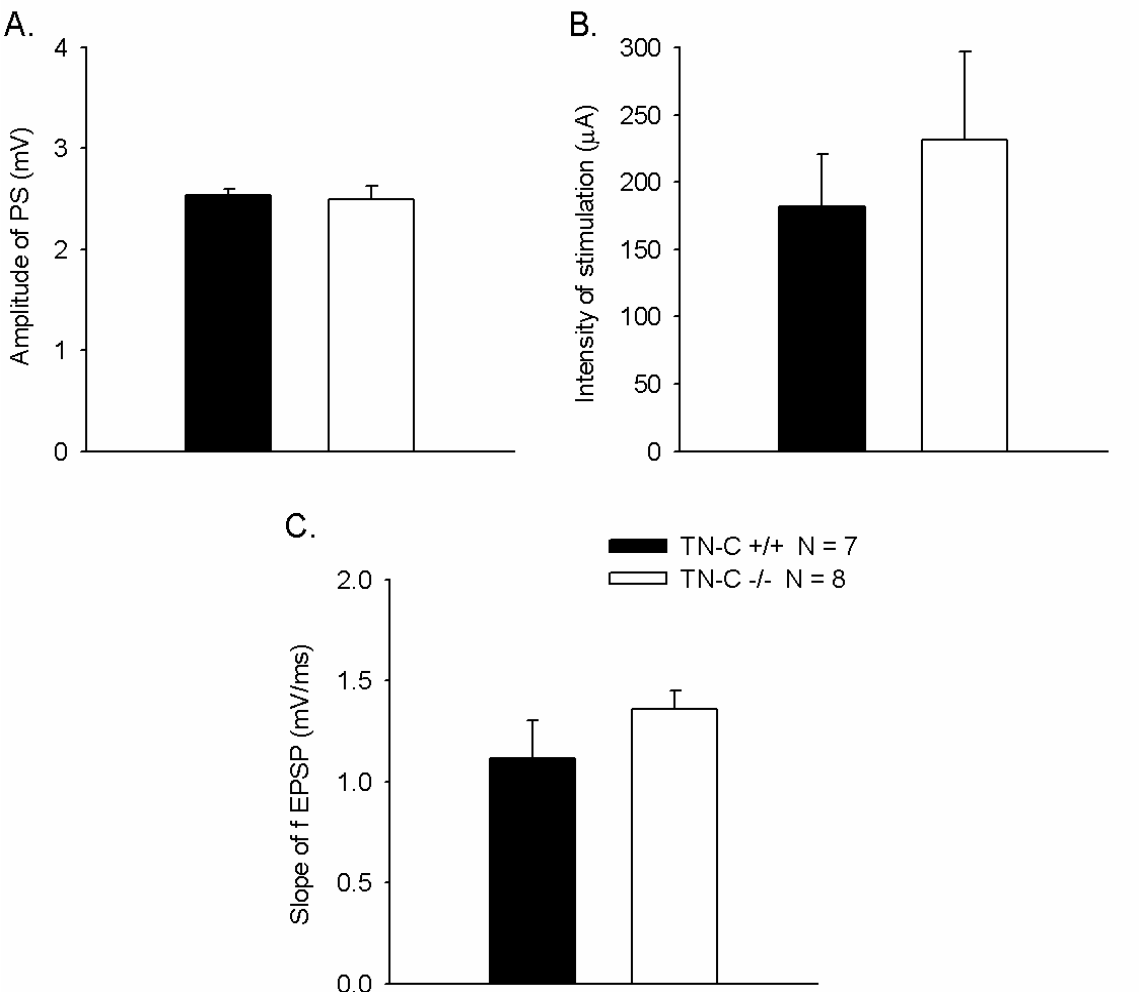

Figure 24. **Normal basal synaptic transmission in the dentate gyrus of TN-C -/- mice.** Data represent mean+SEM of three parameters characterizing basal synaptic transmission: amplitude of population spikes (A), intensity of stimulation used to evoke population spikes of 2-3 mV (B), and the slope of fEPSP (C) collected at the stimulation strength used for baseline recordings. N indicates the number of mice analyzed.

VII.2.3. Long-term potentiation in the dentate gyrus

After assessing the role of the two extracellular matrix molecules in short-term plasticity, we proceed further by investigating their role in long-term potentiation. Recording of a 20 min stable baseline was followed by the application of six trains of theta-burst stimulation using doubled intensity of stimulation as compared with baseline recordings. This elicited a robust LTP both in TN-C -/- and in TN-C +/+ mice (Fig. 26). Immediately after the LTP induction, the levels of post tetanic potentiation in TN-C -/- animals (PTP_{slope} = 125.5 ± 7.6; PTP_{PS} = 231.6 ± 45.4) were not different from levels recorded in the TN-C +/+ mice (PTPslope= 130.8 ± 7.63; PTP_{PS} = 255.2 ± 28.6). Three hours after, the magnitude of population spike amplitude potentiation was 217 ± 18.6 %

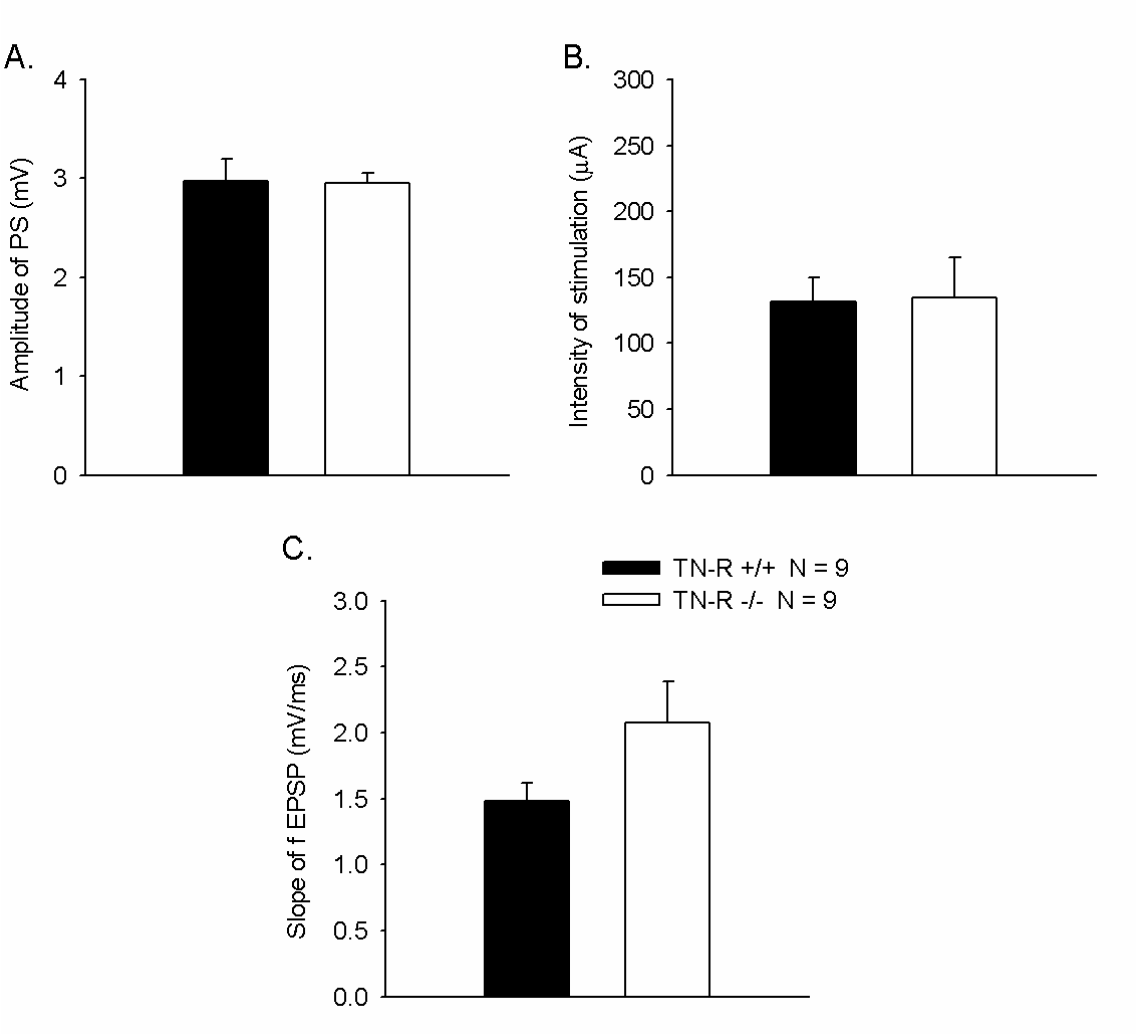

Figure 25. **Normal basal excitatory transmission in the dentate gyrus of TN-R -/- mice.** Data represent mean + SEM of three parameters characterizing basal synaptic transmission: amplitude of population spikes (A), intensity of stimulation used to evoke population spikes of 2-3 mV (B) and slope of fEPSP (C) collected at the stimulation strength used for baseline recording. N indicates the number of mice analyzed and is the same in A, B and C.

in TN-C +/+ and 242.5 ± 32.7% in TN-C -/- mice (Fig. 26A and 26C). The magnitude of potentiation in the slope of fEPSP was 115.61 ± 5.73% in TN-C +/+ and 115.73 ± 5.95% in TN-C -/- mice (Fig. 26B and 26D).

Thus, potentiation of population spikes and fEPSPs was normal in TN-C -/- mice as compared with TN-C +/+ controls. These findings confirm previous *in vitro* results from our laboratory (Evers et al, 2002) which showed that LTPs at both the medial and lateral perforant path were normal in TN-C deficient mice. Since usually *in vitro* recordings are performed in the presence of picrotoxin and *in vitro* and *in vivo* LTP in TN-C -/- mice is normally induced by different stimulation protocols (see section V.2.5.), our results support the view that TN-C does not play an important role at this synapse.

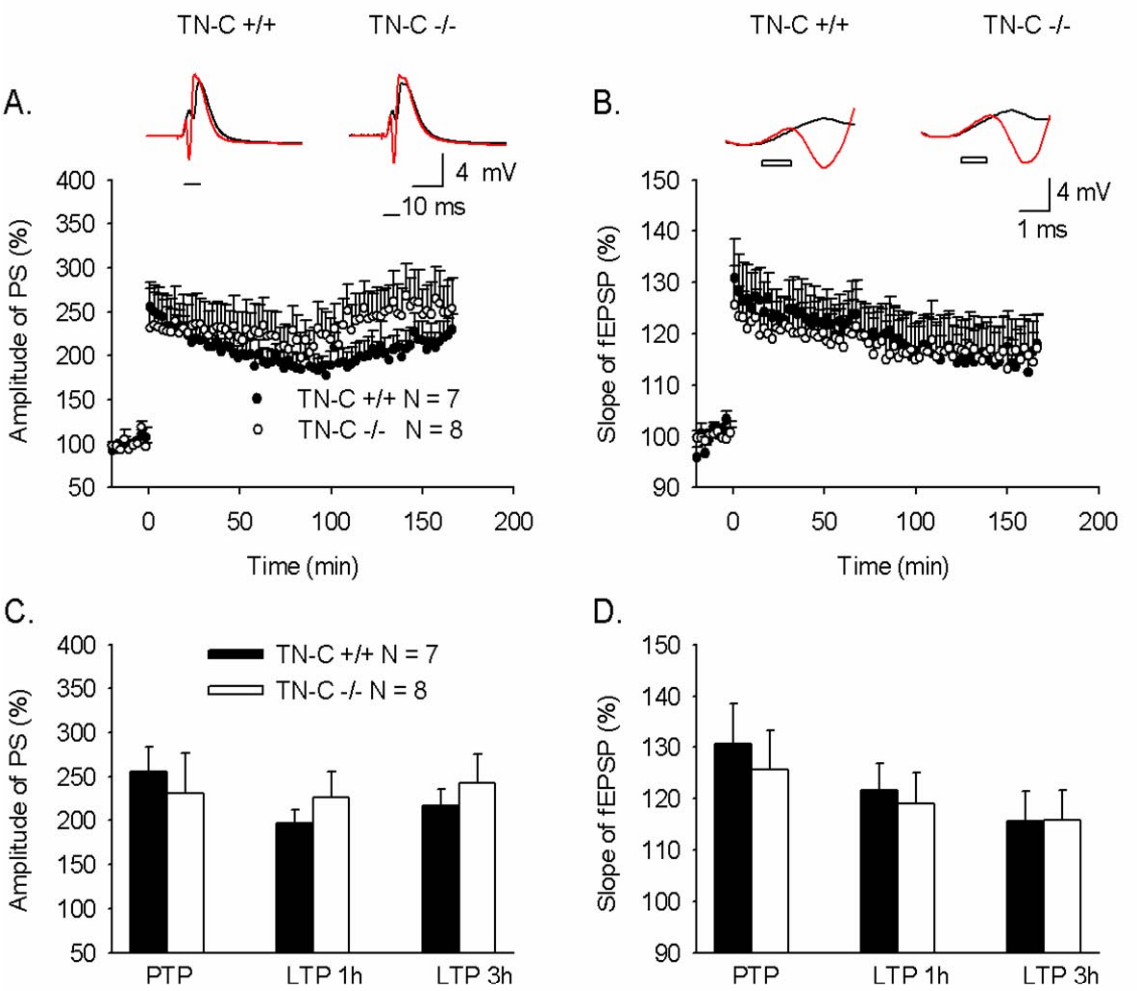

Figure 26. **Normal synaptic plasticity at the perforant path-dentate gyrus synapse of TN-C -/- mice.** Data represent mean + SEM of PS amplitude (A, C) and slope of fEPSPs (B, D) expressed in percentage of the baseline (time from -20 to 0). Theta-burst stimulation (applied at time=0) elicited a robust LTP both in wild type mice and mice deficient in tenascin-C. Examples of responses collected before and after the induction of LTP are shown above LTP profiles. Horizontal bars in A indicate time intervals which are shown with a higher resolution in B. C, D) Cumulative data showing levels of PTP and LTP from all recorded mice at different time points (1h and 3h). N indicates the number of mice analyzed (the same number from A to D).

The second extracellular matrix molecule investigated was TN-R. In the case of this molecule, the situation is completely different. In the first minutes after the LTP induction, the slope levels of post-tetanic potentiation (PTP) were significantly impaired in the TN-R deficient mice whereas no such abnormality was detected when PTP of the population spike was measured. Three hours after LTP induction, the magnitude of potentiation in the slope of fEPSP (Fig. 27B, 27D and table 14) was still significantly impaired in the TN-R -/- as compared to TN-R +/+ mice ($p = 0.033$, *t* test), whereas the

levels of potentiation of the population spike (Figures, 27A, 27C and table 14) was higher in TN-R -/- animals (p = 0.009, *t* test).

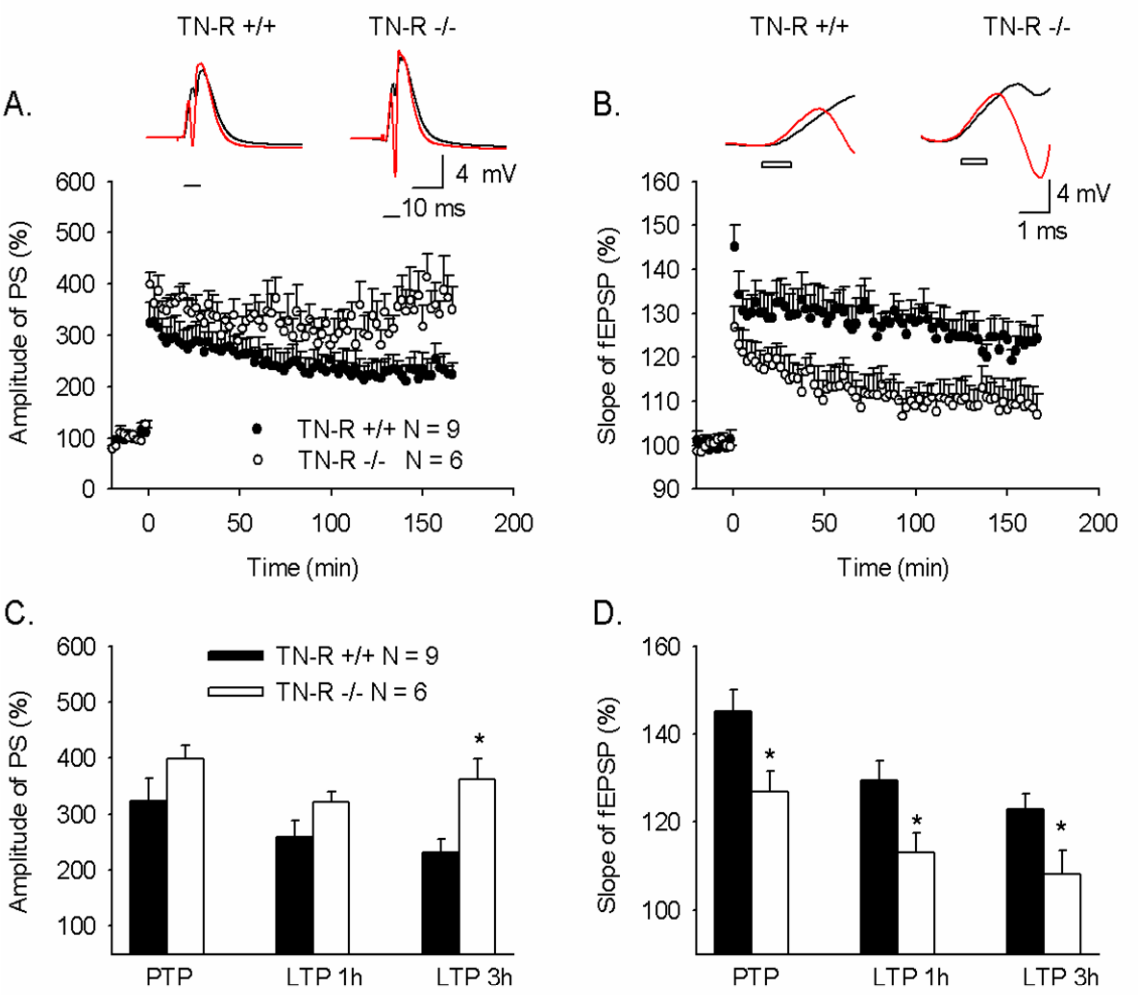

Figure 27. **Abnormal synaptic plasticity at the perforant path-dentate gyrus synapse of TN-R -/- mice.** Data represent mean+SEM of PS amplitude (A,C) and slope of fEPSPs (B,D) expressed as a percentage of the baseline (time from -20 to 0). Theta-burst stimulation (applied at time=0) elicited a robust LTP both in TN-R -/- and TN-R +/+ mice. Examples of responses collected before and after the induction of LTP are shown above LTP profiles. Horizontal bars in A indicate time intervals which are shown with a higher resolution in B. (C, D) Cumulative data showing levels of PTP and LTP from all recorded mice at different time points (1h and 3h). N indicates the number of mice analyzed (the same number from A to D). P < 0.05, t-test.

VII.2.3.1. Effect of muscimol on LTP of tenascin-R wild-type mice

Since our results show that some aspects of synaptic plasticity (i.e., the impairment in the LTP of slope and the enhancement of the population spike at the end of three hours of recording) are abnormal in the TN-R -/- mice, we wanted to find out what could be the possible substrate for such abnormalities. Initial studies investigating the role of the

TN-R in synaptic plasticity showed that LTP in the area CA1 of hippocampus is impaired in mice lacking this molecule. This impairment was accompanied by an increase in the levels of basal excitatory synaptic transmission and a reduced amplitude of unitary perisomatic inhibitory postsynaptic currents recorded from CA1pyramidal cells (Bukalo et al., 2001; Saghatelyan et al., 2001). Furthermore, a strong reduction in the density of the symmetrical perisomatic synapses was observed in the area CA1 (Nikonenko et al., 2003). Combining all these previous findings, a recent study from our group, showed that an *in vivo* administration of muscimol (a $GABA_A$ agonist) 24 hours before the electrophysiological recordings was able to rescue the LTP in area CA1 in TN-R -/- mice (Bukalo et al, 2007). Following the same rationality, we performed the same type of experiment *in vivo* at the perforant path-dentate gyrus synapse. Twenty four hours before the experiments were conducted, the mice have been i.p. injected with muscimol (1mg/kg b.w.) or saline solution. Since, at the beginning we wanted to be sure that such an injection does not disrupt the normal mouse physiology (i.e., due to stress), we first performed experiments with injection of vehicle and muscimol in wild-type animals.

Analysis of short-term synaptic plasticity in TN-R +/+ mice injected either with muscimol or NaCl (Fig. 28) did not reveal any abnormality in the levels of facilitation or inhibition at none of the interpulse intervals studied. The data are summarized in tables 12 (paired-pulse facilitation of fEPSPs) and 13 (paired-pulse modulation of the population spikes).

Inter-pulse interval	10 ms (%)	25 ms (%)	50 ms (%)	100 ms (%)
TN-R +/+ (NaCl)	110.5 ± 5	123.3 ± 2.6	112.3 ± 2	103.3 ± 6
TN-R +/+ (muscimol)	117.6 ± 8.7	120.3 ± 6.3	121 ± 8.	102.5 ± 4

Table 12. **Analysis of paired-pulse facilitation at the perforant path-dentate gyrus synapse in mice injected with muscimol.** The values represent the levels of facilitation of the second slope of fEPSP and they are expressed as percentage of the first slope ± SEM. Number of tested animals is the same as shown in figure 28A.

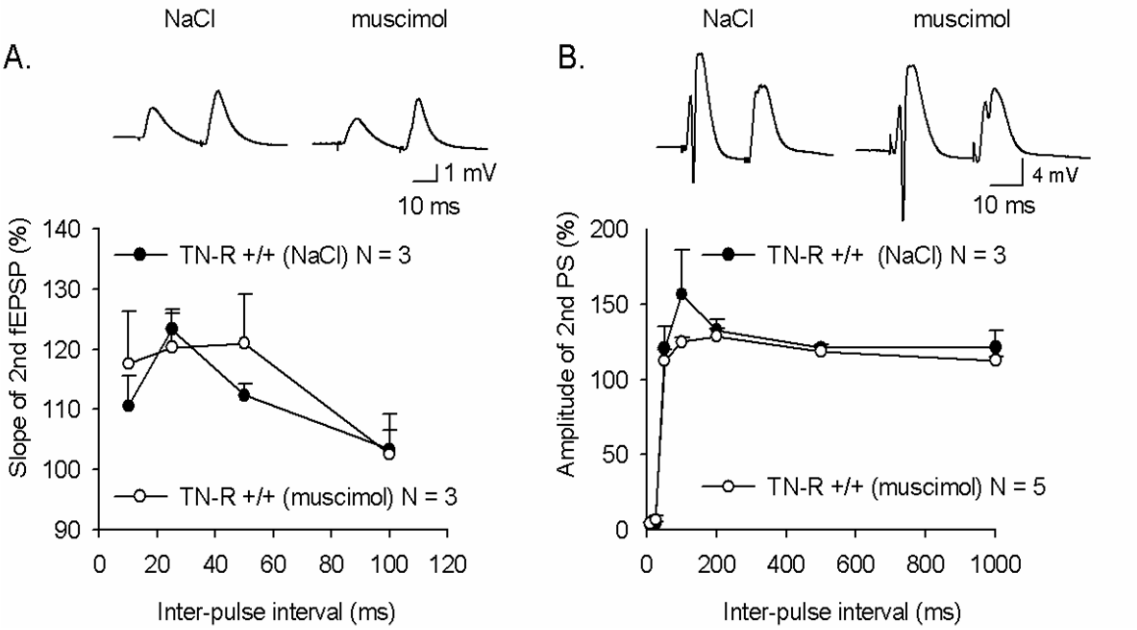

Figure 28. **Normal short-term plasticity in TN-R +/+ mice injected with muscimol.** Intraperitoneal injection of NaCl or muscimol 24h before the experiment does not affect paired-pulse facilitation of fEPSPs(A) or paired-pulse modulation of the population spike(B). Upper are shown example traces at 25 ms inter-pulse interval from TN-R +/+ mice injected either with NaCl or with muscimol. N represents the number of mice analyzed.

Inter-pulse interval	TN-R +/+ (NaCl)	TN-R +/+ (muscimol)
10 ms	3.3 ± 1.4	4.2 ± 0.7
25 ms	3.8 ± 1.5	6.5 ± 3
50 ms	120.8 ± 14.4	112.4 ± 7.2
100 ms	156.7 ± 29	125 ± 3.2
200 ms	132.5 ± 7.4	128.7 ± 5
500 ms	120.8 ± 2.3	118.7 ± 2.5
1000 ms	121.5 ± 11.3	112.7 ± 2.5

Table 13. **Analysis of paired-pulse modulation of population spikes at the perforant path-dentate gyrus synapse in mice injected with muscimol.** The values represent the levels of depression/facilitation of the second PS and they are expressed as a percentage of the first PS ± SEM. Number of tested animals is the same as shown in Fig. 28B.

We further investigated the basal synaptic transmission in the TN-R +/+ mice injected with muscimol. The PS values were in the predicted range (i.e., 2-3mV) for both groups of mice injected either with muscimol or NaCl (Fig. 29A) and there was also no difference in the stimulation intensity necessary to evoke such a PS (Fig. 29B). Likewise, measurements of the slope of fEPSP (Fig. 29C) did not reveal any difference between the two groups suggesting thus that administration of muscimol 24 hours before the experiment does not affect the basal excitatory synaptic transmission in the wild-type mice.

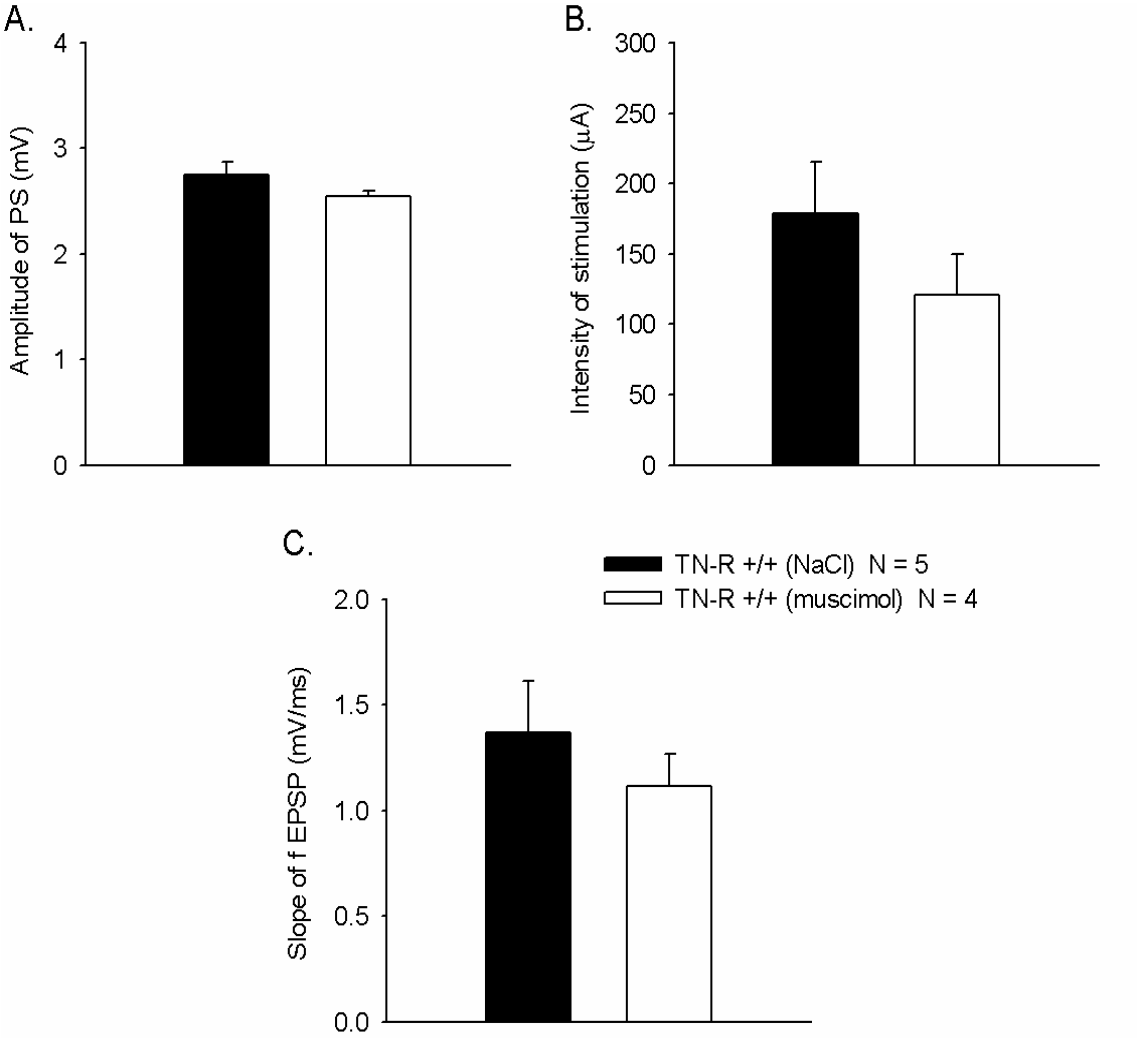

Figure 29. **Normal basal excitatory transmission in the dentate gyrus of TNR +/+ mice injected with muscimol.** Data represent mean+SEM of three parameters characterizing basal synaptic transmission: amplitude of population spikes (A), intensity of stimulation used to evoke population spikes of 2-3 mV (B) and slope of fEPSP (C) collected at the stimulation intensity used for baseline recording. N indicates the number of mice analyzed and is the same in A, B and C.

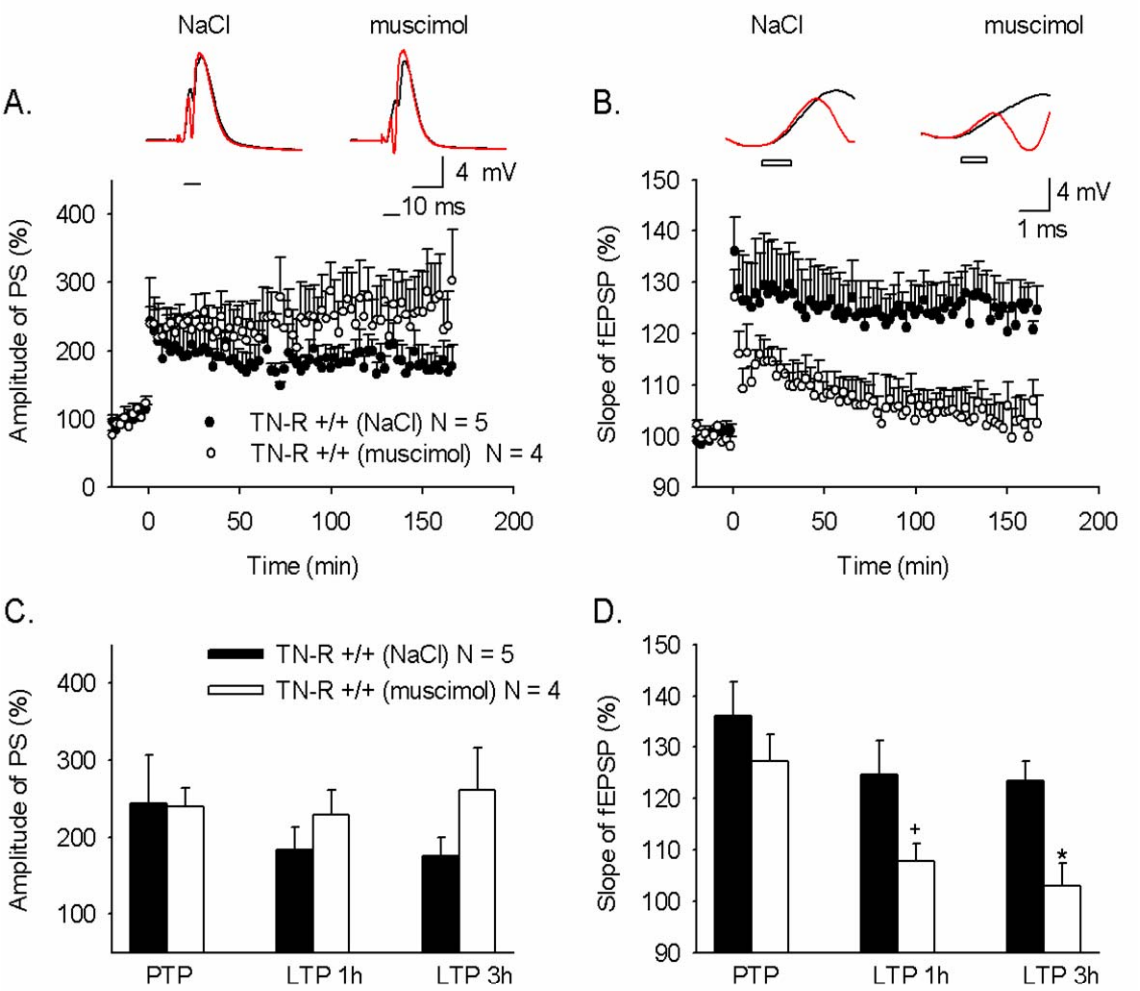

Figure 30. **Injection of muscimol 24h before the experiment impairs long-term synaptic plasticity at the perforant path-dentate gyrus synapse in the TN-R +/+ mice.** Data represent mean + SEM of PS amplitude (A,C) and slope of fEPSPs (B,D) expressed in percentage of the baseline (time from -20 to 0). Theta-burst stimulation elicited a robust LTP of PS in wild-type mice injected either with NaCl or with muscimol (A,C), however LTP of slope of fEPSP is impaired in the TN-R +/+ mice injected with muscimol (B,D). Examples of responses collected before and after the induction of LTP are shown above LTP profiles. Horizontal bars in A indicate time intervals which are shown with a higher resolution in B. C) Cumulative data showing normal levels of PTP and LTP in the case of PS. D) Cumulative data showing normal levels of PTP and impaired LTP at the end of three hours of recoding in the case of slope of fEPSP. N indicates the number of mice analyzed (the same number from A to D). $P < 0.05$, t-test.

Application of 6 trains of theta-burst stimulation induced a robust potentiation of the population spike in both groups injected either with NaCl or with muscimol (Fig. 30A and C). Surprisingly, although the levels of potentiation for the slope of fEPSP were normal in the mice injected with saline solution, at the end of three hours of recording they were drastically impaired in the mice injected with muscimol (data shown in table 14). Since muscimol impaired dentate gyrus LTP in wild-type mice, we argued that it is unlikely that muscimol would improve LTP in TN-R -/- mice. The completion of this

series of electrophysiological recordings also coincided with completion of immunohistochemical analysis that revealed increases in the number of parvalbumin-expressing interneurons and their terminals on granule cells of TN-R -/- mice. The latter is in contrast to CA1, thus, suggesting that mechanisms mediating impairment of LTP in the CA1 region and dentate gyrus are distinct and there is no reason to perform experiments with muscimol in TN-R -/- mice.

VII.2.3.2. Rescue of LTP in TN-R -/- mice by bicuculline

Research conducted in our laboratory showed that both the number and the density of the parvalbumin-positive interneurons are significantly increased (Fig. 31A) in the dentate gyrus of the TN-R -/- mice (Dr. Elena Sivukhina, unpublished observation). Furthermore, densities of both PV^+ and PV^- perisomatic puncta were higher compared with TN-R +/+ littermates (+11% and +16%, respectively, Fig. 31B) and there was no difference in granule cell soma area between the genotypes. Thus, morphological data pointed out that a single granule cell is innervated by a higher-then-normal number of inhibitory interneurons suggesting that the impairment observed in the synaptic plasticity in the TN-R -/- mice might be due to an increased inhibition in this area.

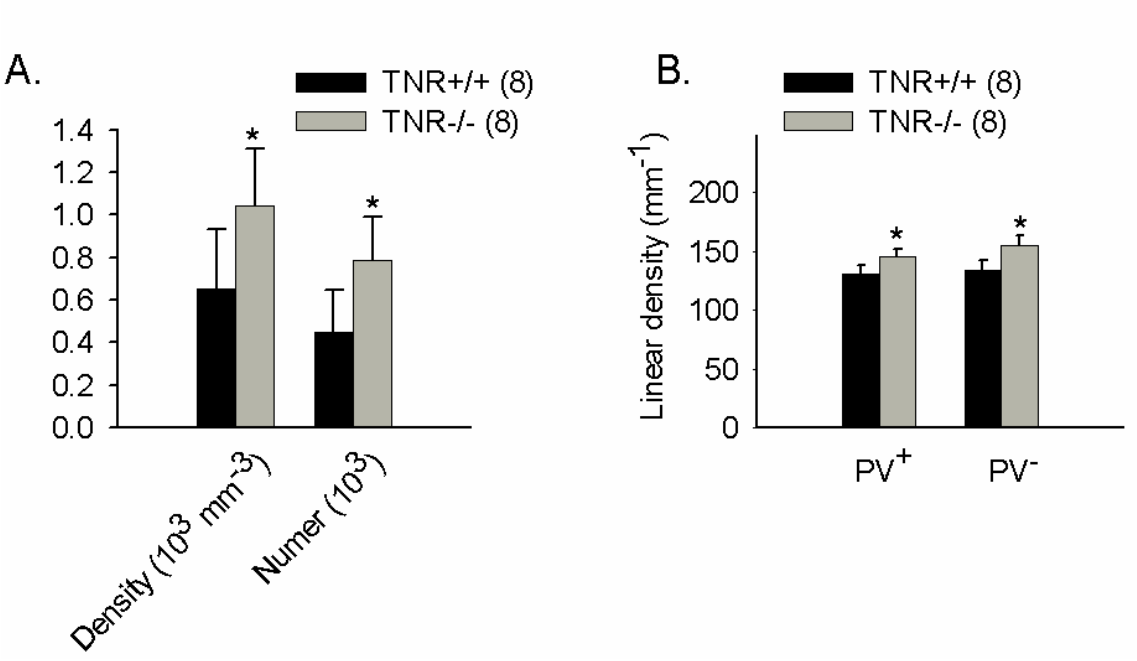

Figure 31. **Increased number of the inhibitory interneurons in the dentate gyrus of the TN-R -/- mice.** A) Significantly increase in both the density and the number of PV^+ interneurons in the DG of tenascin-R deficient mice. B) Increased densities for both PV^+ and PV^- perisomatic puncta around granule cell bodies in the DG. Data are presented as mean values + SD. * $p < 0.05$, t test. Experiments were performed by Dr. Elena Shivukhina.

In order to test this hypothesis, we further recoded LTP at the PP-DG synapse in the presence of bicuculline (Bcc), a $GABA_A$ inhibitor. Shortly, the experiments in the presence of bicuculline were conducted in the same way as in untreated mice, with the only difference that instead of the metal recording electrode, a glass pipette filled with bicuculline was used to deliver the drug, and in order to avoid epileptic activity, a PS of 1-2 mV was chosen for baseline recordings. Since the responses in the presence of the $GABA_A$ antagonist are smaller and often can be contaminated by spiking activity even in the wild-type animals (Nosten-Bertrand et al., 1996), short-term plasticity was not recorded for this type of experiment. Analysis of the basal synaptic transmission did not reveal any differences between the two genotypes in the presence of bicuculline.

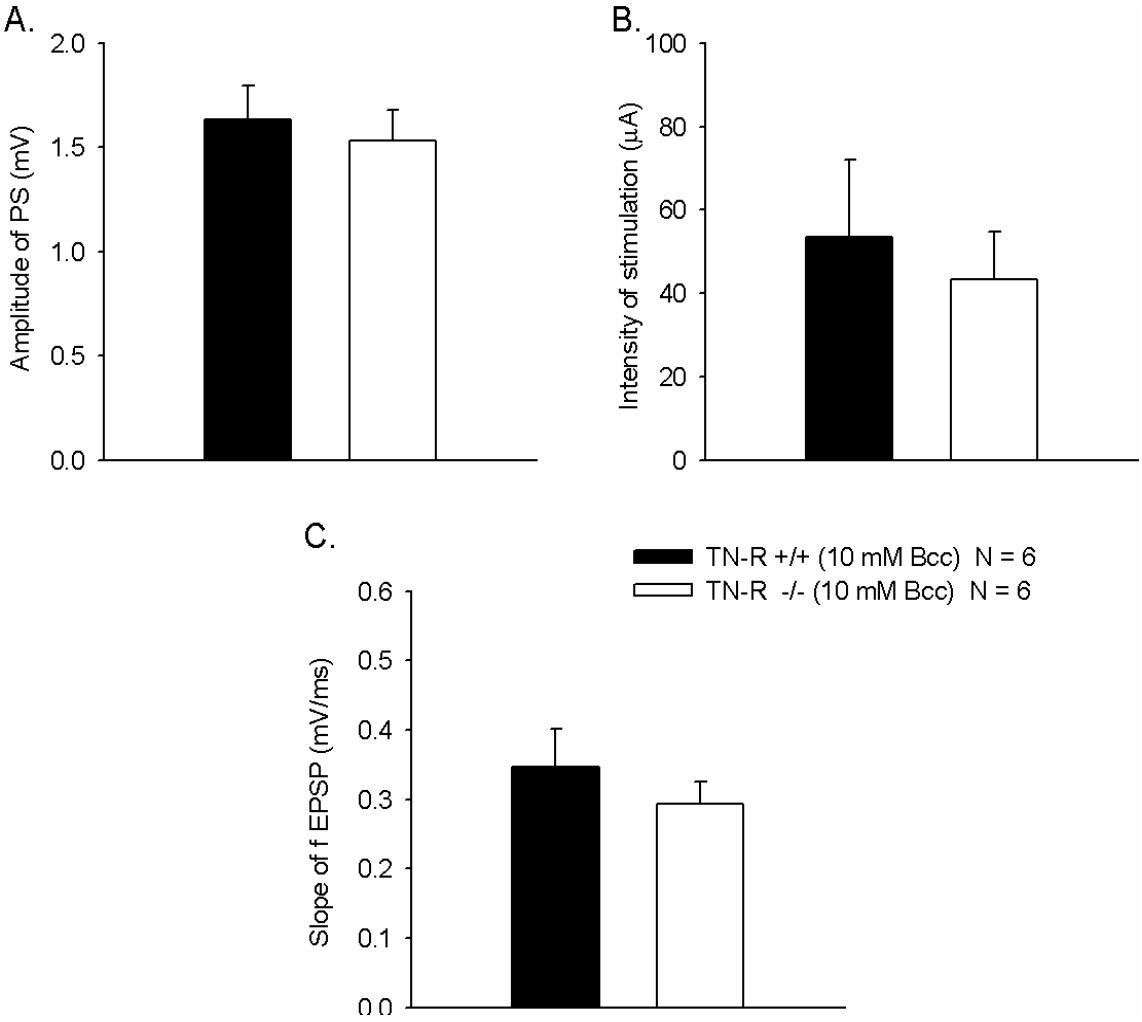

Figure 32. **Normal basal excitatory transmission in the dentate gyrus of TNR -/- mice recorded in presence of 10 mM bicuculline in the recording pipette.** Data represent mean + SEM of three parameters characterizing basal synaptic transmission: amplitude of population spikes (A), intensity of stimulation used to evoke population spikes of 1-2 mV (B) and slope of fEPSP (C) collected at the stimulation strength used for baseline recording. N indicates the number of mice analyzed and is the same in A, B and C.

As a result of the Bcc infusion and the fact that PS values were smaller (Fig. 32A) than in the previous experiments, the intensities used for evoking the desired population spike amplitude (Fig. 32B) were also significantly lower than in the untreated animals but to the same extent in both genotypes. The same was also true in the case of slope of fEPSP measurements (Fig. 32C).

Application of theta-burst stimulation, induced a robust potentiation for both parameters measured, although the potentiation of the PS (Fig. 33A and C) was significantly smaller in both genotypes compared with the pharmacologically untreated mice. This effect has been previously reported in the wild-type mice (Nosten-Bertrand et al., 1996).

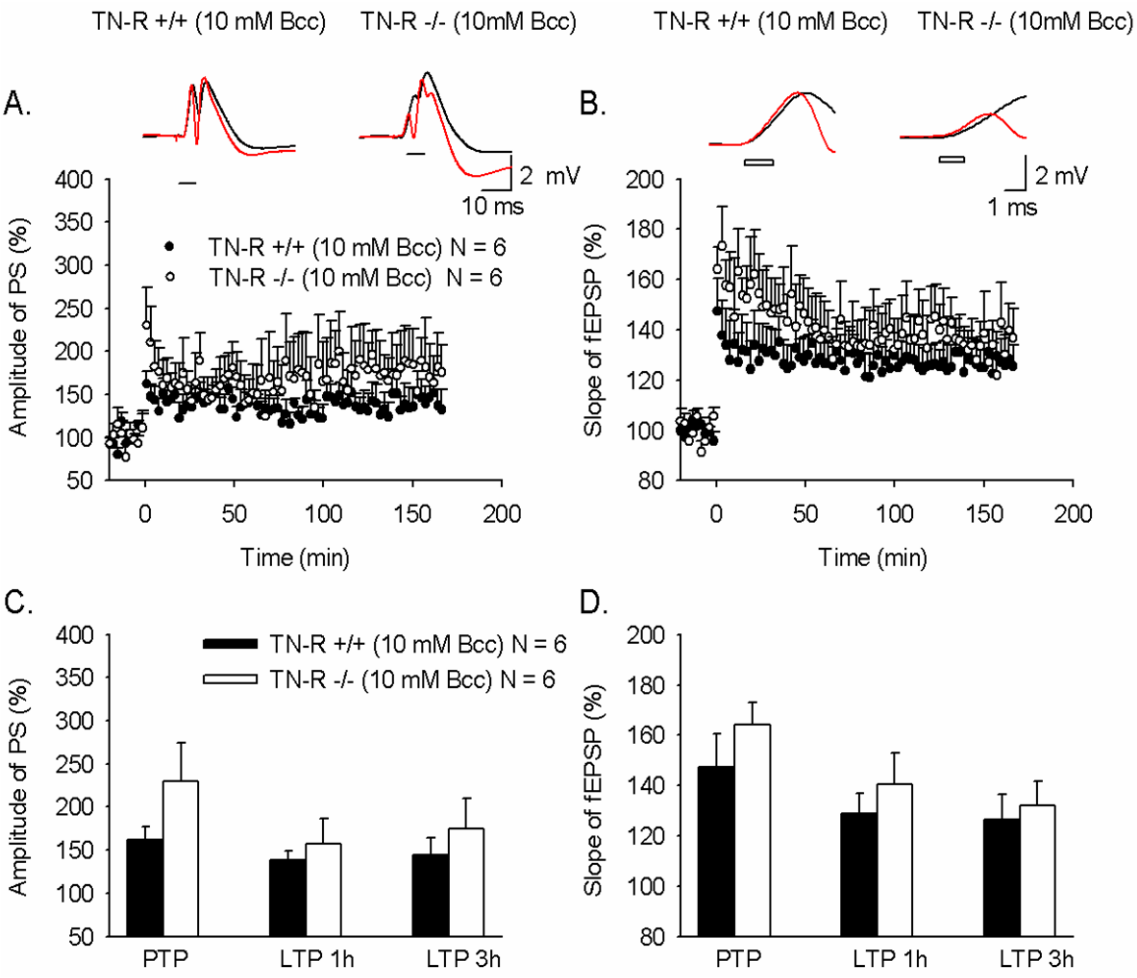

Figure 33. **Rescue of LTP in TN-R -/- mice by suppression of GABAergic inhibition.** LTP was induced in the presence of 10 mM bicuculline in the recording pipette. Data represent mean + SEM of population spike (PS) amplitude (A, C) and slope of fEPSPs (B, D) expressed in % regarding the baseline. TBS protocol elicited a robust LTP both in TNR +/+ and TNR -/- mice. Examples of responses collected before and immediately after the LTP induction are shown above LTP profiles. Please note the change in the responses appearance due to disinhibition (see also figure 27). Horizontal bars in A indicate time intervals which are shown with a higher resolution in B. C, D) Cumulative plots showing normal PTP and LTP for both parameters in TN-R -/- mice in the presence of 10 mM bicuculline. N indicates the number of mice analyzed (the same number from A to D).

The levels of post-tetanic potentiation for both PS amplitude and slope of fEPSP were normal in the TN-R -/- mice. The same was also true for the levels of potentiation at the end of three hours of recording (data are summarized in table 14). Thus, bicuculline infusion fully restored short- and long-term potentiation of fEPSPs to normal levels in TNR -/- mutants (Fig. 33B and 33D). These data indicate that population spike amplitude potentiation is mostly mediated by theta-burst induced long-term disinhibition in the dentate gyrus that can be occluded by infusion of $GABA_A$ receptor antagonists.

Mice	PTP (%)		LTP 1h (%)		LTP 3h (%)	
	Slope	PS	Slope	PS	Slope	PS
TN-R +/+	145.1 ±4.9	324 ±39.8	129.5 ±4.3	257.9±30.1	122.8 ±3.6	230.9±24.6
TN-R -/-	126.9 ±4.5	399.5±23.3	113.1 ±4.3	321.1±18.7	108.2 ±5.3	361.7±37.7
TN-R +/+ (NaCl)	136 ±6.6	243.4±63.5	124.7 ±6.4	183 ±29.8	123.4 ±3.7	175.5±23.4
TN-R +/+ (muscimol)	127.2 ±5.2	239.1±24.7	107.8 ±3.3	229.2 ±32	102.9 ±4.5	261.4±54.5
TN-R +/+ (bicuculline)	147.4 ±13.2	162.3±15.2	128.7 ±8.1	138.4 ±11	126.3 ±0.1	143.7±20.2
TN-R -/- (bicuculline)	164.1 ±8.7	230.2±43.9	140.4±12.3	156.7±29.1	132.04±9.8	174.7±35.1

Table 14. **Summarized data for short- and long-term potentiation of fEPSP and population spike in the dentate gyrus of tenascin-R -/- mice**. Data are presented in % of the baseline. Error bars are SEM

VII.2.4. LTP recording at CA3-CA3 synapse of tenascin-R deficient mice

As mention above (see chapter VII.2.3.1), previous electrophysiological studies from our laboratory showed that LTP is impaired in the area CA1 of hippocampus. The electrophysiological data correlated nicely with morphological findings in the area CA1 (Nikonenko et al, 2003). In the present study, we found that also LTP at the perforant path-dentate gyrus is impaired in the tenascin-R mutants, deficits which could be rescue by blocking GABAergic inhibition in the area of recording (see chapter VII.2.3.2). The third important subfield of hippocampus is the CA3 region. Experiments performed in our group showed that LTP at the mossy fibers–CA3 synapse is normal (unpublished data from Dr. Olena Bukalo). CA3 pyramidal cells not only receive mossy-fiber input but also are interconnected via associational/commissural axons. In order to see if this

major input to the CA3 cell is affected by the lack of TN-R, we further investigated LTP at this synapse.

Recording of amplitudes of fEPSPs at different stimulation intensities followed by two-way ANOVA for repeated measurements did not reveal any differences between TN-R +/+ and TN-R -/- mice with regard to stimulus-response curves (Fig. 34A). Paired-pulse facilitation recorded at 10, 20, 50, 100 and 200 ms inter-pulse interval revealed no abnormalities at the presynaptic level in these mutants (Fig. 34B). Following a 10 min baseline recording, a robust LTP was induced in both genotypes by two trains of high frequency stimulation (Fig. 34C). Thus, the levels of short-term potentiation were 201 ± 10.8 % in TN-R +/+ mice and 202.24 ± 10 % in TN-R -/- mice. The levels of LTP were 140 ± 3.8 % in TN-R +/+ and 148.44 ± 5 % in TN-R -/- mice (Fig. 34D).

Mice/Region/Condition	Paired-pulse facilitation of fEPSPs	Paired-pulse modulation of population spike	Basal synaptic transmission	LTP of fEPSPs	LTP of population spikes
TN-R -/- DG *in vivo*	↓25,100ms	=	=	↓	↑
TN-R +/+ DG *in vivo* muscimol	=	=	=	↓	=
TN-R -/- DG *in vivo* bicuculline	n.d.	n.d.	=	=	=
TN-R -/- CA3 *in vitro*	=	n.d.	=	=	n.d.

Table 15. **Summary of electrophysiological findings in the mice deficient in TN-R molecule.** All the findings are expressed relatively to the levels found in wild-type mice. Abbreviations: PS, population spike; =, not different from wild-type mice; ↓, impaired compared with the wild type situation; ↑ increased compared with the controls mice; n.d, not determinated.

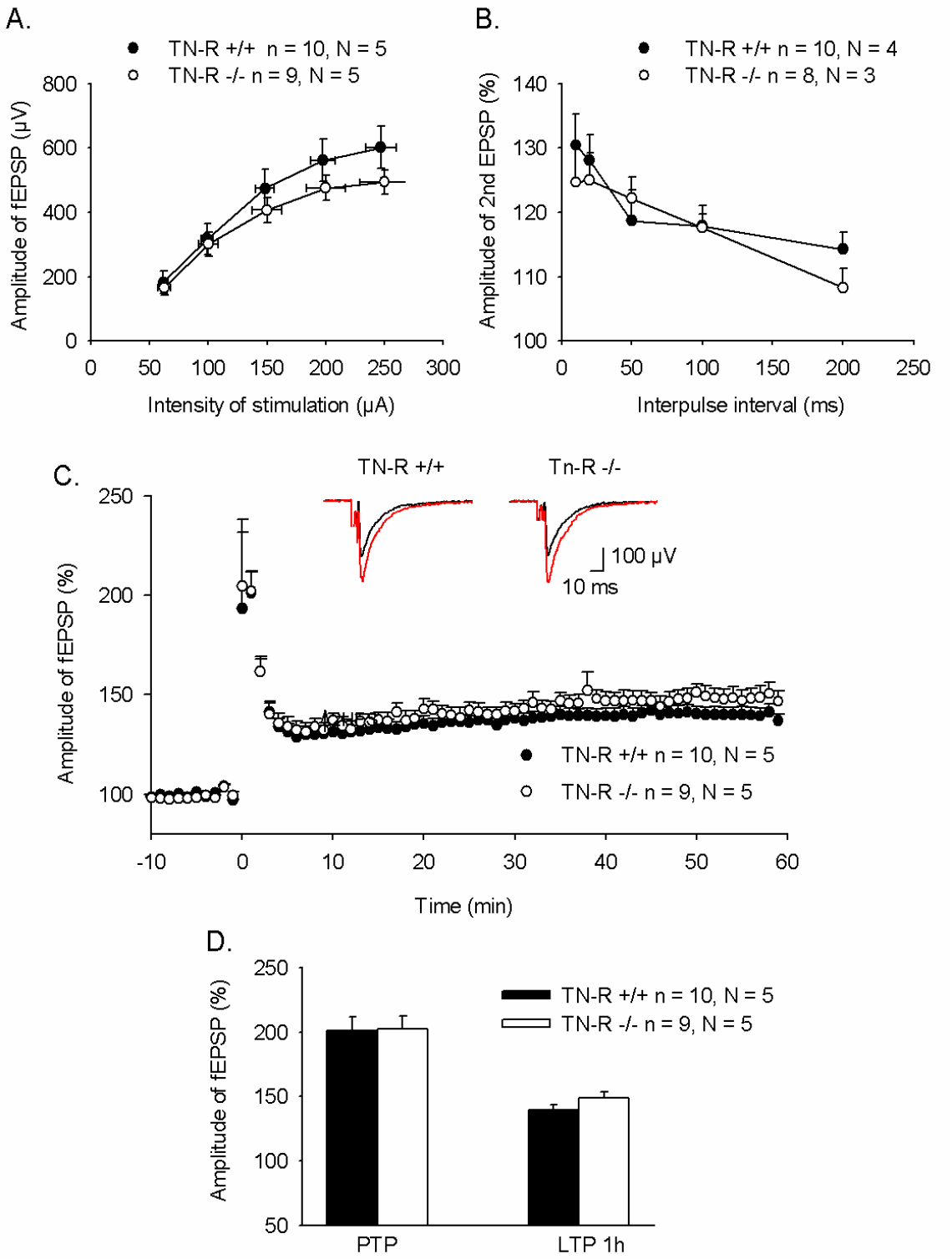

Figure 34. **Normal LTP at the A/C synapse in the TN-R -/- mice.** A) Input-output curves for amplitudes of fEPSPs evoked by stimulation of associational/commissural projections to the CA3 field at different stimulation intensities. No difference between genotypes was detected. B) Normal paired-pulse facilitation at different inter-pulse intervals. C) Two trains of HFS induce similar levels of short- and long-term potentiation in both genotypes. Upper panel shows fEPSPs recorded before and 50-60 min after HFS for both TN-R +/+ and TN-R -/- mice. D) Cumulative plots showing normal levels of PTP and LTP in the tenascin-R mutants.

Statistically significant differences were found neither for short- nor for long-term potentiation. Thus, although the long-term plasticity is affected at the areas CA1 and DG of the hippocampus, the NMDAR-dependent LTP at associational/commissural connections in the CA3 subfield is normal in TN-R -/- mice.

In conclusion, the TN-R -/- mice exhibit regional specific impairments in synaptic plasticity, most of these findings being correlated also at the morphological level (data not shown). The findings of the present study are summarized in table 15.

VIII. DISCUSSIONS

PROJECT 1. The roles of NCAM and PSA in synaptic plasticity

VIII.1.1. NCAM and PSA deficiency causes minor abnormalities in paired-pulse modulation in the dentate gyrus

To assess short-term synaptic plasticity in NCAM- and PSA deficient mice, we analyzed facilitation of transmitter release elicited by paired-pulse subthreshold stimulation of the perforant path. Only PST-/- mice showed slightly but significantly abnormal paired-pulse facilitation of fEPSPs (see table 4). This abnormality did not result in altered modulation of population spikes in PST -/- mice in response to paired-pulse supramaximal stimulation; this is not surprising as this parameter is known to reflect excitability of neurons and synchronization of spiking activity in the population of granule cells rather than modulation of transmitter release. Excitability and synchronization depend on intrinsic properties and network activity, including the activity of GABAergic interneurons. In contrast to PST -/- mice, we found a slightly abnormal paired-pulse modulation of population spikes, although not of synaptic responses, in NCAM -/- and STX -/- mutants. In summary, these data suggest that polysialylation of NCAM in immature neurons possibly shapes the organization of the neural network in the dentate gyrus and, thus, affects the firing pattern of granule cells, whereas polysialylation of NCAM in synapses of mature neurons modulates transmitter release in synapses formed on these cells by yet unknown mechanisms.

VIII.1.2. Impairment in basal synaptic transmission in the dentate gyrus of STX deficient mice

Another finding of the present study is that basal synaptic transmission in the perforant path-dentate gyrus connection is impaired in STX -/- mice, i.e. in mice with deficient polysialylation of immature neurons that are generated continuously in this hippocampal region in adult mice. As presynaptic stimulation elicited normal population spike activity in the dentate gyrus of STX -/- mice, while fEPSPs were reduced, the mutants

display a shift in the E-S curve that is indicative of an increase in excitability of granule cells. This phenomenon might result from a secondary homeostatic / adaptive event to maintain spiking activity of granule cells at normal levels despite reduced synaptic input. As paired-pulse facilitation of fEPSPs, which is related to the probability of release, is normal in STX -/- mice, it is unlikely that probability of release is reduced in this mutant. More plausible reasons for the reduction in the basal synaptic transmission in STX -/- mice would be a reduction in the number of synapses formed by perforant path axons in the dentate gyrus or smaller postsynaptic responses elicited by single vesicle release. The hypothesis that PSA synthesized by the developmentally early acting polysialyltransferase STX might be important for proper synaptogenesis in the dentate gyrus is supported by recent *in vitro* data showing that polysialylation of immature hippocampal neurons promotes synapse formation in a choice situation when NCAM deficient and PSA-NCAM-expressing neurons are cocultured (Dityatev et al., 2000; Dityatev et al., 2004). In the dentate gyrus, a choice situation is created by the progenitor cell-derived immature neurons, which are highly polysialylated by STX (Hildebrandt et al., 1998; Angata et al., 2004) and situated in a cellular territory with a lower expression of PSA by mature granule cells. This scenario favors preferable synapse formation on PSA-enriched immature neurons in wild-type mice. In STX -/- mice preferential PSA-mediated synapse formation might no longer take place and immature granule cell neurons would thus receive less synaptic input. As our recordings revealed impaired basal synaptic transmission in adult STX -/- mice, we have to postulate that the deficit in formation of synapses on immature granule cells might have long-lasting consequences and result in reduced synaptic coverage of mature granule cells. As basal synaptic transmission is normal in the dentate gyrus of NCAM -/- mice, the deficit in synaptic transmission in STX mutants highlights the interesting possibility that abnormal polysialylation of NCAM leads to a more severe abnormality of synaptic transmission than the absence of the NCAM glycoprotein itself. This interpretation is consistent with data on mutant mice deficient in both PST and STX, which exhibit numerous abnormalities in axonal projections that are not observed in single NCAM -/- mice or mice deficient in the three genes PST, STX and NCAM (Weinhold et al., 2005). As PSA is a strongly anti-adhesive structure, a PSA-negative (although NCAM glycoprotein-positive) environment in which immature granule cells are situated in STX deficient mice might be too adhesive for optimal axonal outgrowth and remodeling,

processes that would appear necessary for synapse formation on newly generated granule cells.

VIII.1.3. Impaired LTP in the dentate gyrus in NCAM deficient mice

Our results provide electrophysiological evidence that NCAM regulates synaptic plasticity *in vivo* and, thus, close a gap between previous in vitro data on the role of NCAM in LTP (Bukalo et al., 2004; Dityatev et al., 2000; Luthi et al., 1994; Muller et al., 1996) and behavioral studies, demonstrating the importance of NCAM in hippocampus-dependent learning and memory in the Morris water maze and contextual fear conditioning (Bukalo et al., 2004; Cremer et al., 1994; Stork et al., 2000).
Although a wealth of information has been accumulated on the contributions of cell adhesion on synaptic plasticity in vitro, experiments *in vivo* are very rare. For instance, normal dentate gyrus LTP was found *in vivo* in anaesthetized mice deficient in the neural cell adhesion molecule L1 (Bliss et al., 2000), whereas mice deficient in the cell adhesion molecule Thy-1 show impaired dentate gyrus LTP in both anaesthetized and freely moving mice (Errington et al., 1997; Nosten-Bertrand et al., 1996). This impairment was further shown to be due to an increase GABAergic activity (Nosten-Bertrand et al., 1996). Unfortunately, up to now there are no data available on GABAergic transmission in the dentate gyrus of NCAM -/- mice, but transgenic mice overexpressing the secreted extracellular domain of NCAM exhibit a striking reduction in inhibitory synapses in the cingulate and frontal association cortices and in the amygdala (Pillai-Nair et al., 2005), thus suggesting a link between inhibitory interneurons and NCAM. Our present data show that paired-pulse inhibition of population spikes, a measure of feedback inhibition, was not higher in NCAM -/- mice when compared with the wild-type mice. In fact, there was a tendency in both NCAM-/- and STX -/- mice to exhibit lower modulation of population spikes, as compared with their controls. It is therefore more likely that the number of GABAergic interneurons or their synapses/synaptic activity is decreased, rather than increased, in the dentate gyrus of NCAM -/- or STX -/- mice. Still, lower amplitudes of population spikes elicited by theta-burst stimulation in NCAM -/- mice, as found in the present study, could reflect an increased recruitment of GABAergic interneurons or facilitation of inhibitory currents in response to theta-burst stimulation. Also the fact that LTP in the dentate gyrus was normal in the presence of a $GABA_A$ receptor antagonist in vitro, support the idea about possible abnormalities in GABAergic transmission in the dentate gyrus.

Another hypothesis to explain the observed deficit in LTP of NCAM -/- mice would be that LTP is already saturated at dentate gyrus synapses because of prior potentiation. However, in this case one would expect to find elevated levels of basal synaptic transmission in NCAM mice, as, for example, was observed in mice deficient in the extracellular glycoprotein tenascin-R (Saghatelyan et al., 2001). Our data, however, show normal basal synaptic transmission in NCAM -/- mice and, thus, argue against this idea. Another possibility is that NMDA receptor activity is reduced in these animals. The NMDA receptor is critical for induction of LTP in the dentate gyrus of mice (Kleschevnikov and Routtenberg, 2001). The functional cross-talk between adhesion molecules and neurotransmitter receptors is not new. Exciting examples linking cell adhesion molecule function and NMDA receptors are provided by EphB receptors, integrins and alphaneurexin (Kattenstroth et al., 2004; Lin et al., 2003; Takasu et al., 2002). In this respect, it is noteworthy that tetanic stimulation of the perforant path results in an increased proportion of axospinous synapses expressing NCAM-180, the largest isoform of NCAM with the longest cytoplasmic domain of all splice variants, that interacts with spectrin enriched in postsynaptic densities (Schuster et al., 1998; Sytnyk et al., 2002). Also NCAM-180 and the NR2A subunit of NMDA receptors colocalize before, and coredistribute to, the periphery of postsynaptic densities after induction of dentate gyrus LTP (Fux et al., 2003). It is not known how these activity-dependent changes in NCAM expression and cellular localization contribute to synaptic plasticity in the dentate gyrus but it is conceivable that an association between NCAM-180 and NMDA receptors could be critical for NMDA receptor activity during the induction of LTP. Additional indirect support for this notion comes from our *in vitro* experiments in which CA1 LTP is restored by an induced increase in Ca^{2+} influx via NMDA receptors, such as elevation of extracellular Ca^{2+} concentration (Bukalo et al., 2004) or lowering of extracellular Mg^{2+} concentration (Gaga Kochlamazashvili, unpublished observations), which is known to facilitate activation of NMDA receptors.

VIII.1.4. Normal LTP in the dentate gyrus of PSA deficient mice

Besides study on the involvement of NCAM molecule in the synaptic plasticity at the perforant path-DG synapse, we also characterized the role of PSA at this synapse. Our data suggest that in this area of the hippocampus, LTP is NCAM- but not PSA-dependent (table 8). These features observed in the DG resemble those of mossy fibers LTP. In both cases, induction of LTP requires NCAM but not PSA (Angata et al., 2004;

Cremer et al., 1998; Eckhardt et al., 2000; Fux et al., 2003). Thus, in respect to PSA-dependence, LTP in the dentate gyrus is different from LTP and LTD in the CA1 region, which are both PSA dependent. Interestingly, we did not detect any abnormalities in dentate gyrus LTP in PST -/- or STX -/- mice. However, the number of immature granule cells in which polysialylation of NCAM is driven by STX is quite small, and therefore even strong abnormalities in LTP in this subpopulation of granule cells might not be well detected using field recordings, which provide a measure of activity in large neuronal cell populations. Because the highly polysialylated subpopulation of newly generated granule cells shows particularly high levels of LTP (Schmidt-Hieber et al., 2004), it will be of interest to compare LTP in these neurons and mature granule cells in STX -/- and NCAM -/- mice using single-cell recordings.

The expression of the two enzymes is not overlapping: at earlier stages of development STX is active, whereas the later stages PST is coming into play paralleling the decrease in STX activity. Thus, we cannot exclude the fact that residual PSA made by the complementary enzyme is enough and sufficient for normal synaptic plasticity at this hippocampal subregion. In this respect, it will be interesting to study synaptic plasticity in the double knock out mouse, which lack both PST and STX enzyme, or in endo-N treated brains or slices.

VIII.1.5. LTP in newly generated granule cells in the dentate gyrus depends on NR2B-containing NMDA receptors

LTP at the MPP-DG connection has proven difficult to be induced in slice preparation because of the very strong GABAergic innervation in this hippocampal subfield (Wingstrom and Gustafsson, 1983). Despite this impediment, recent studies in rats have identified a small but stable form of LTP that can be elicited in the absence of $GABA_A$ antagonists and is specifically affected by gamma irradiation (Snyder et al., 2001; Wang et al., 2000). Furthermore, this LTP requires activation of NR2B-containing NMDA receptor subtype that is known to be present in the developing brain but is largely replaced by the NR2A-containing NMDA subtypes during the maturation (Monyer et al., 1994; Williams et al., 1993). Although some NR2B immunolabeling persists in the adult brain of mice, its highest levels are present at 15-21 days postnatal (Okabe et al., 1998), approximately the age of the neurons that we think are responsible for this form of LTP. In agreement with these results, in our experiments we also observed a small LTP in response to the MPP stimulation that did require the use of $GABA_A$ receptor

blockers. Moreover, this form of LTP could be abolished by Ro 25-6981, a specific NR2B antagonist, suggesting thus that the observed potentiation is due to activation of immature neurons in the DG.

VIII.1.6. Normal LTP of immature granule cells in NCAM deficient mice

After establishing this type of LTP in our laboratory, we investigated the role of NCAM in synaptic plasticity of immature neurons in the dentate gyrus. Since we showed that *in vivo* situation LTP is impaired at the PP-DG synapse, the finding that LTP elicited in immature neurons is normal came as a surprise. However, although it is well accepted that immature neurons highly express PSA-NCAM, up to now, there was no direct evidence in support of idea that NCAM is important for the synaptic plasticity of these neurons. Thus, our data are not in conflict with other facts. Furthermore, newborn DGCs exhibit distinct electrophysiological properties compared with their mature neighbours in the adult brain, such as enhanced excitability and lower threshold for LTP induction (Schmidt-Hieber et al, 2004). Thus, it is easier to induce LTP on immature neurons than on mature ones and a deficit of NCAM may be compensated by other molecules. For example, the T-type Ca^{2+} channels have been shown to be more active in the immature granule cells and if a deficit in NCAM may lead to a deficit in NMDA receptor-mediated synaptic plasticity, these channels may compensate for it. Finally, it is noteworthy that in our *in vivo* paradigm it is impossible to distinguish between LTP in mature and immature neurons, since the levels of the observed potentiation are a sum of the changes induced in two neuronal populations. Thus, if the stimulation protocol used for induction of LTP would predominantly elicit synaptic changes in mature wild-type neurons but fail to induce LTP in mature NCAM -/- cells, we would see a strong difference between genotypes, as found in our *in vivo* experiments.

The only abnormality that we detected in the NCAM -/- mice using the protocol for induction of LTP in immature neurons was the impaired PTP. Since it is believed that PTP is mainly presynaptic phenomenon (Volianskis and Jensen, 2003), this impairment could be due to abnormal function of the adult entorhinal neurons that send their axons to the dentate gyrus (i.e. presynaptic components) rather than due to abnormalities in postsynaptic immature neurons. If this is indeed the case, remains to be investigated.

VIII.1.7. Normal *in vitro* LTP at MPP-DG synapses in the presence of a $GABA_A$ receptor antagonist

The normal LTP exhibited by immature neurons in the dentate gyrus motivated us to further check how NCAM deficiency affects synaptic plasticity in the presence of $GABA_A$ antagonists (the conventional protocol for LTP recording in vitro). Since LTP generated by immature neurons can be evoked just by stimulation of the medial perforant path, we recorded synaptic plasticity in disinhibited slices from the same pathway (MPP-DG). Also, in these experiments, we found that a deficiency in NCAM did not affect LTP, as reflected by normal levels of potentiation recorded in the NCAM -/- mice, supporting the view that NCAM necessary not for all forms of plasticity. The stimulation protocol used in the *in vitro* experiments in disinhibted slices (short trains of high-frequency stimulation) is rather mild compared with the normal high-frequency stimulation or even with the theta-burst stimulation. In our *in vivo* recordings, the differences between the NCAM +/+ and NCAM -/- became obvious after the second burst (see figure 18D), suggesting thus that a longer stimulation might be a precondition to reveal a differences between genotypes. Other interpretations are also possible. We can not ruled out that slices have an overall loss of connectivity and an altered milieu compared to *in vivo* preparations, both of these factors may influence synaptic plasticity and cell excitability. Particularly, the observed difference in findings in *in vitro* and *in vivo* preparations might be due to a deficiency in input from some afferents in the hippocampal slices, which provide neuromodulatory regulation of the dentate granule cell excitability under *in vivo* conditions.

PROJECT 2. The roles of tenascin-C and tenascin-R in synaptic plasticity

VIII.2.1. Normal basal synaptic transmission, short- and long-term plasticity in the dentate gyrus of tenascin-C deficient mice

Previous studies performed in rats showed that kainate injections (Nakic et al., 1996) and electrical stimulation at the perforant path-dentate gyrus synapse (Nakic et al., 1998) results in upregulation of TN-C expression both at the mRNA and protein level. Furthermore, the fact that injection of TN-C fragments (fibronectin type III domains 6–8 but not domains 3–5) into the CA1 region of acute hippocampal slices from wild-type mice reduced LTP argue in favor of a direct involvement of TN-C in synaptic plasticity in CA1 in vitro (Strekalova et al., 2002). Here, we attempted to study if TN-C plays a role in synaptic plasticity at the PP-DG synapse *in vivo*.

Our present experiments revealed that, basal synaptic transmission and both short- and long-term plasticity at this hippocampal subfield are normal in the TN-C deficient mice, indicating that presynaptic calcium release, firing of the neurons and the flow of information from the entorhinal cortex to the dentate gyrus is normal in the absence of the TN-C molecule. These data nicely correlate with morphological findings, which show that the dentate gyrus of TN-C deficient mice appears indistinguishable when compared with the wild-type control (Evers et al., 2002). Furthermore, our present *in vivo* data are in agreement with the results of previous in vitro recordings in the dentate gyrus, which failed to reveal any difference between TN-C genotypes (Evers et al., 2002). Since i) different protocols were used for LTP induction in vitro (SHFS) and *in vivo* (TBS) and ii) dentate gyrus LTP in vitro has been studied in the presence of a $GABA_A$ receptor antagonist, whereas present recordings were done without disinhibition of the dentate gyrus, the combined results indicate that synaptic plasticity in the dentate gyrus of TN-C -/- mice is normal irrespectively of the induction protocol used or the state of GABAegric inhibition.

To summarize the previous and the present studies, we can say that the abnormalities observed at the Schaffer collaterals-CA1 synapse (Evers et al., 2002) appear to be region-specific, since both in vitro LTP at the mossy fibers synapse and *in vivo* LTP at the perforant path-dentate gyrus synapse (present study) are normal in these mutants (Evers et al., 2002).

VIII.2.2. Normal synaptic transmission and paired-pulse modulation but abnormal facilitation in tenascin-R deficient mice

In order to investigate the role of TN-R molecule in short-term plasticity, two different protocols were applied to study paired-pulse facilitation of fEPSPs and paired-pulse modulation of population spikes.

As shown also for the other extracellular matrix molecule investigated, TN-R deficiency does not result in abnormal population spike inhibition/facilitation, suggesting thus the feedback GABAergic inhibition (activated by a single stimulus) in the dentate gyrus is normal in these mutants despite the fact that the number of the parvalbumin-positive inhibitory interneurons is increased in the dentate gyrus (Dr. Elena Shivukhina, unpublished data). One possible explanation for this finding could be that inhibitory activity elicited by the supramaximal stimulation is sufficient to almost fully suppress the second population spike at the short tested intervals. Thus, it may be difficult to reveal the effect of stronger inhibition in TN-R -/- mice, since inhibition is strong and close to saturation even in wild-types. However, since LTP was impaired in TN-R -/- mice in a $GABA_A$ receptor-dependent manner (see below), the inhibitory input to granule cells recruited during submaximal theta-burst stimulation is expected to be increased. A better understanding of this phenomenon would be possible by performing in vitro whole cell recordings of inhibitory activity using the same kind of paradigm as we used for our *in vivo* study.

In contrast to the above mentioned protocol, paired-pulse facilitation of fEPSPs was reduced at 25 and 100 ms interval, although the levels of facilitation were normal at 10 and 50 ms inter-pulse interval. As paired-pulse facilitation is generally considered to be an indicator of presynaptic release probability, it is plausible to argue that TN-R deficiency affects presynaptic processes. The deficit in paired-pulse facilitation in the dentate gyrus appears to be hippocampal subregion specific, since it is normal in the CA1 region of TN-R -/- mice (Bukalo et al., 2001; Saghatelyan et al., 2001). How TN-R may affect presynaptic properties of synapses formed by entorhinal cortex axons on dentate gyrus granule cells remains to be investigated, since more advanced analyses could not be performed using *in vivo* recordings.

Although the basal synaptic transmission has been shown to be elevated in CA1, presumably due to reduction in perisomatic inhibition of pyramidal cells (Saghatelyan et

al., 2001; Bukalo et al., 2007), in the dentate gyrus this parameter was normal, pointing thus to another region-specific difference.

VIII.2.3. Impaired LTP at perforant path-dentate gyrus synapse in tenascin-R deficient mice

This study provides the first evidence that an extracellular matrix molecule is essential for synaptic plasticity in the dentate gyrus *in vivo*. Recordings in anaesthetized TN-R -/- mice showed a deficit in LTP of the fEPSP and a tendency of the population spike to be higher in the mutant animals from the first minutes of recording but this difference became significant only at the end of three hours of recording. One possible reason for this slow increase of population spike amplitudes may be a time-dependent suppression of various neuronal projections to and from the hippocampus by urethane anesthesia (Gilbert and Mack, 1999). The opposite might also be true, that with time, the levels of anesthesia were not so deep anymore and neurons become more excitable. However, this effect was seen only in knockout mice and not in wild-types and the tendency was observed from the first minutes after the application of the TBS protocol. Thus, it is plausible to assume that this increase in the granule cells discharging might reflect changes in the excitability induced by the tetanization protocol.
After discovering that LTP of fEPSP is impaired in TN-R -/- mice, we wanted to further investigate what could be the reasons for this impairment and further proceeded with a pharmacological approach.

VIII.2.4. Muscimol impairs LTP in wild-type mice

Previous experiments performed in the CA1 region of hippocampus showed that the HNK-1 carbohydrate carried by TN-R is involved in regulation of perisomatic, but not dendritic inhibition of pyramidal cells via reduction of evoked GABA release (Saghatelyan et al., 2000). Reduction in perisomatic inhibition in TN-R -/- mice coincided with an impairment of the synaptic plasticity and increased basal excitatory synaptic transmission (Bukalo et al., 2001; Saghatelyan et al., 2001). In addition, ultrastructural analysis of inhibitory perisomatic terminals on pyramidal cells in the CA1 regions in TN-R -/- mice revealed a reduction in the density of perisomatic synapses and abnormalities in their architecture, including a reduction in the size of active zones and distribution of synaptic vesicles (Nikonenko et al., 2003). A recent

study from our laboratory showed that the impaired LTP in the CA1 region of TN-R deficient mice could be restored by both *in vivo* and *in vitro* pharmacological treatments with muscimol (Bukalo et al, 2007). Since it has been previously shown that a concentration of 2.5 μM inhibits LTP in this area, it is important to mention that the concentration of muscimol used in the study of Bukalo and colleagues is less then twice smaller (1 mg/kg of body weight in for *in vivo* injections or 1μM for in vitro experiments) and did not affect LTP recorded in wild-type mice.

Application of muscimol is known to increase $GABA_A$ receptor-mediated currents and reduce spiking and excitatory postsynaptic potentials (Rovira et al., 1984; Steele and Mauk, 1999; Yoshioka and Sakurai, 1995). Also, it has been recently shown that local microinjection of muscimol into thalamus decreased the frequency of the slow oscillations (Doi et al., 2007).

Initially we thought that this mechanism could be the reason of impaired LTP in the dentate gyrus of TN-R -/- mice. Before testing this idea in mutant animals, we started by performing the experiments just in wild-type mice to see if muscimol injection has any effect on the recorded electrophysiological parameters. Intraperitoneal injection of muscimol or NaCl 24 hours before the electrophysiological recording did not affect the basal synaptic transmission or short-term plasticity at the perforant path-dentate gyrus synapse. However, enhancing the inhibition resulted in a significantly decreased level of potentiation of fEPSPs in wild-type mice, without affecting the potentiation of population spikes. These data suggest that a transient increase in GABAergic inhibition by muscimol has more long-lasting effects on synaptic plasticity then on the cell excitability. Also these results argued that it would be very unlikely to enhance LTP in TN-R -/- mice by muscimol.

VIII.2.5. Bicuculline rescues LTP in the dentate gyrus of TN-R deficient mice

In parallel with our electrophysiological recordings, morphological investigations were conducted in our laboratory. Unpublished observations of Dr. Elena Shivukhina showed that, in contrast to CA1 region, increased linear densities of GABAergic perisomatic synapses are seen in the dentate gyrus of TN-R -/- mice. Mostly this phenomenon is due to highly elevated number of GABAergic interneurons, whereas the average number of terminals formed by a single PV^+ interneuron on granule cell somata in the dentate gyrus is reduced by more than 20% in TN-R -/- versus TN-R +/+ mice. In this context,

one would expect that the impairment observed in our initial experiments is not due to a reduction in the inhibition levels, as seen in the CA1 region, but rather is due to an increase in the inhibition.

A combination of electrophysiological and pharmacological analyses *in vivo* in mice is rather rare due to a number of technical difficulties. One of the few studies in the literature addressing this issue *in vivo* involved the Thy-1 deficient mouse, which exhibits impaired dentate gyrus LTP in both anaesthetized and freely moving mice (Nosten- Bertrand et al., 1996; Errington et al., 1997). As Thy-1 deficient mice show an increase in inhibitory postsynaptic currents in the dentate gyrus (Hollrigel et al., 1998) and because levels of LTP are normalized in mutants towards wild-type levels in vitro and in anaesthetized mice *in vivo* by a $GABA_A$ receptor antagonist (Nosten-Bertrand et al., 1996; Errington et al., 1997), it is conceivable that abnormally high GABAergic activity in the dentate gyrus causes the impairment of LTP in Thy-1 deficient mice. Up to present there are no available data regarding GABAergic transmission in the dentate gyrus of TN-R deficient mice, but in the light of the recent morphological observations from our laboratory, we also performed the same kind of experiment as was done by Nosten- Bertrand and colleagues. Thus, local infusion of $GABA_A$ receptor antagonist bicuculline restored LTP in TN-R -/- mice to normal levels, indicating that enhanced GABAergic transmission causes the abnormalities in LTP in TN-R -/- mice. This is in line with the finding of increased numbers of paravalbumin-expressing interneurons and densities of perisomatic GABAergic synapses in the dentate gyrus of TN-R -/- mice. Bicuculline also reduced and equalized potentiation of population spike amplitudes in TN-R -/- and TN-R +/+ mice. These data are consistent with the hypothesis that enhanced excitability in TN-R -/- in the absence of bicuculline is due to higher levels of theta-burst induced disinhibition, reflecting higher levels of baseline GABAergic inhibition. Thus, different mechanisms appear to underlie impairment of NMDA receptor-dependent LTP in the CA1 region and the dentate gyrus in TN-R-/- mice. While diminished inhibition in CA1 appears to result in a homeostatic/metaplastic modulation of LTP (Bukalo et al., 2007), LTP in the dentate gyrus is reduced due to elevated inhibition.

Another interesting similarity between Thy-1 and TN-R knockout mice is that both of them have normal spatial learning and memory (Montag-Sallaz and Montag, 2003; Nosten-Bertrand et al., 1996) despite the fact that LTP in the dentate gyrus is impaired. In the case of TN-R -/- mice, the synaptic plasticity impairment observed in

the dentate gyrus is believed to be a physiological substrate of the improved re-learning observed in these mice (Dr. Fabio Morellini, unpublished observations). Taken together, these observations suggest that LTP at the entorhinal cortex-dentate gyrus synapse may not be necessary for spatial learning but is required for re-learning.

VIII.2.6. Normal LTP at the CA3-CA3 synapse in tenascin-R deficient mice

In order to extend analysis of synaptic transmission and plasticity in the hippocampus of the TN-R deficient mice, we further investigated synaptic plasticity at the associational/commissural synapse in the CA3 region. Induction of LTP in this area revealed no difference between the genotypes, suggesting that plasticity is not affected at this particular synapse. Furthermore, the basal synaptic transmission and the levels of facilitation at different time intervals are also normal in TN-R -/- animals. Investigations of the same parameters at the mossy fibers-CA3 synapses show again lack of function of TN-R in the CA3 region, with synaptic transmission and LTP being normal (Dr. Olena Bukalo, unpublished observations). The present data are also supported by the morphological observations. Analysis of synaptic coverage of paravalbumin-expressing interneurons on CA3 principal cells show no difference between the TN-R +/+ and TN-R -/- mice (Dr. Elena Shivukhina, unpublished data).

From past and present experiments performed in our laboratory, we can conclude that induction of LTP shows remarkable subregion specific features, being normal in the CA3 subfield (both at the mossy fibers and at the A/C synapses), but abnormal in CA1 (Saghatelyan et al., 2001) and in the dentate gyrus of TN-R -/- mice (present study).

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X. ACKNOWLEGMENTS

This study has been performed in the Institute for Biosynthesis of Neuronal Structures of the Center for Molecular Neurobiology (ZMNH) at the University of Hamburg. For this, I would first like to thank Prof. Melitta Schachner who offered me all the necessary conditions and facilities for performing this study. I am extremely grateful to Dr. Alexander Dityatev for the helpful discussions, constant support and guidance through the first chapter of the scientific journey.

I would also like to thank to Torsten and Fritz for always having a solution for my technical problems, Dr. Oleg Senkov for helping me in the initial phase of the project, former and present members of the lab for making every working day more colorful. I am thankful to all members of the 'Synaptic plasticity' club and to the lately developed *lunch group*. My greatest thanks to the members of the former Wednesday evening Stammtisch, Julia and Eka ☺ .

…….I am thankful to all the people, who in a way or another were involved in the correction of this thesis: Apu, Iurie, Rod and especially Martin.

…….Although hundreds of kilometers away, I did not forget about the people who flavored my life with their existence, the ones who truly stood by me over the years: Razvan and Cosmina. I am of course deeply grateful to my aunt, my family and especially my beloved brother, Leo for keeping my eyes and my mind open.

Last, but not least, I would like to express my deepest gratitude to the person who over the last years supported me in all possible ways, Domnul Radu Popescu.

XI. LIST of PUBLICATIONS

The present dissertation is mainly based on the following articles and abstracts.

Articles

Stoenica L., Senkov O., Gerardy-Schahn R., Weinhold B., Schachner M. and Dityatev A. (2006) In vivo synaptic plasticity in the dentate gyrus of mice deficient in the neural cell adhesion molecule NCAM or its polysialic acid. Eur. J. Neurosci. 23, 2255–2264.

Gurevicius K., Kuang F., Stoenica L., Irintchev A., Gureviciene I., Iivonen H., Dityatev A., Schachner Melitta, Tanila H. Specific hypoplasia of hippocampal CA1 subregion in mice deficient in the extracellular matrix glycoprotein tenascin-C (submitted).

Sivukhina E., Stoenica L., Oulianova E., Bukalo O., Morellini F, Dityatev A., Irintchev A. and Schachner M. Hippocampal hyperplasia and area-specific aberrations of inhibitory inputs to principal neurons in tenascin-R deficient mice: potential substrates of altered synaptic plasticity and behavior (submitted).

Abstracts

Stoenica L., Schachner M., Dityatev A. In vivo synaptic plasticity in the dentate gyrus of anesthetized mice deficient in the extracellular matrix glycoproteins tenascin-C or tenascin-R. FENS Abstr., vol. 3, A049.19, 2006

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Bestätigung der Korrektheit der Englischen Sprache in der Dissertation von Luminata Stoenica mit dem Thema "Synaptic plasticity in mice (Mus musculus L., 1758) deficient in cell adhesion or extracellular molecules: *in vivo* and *in vitro* electrophysiological analysis".

Hiermit bestätige ich, Martin Hammond, die Korrektheit der Englischen Sprache in Wort und Schrift.

Martin Hammond, B.Sc. (Hons), M.Sc.