Functional characterization of neuron-NG2 cell synaptic transmission

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“What is 'real'? How do you define 'real'? If you're talking about what you can feel, what you can smell, what you can taste and see, then 'real' is simply electrical signals interpreted by your brain.”

Morpheus in “The Matrix” (1999)

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List of abbreviations

-/-	homozygous NG2 knockout
+/-	heterozygous NG2 knockout
4-AP	4-aminopyridine
ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AN2	NG2 homologue in mice
as	antisense
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BDNF	brain-derived neurotrophic factor
bp	base pairs
CA	Cornu Ammonis
CCD	charge-coupled device
CGP	(2S)-3-[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxy-propyl](phenylmethyl)phosphinic acid
CKAMP44	cysteine-knot AMPA receptor modulating protein 44
$[Cl^-]_i$	intracellular Cl^- concentration
C_m	membrane capacitance
CNIH	cornichon related protein
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CSPG	chondroitin sulfate proteoglycan
DEPC	diethyl pyrocarbonate
DIC	differential interference contrast
DMCM	methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
DZ	diazepam
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
ePSC	evoked postsynaptic current
EYFP	enhanced yellow fluorescent protein
Fig.	figure
GABA	γ-aminobutyric acid
$GABA_A$ receptor	ionotropic GABA receptor

$GABA_B$ receptor	metabotropic GABA receptor
GAG	glycosaminoglycan
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GluA	AMPA receptor subunit
GluR	glutamate receptor
GluT	glutamate transporter
GRIP	glutamate receptor-interacting protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hGFAP	human glial fibrillary acidic protein
I	current
KCC2	K^+-Cl^- cotransporter 2
kDa	kilo Dalton
K_{ir}	inwardly-rectifying K^+
LNS	laminin G/neurexin/sex hormone binding globulin
LOR	loreclezole
LTP	long-term potentiation
mGAT	mouse GABA transporter
mPSC	miniature postsynaptic current
mRNA	messenger ribonucleic acid
n	number of samples
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NCX	Na^+/Ca^{2+} exchanger
NG2	nerve/glia antigen 2, a chondroitin sulfate proteoglycan
NKCC1	Na^+-K^+-Cl^- cotransporter 1
NLG	neuroligin
NMDA	N-methyl-D-aspartic acid
NRX	neurexin
OPC	oligodendrocyte precursor cell
p	postnatal day
PBT	pentobarbital
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGF-AA	ligand of the PDGFα receptor
PDGFαR	platelet-derived growth factor α receptor
PDZ	postsynaptic density 95/discs large/zonula-occludens-1
PPR	paired-pulse ratio
PSC	postsynaptic current
Q	charge
R_m	membrane resistance

RNA	ribonucleic acid
R_s	series resistance
RT	reverse transcription
SD	standard deviation
se	sense
Tab.	table
TARP	transmembrane AMPA receptor regulatory protein
TTX	tetrodotoxin
U	enzyme units
V	voltage
V_{hold}	holding potential
V_{rest}	resting potential
wt	wild type
ZPD	zolpidem

1 Introduction

Our understanding of the role of glial cells in the central nervous system (CNS) has drastically changed in the past decades. The term glia was introduced in 1856 by the famous neuropathologist Rudolf Virchow. Glia is the Greek expression for 'glue' which illustrates the initial function assigned to these cells: structural and trophic support of neurons. As a consequence, research largely concentrated on the role and function of neurons which facilitate the fast signal processing in the brain. Nowadays we know that glial cells are not merely passive elements supporting neurons. Instead, they actively participate in signal processing and higher brain functions (Perea and Araque, 2010).

The present study focusses on NG2 cells which are today considered as an own class of glial cells that display some remarkable properties distinct from other cell types (Bergles et al., 2010). The following introduction will give an overview on the current scientific knowledge relevant for the present study.

1.1 The hippocampus

The hippocampus is located in the medial temporal lobe of the cerebrum and belongs to the limbic system. Especially in rodents, it occupies a substantial portion of the forebrain (Fig. 1.1A) (Amaral D.G. and Witter M.P., 1995). The hippocampus is made up of three main structures: the dentate gyrus, the Ammon's horn (Cornu Ammonis, CA) and the subiculum. In the dentate gyrus, three different layers are distinguished. The stratum granulare contains densely packed granule cells that project their dendrites in the above lying stratum moleculare and their axons via the hilus which comprises a polymorphic layer to the Ammon's horn. The Ammon's horn is subdivided into the three regions CA1, CA2 and CA3, each of which consists of the four layers stratum oriens, stratum pyramidale, stratum radiatum and stratum lacunosum moleculare (Fig. 1.1B). The principle cells of the CA regions are the excitatory pyramidal neurons whose cell bodies lie densely packed in the stratum pyramidale. The other strata mainly contain inhibitory interneurons that participate in the local signal processing and different types of glial cells (Amaral D.G. and Witter M.P., 1995).

The main communication partner of the hippocampus is the entorhinal cortex. Neurons from layer II of the entorhinal cortex send their projections via the perforant path to granule cells of the dentate gyrus. The granule cells convey the information via the mossy fiber tract to the pyramidal cells of the CA3 region. From here axons project through the Schaffer collaterals to the dendrites of CA1 pyramidal cells that themselves send the information via the subiculum back to deeper layers of the entorhinal cortex before they finally reach the neocortex (Fig. 1.1C) (Deng et al., 2010).

Therefore, information flow in the hippocampus principally follows a unidirectional excitatory trisynaptic loop. However, certain regions also receive direct inputs from the entorhinal cortex. The CA3 region is targeted by some axons of the perforant path without any processing by the dentate gyrus. Similarly, the CA1 region receives direct inputs from layer III neurons of the entorhinal cortex via the temporoammonic pathway (Fig. 1.1C). In addition, each subregion of the hippocampus contains intrinsic circuits of inhibitory and excitatory cells that process the information before forwarding them (Amaral D.G. and Witter M.P., 1995; Deng et al., 2010).

Together with the entorhinal cortex, the hippocampus has an essential role in transferring information from the short-term memory to the long-term memory. This was impressively proven by a human patient that was subjected to a bilateral medial temporal lobe resection which resulted in an anterograde amnesia. This patient known as H.M. was unable to form any new declarative memory. However, his memory of events that happened in the past was almost unaffected (Scoville and Milner, 1957). In this context, Bliss and Lomo (1973) discovered that certain activity-patterns in the hippocampus may increase the synaptic transmission. This phenomenon, known as long-term potentiation (LTP) is today regarded as the cellular counterpart of learning and memory. Besides this crucial function in memory consolidation, the hippocampus was also shown to be involved in spatial navigation. In mice, special place cells were described that increase their activity when the animal resides in a certain location (O'Keefe and Dostrovsky, 1971).

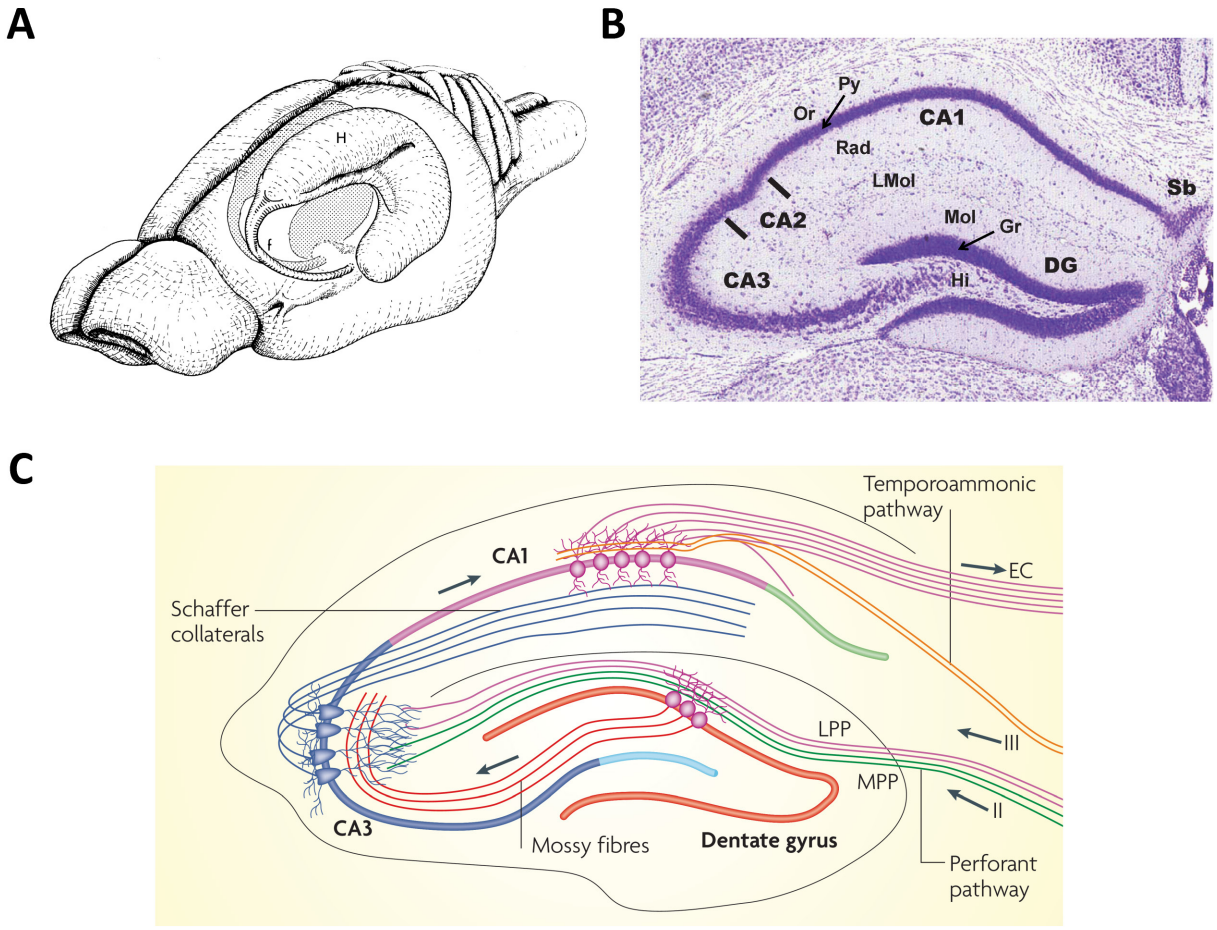

Fig. 1.1 Anatomy and synaptic connections of the rodent hippocampus. (A) Drawing of a rat brain illustrating the anatomical location of the hippocampus (H) (Amaral D.G. and Witter M.P., 1995). **(B)** Nissle-staining of a mouse brain section labeling cell nuclei in blue. The dentate gyrus (DG) is composed of the three layers stratum moleculare (Mol), stratum granulosum (Gr) and hilus (Hi). The Ammon's horn (Cornu Ammonis, CA) is divided into CA1, CA2 and CA3 region each composed of the four layers stratum oriens (Or), stratum pyramidale (Py), stratum radiatum (Rad) and stratum lacunosum moleculare (LMol). The subiculum (Sb) lies between the CA regions and the entorhinal cortex (not shown) (modified from Paxinos and Franklin, 2001). **(C)** Schematic of the synaptic circuitry in the hippocampus. Signals from the entorhinal cortex (EC) enter the hippocampus via the lateral and medial perforant path (LPP and MPP, respectively) projecting to the dentate gyrus. Information is then relayed via the mossy fibers to the CA3 region which sends axon fibers (Schaffer collaterals) to the CA1 area. The processed information finally leaves the hippocampus via back-projections to the EC. Besides this trisynaptic circuit, CA3 also receives direct information from the EC via the LPP and MPP and the CA1 receives direct information via the temporoammonic pathway (Deng et al., 2010).

Because of its fundamental role in learning and memory as well as its highly organized structure and clear synaptic connections, the hippocampus is commonly used to study synaptic transmission (Malenka and Nicoll, 1999).

1.2 The barrel cortex

In 1970, Woolsey and Van der Loos discovered that the whiskers of rodents are represented by well-defined barrel-like structures in layer IV of the somatosensory cortex. Intriguingly, the pattern of barrels almost exactly replicates the arrangement of whiskers on the contralateral face of the animal. Each barrel corresponds to one particular whisker and receives and processes tactile information only from this whisker (Fig. 1.2A, B). This highly defined relationship of a sensory input and a specific representation in the brain makes the barrel cortex an ideal model system to study development and plasticity in the brain in response to sensory experience (Petersen, 2007; Fox, 2008).

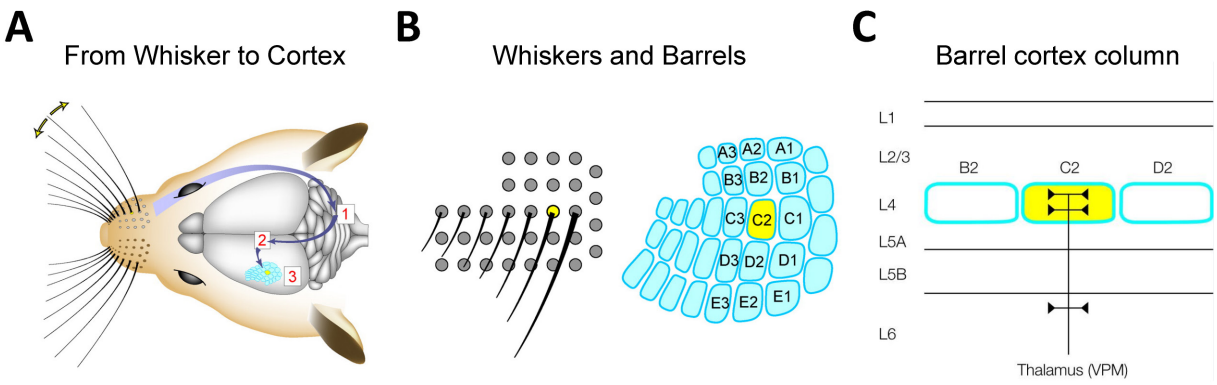

Fig. 1.2 Synaptic pathways in the rodent barrel cortex. (A) Scheme of the trisynaptic excitatory pathway from whiskers to barrel cortex. Sensory neurons detect mechanical stimulation and send the information to the brain stem (1). Neurons of the brain stem project to the thalamus (2) from where the signals are delivered to the corresponding barrel in the somatosensory cortex (3). **(B)** Representation of the whisker field in the barrel cortex (top view). (A) and (B) from Petersen (2007). **(C)** The barrel cortex consists of six layers (lateral view). Thalamic afferents mainly terminate in the barrels located in layer IV. Modified from Aronoff and Petersen (2008).

The cortex is a six-layered structure with the barrels located in layer IV (Fig. 1.2C). An excitatory trisynaptic pathway connects the whiskers with the barrels. Deflection of a specific whisker opens mechano-gated ion channels in nerve endings of sensory neurons. These neurons send the information to the trigeminal nuclei of the brain stem. From there the signals are conveyed to the ventral posterior medial nucleus of the thalamus. Thalamic neurons project directly to the corresponding barrel in layer IV of the somatosensory barrel cortex (Fig. 1.2A, C) (Petersen, 2007).

The barrels can easily be visualized even in unstained living brain slices owing to their specific structure. In general, thalamic afferents innervate layer IV of the primary somatosensory cortex with a relatively homogeneous distribution. In the barrel cortex, however, these afferents display a much more defined localization. Axons representing a specific barrel form discrete batches that are clearly separated on all sides by so called septa. These septa constitute cell-rich borders between the barrels that surround the cell-sparse core filled with thalamic afferents. Neurons located in the septa send their dendrites into the core of the barrel where they receive the information conveyed by the thalamic afferents. A certain neuron always belongs to a certain barrel, therefore receiving information from one whisker only (Petersen, 2007; Fox, 2008).

1.3 Glial cells in the CNS

Glial cells constitute the majority of cells in the CNS. The seminal work of Ramón y Cajal and Pío del Río-Hortega introduced the classical view, that glial cells are subdivided into astrocytes, oligodendrocytes and microglia with the latter being the only group derived from the mesoderm while all others being of ectodermal origin (Somjen, 1988). However, in the past decades another cell type emerged as an own class of glial cells, namely NG2 cells, which are in focus of the present study (Bergles et al., 2010; Trotter et al., 2010).
This section will give an overview of the properties and functions of glial cells in the CNS with main emphasis on NG2 cells.

1.3.1 Astrocytes

Astrocytes are morphological and functionally divers depending on the demands of their local environment. Based on their morphology, protoplasmic astrocytes of gray matter regions that extent radial processes are differentiated from fibrous astrocytes in white matter regions that display rather longitudinal orientated processes (Reichenbach and Wolburg, 2013). Apart from these regional differences, virtually all astrocytes exhibit highly ramified processes that make contact with blood vessels via specialized protrusions called endfeet. These endfeet contain a high density of glucose transporters, water-selective aquaporin channels and inwardly-rectifying K^+ (K_{ir}) channels. Astrocytes thereby take up

glucose from the blood and provide it to neurons. Furthermore, they contribute to ion homeostasis and osmoregulation of the brain (Abbott et al., 2006).

Another common feature of astrocytes is the presence of gap junction channels. These are specialized intercellular connections that enable the exchange of ions and molecules < 1 kDa. In this way, astrocytes form extensive networks via which they distribute and share the nutrients taken up from the blood (Ransom and Giaume, 2013). Moreover, gap junctions are important for the spatial buffering of extracellular K^+ released during neuronal activity (Wallraff et al., 2006).

The fine processes of astrocytes were also shown to ensheath the majority of synapses in the brain. These processes contain a high density of neurotransmitter transporters, which facilitate the fast removal of neurotransmitters from the synaptic cleft during neuronal activity. Especially the glutamate uptake was extensively studied. It was shown that astrocytes convert glutamate to glutamine and release it. The glutamine is taken up by nearby neurons and converted back to glutamate to refill the vesicles (Schousboe et al., 2013). Moreover, by taking up the neurotransmitters from the synaptic cleft, astrocytes control extracellular levels of neurotransmitters and are able to shape the postsynaptic receptor currents (section 1.4.2) (Jonas, 2000). Thereby astrocytes actively modulate synaptic communication, a view that was further supported by studies demonstrating that they are even able to release so called “gliotransmitters” like glutamate, adenosine triphosphate (ATP) and D-serine (Volterra, 2013). Intriguingly, the astrocytic release of D-serine was recently shown to be essential for hippocampal LTP which is considered to be the cellular correlate of learning and memory (Henneberger et al., 2010). The tight connection of astrocytic processes with synapses and the fact that astrocytes directly modify synaptic transmission led to the concept of the tripartite synapse, considering astrocytes as elementary synaptic elements (Perea et al., 2009).

Despite these interactions, astrocytes are considered to be electrically non-excitable. The electrophysiological current pattern in response to voltage steps is referred to as “passive” as it is dominated by time- and voltage-independent K^+ conductances (Steinhäuser et al., 2013). However, astrocytes display extensive Ca^{2+} signaling via which they mediate several of their remarkable properties (Volterra, 2013).

1.3.2 Oligodendrocytes

Oligodendrocytes form myelin sheaths around axons of the CNS which increase the propagation speed of action potentials. Myelin sheaths are multilayered and compact cell protrusions that are mainly composed of lipids. These sheaths are interrupted by the nodes of Ranvier which are small unmyelinated regions with a high density of voltage-gated Na^+ channels (Butt, 2013). Due to the strong electrical insulation by the myelin sheaths, action potentials are propagated from node to node, a process known as saltatory conduction. This does not only lead to a drastic increase in conduction velocity but also saves energy since the area of depolarization is confined to the nodes of Ranvier and energy consuming restoration of the resting potential is therefore locally restricted (Nave, 2010). Besides acting as an insulator, myelin sheaths also have neuroprotective and neurotrophic functions, as it was shown that oligodendrocytes supply axons directly with energy, proteins and RNA (White and Krämer-Albers, 2014).

Based on the axon diameter and the number of axons they myelinate, two groups of oligodendrocytes are distinguished. These are named type I/II and type III/IV referring to the original classification by Pío del Río-Hortega. Type I/II oligodendrocytes comprise the larger group frequently present in gray and white matter. They exhibit small somata and myelinate 10-30 small axons with a diameter of less than 2 µm. Type III/IV oligodendrocytes have larger cell bodies and myelinate only 1-5 axons with large diameters (Butt, 2013).

1.3.3 Microglia

Microglial cells are abundantly distributed in the entire brain and constitute the immune system of the CNS. Under physiological conditions they exhibit a small cell soma with highly ramified processes (Kirchhoff, 2013). These processes are very motile and equipped with a variety of neurotransmitter and cytokine receptors enabling microglia to survey their local environment (Noda and Verkhratsky, 2013). Whenever a pathogenic condition is detected, microglia get activated. In this state, their morphology gets hypertrophic with thicker processes. Furthermore, they proliferate to increase their number, migrate to the origin of interference, produce and release cytokines and phagocytize foreign bodies to act as antigen-presenting cells for invading T-cells (Miller et al., 2013; Owens, 2013). However,

microglial cells also serve important functions in the healthy brain where they remove cellular debris and regulate synapse formation and synaptic efficacy (Kirchhoff, 2013).

1.3.4 NG2 cells

In the past decades, NG2 cells emerged as a fourth type of glial cells. Initially, NG2 cells were discovered in cell culture experiments as a group of cells that displayed neuronal (N) and glial (G) properties as they exhibited voltage-gated ion channels but were unable to fire action potentials. As these NG cells were specifically detected by the NG-antiserum number 2, they were referred to as NG2 cells (Wilson et al., 1981; Stallcup, 1981). The target of this antiserum was later found to be the NG2 protein, a cell-surface proteoglycan (detailed information about the NG2 protein in section 1.7) (Nishiyama et al., 1991). Today it is known that the NG2 protein is also expressed by vascular pericytes in the CNS and by different immature mesenchymal stem cells of other organs (Nishiyama et al., 2009). However, in the brain NG2 cells may easily be distinguished from pericytes by their location close to blood vessels and their different morphology.

NG2 cells are abundantly distributed in white and gray matter areas of the developing and adult CNS. They constitute 8-9% of cells in white matter and 2-3% in gray matter regions (Dawson et al., 2003). Their morphology resembles in many ways that of microglia. In gray matter, they typically display a small and round soma with a diameter of about 10 µm with several radial processes that were shown to be highly dynamic (Haberlandt et al., 2011; Hughes et al., 2013). In white matter areas, NG2 cell somata are more elongated and their processes orientate along axonal projections. The morphology of a gray matter NG2 cell as compared to an astrocyte is depicted in Fig. 3.1 (section 3.1.1).

During development, NG2 cells populate the entire forebrain in three waves where they migrate over considerable distances from different growing zones. Intriguingly, if one of these populations is ablated, the remaining cells increase proliferation and finally reach a density comparable to controls (Kessaris et al., 2006). This illustrates one of the remarkable features of NG2 cells. Throughout life, they keep the ability to proliferate and differentiate. As a consequence, NG2 cells are frequently referred to as oligodendrocyte precursor cells (OPCs), since numerous studies demonstrated their potential to generate oligodendrocytes during postnatal myelination and in response to demyelinating diseases (Kang et al., 2010;

Zhu et al., 2011). Other studies even showed that NG2 cells are able to generate astrocytes or neurons under certain circumstances (Zhu et al., 2008; Rivers et al., 2008; Dimou et al., 2008). Due to this fact, they were initially considered to be solely progenitor cells. However, the density of NG2 cells stays constant throughout life and they retain the ability to proliferate and differentiate even after myelination is complete. Thus, NG2 cells comprise by far the biggest population of proliferating cells outside the neurogenic niches in the adult CNS (Dawson et al., 2003). This supports the notion that NG2 cells display an own class of glial cells with a yet unknown function in the healthy adult brain.

The electrophysiological properties of NG2 cells were studied by several groups in detail. It was shown that NG2 cells express different types of voltage-gated K^+ and Na^+ channels albeit at lower densities compared to neurons (Kressin et al., 1995; De Biase et al., 2010; Maldonado et al., 2013). This set of ion channels accounts for the characteristic complex current pattern of NG2 cells (Steinhäuser et al., 1994a; Steinhäuser et al., 1994b). During development, an increase in the expression of K_{ir} channels was observed (Kressin et al., 1995; Bordey and Sontheimer, 1997; Maldonado et al., 2013). Due to this comparably high K^+ conductance, the resting potential of NG2 cell is hyperpolarized compared to neurons (around -80 mV). Although it was suggested that there is a class of spiking NG2 cells that are able to fire action potentials (Karadottir et al., 2008), this property was never observed by plenty of other studies and is therefore not recognized as a common feature of NG2 cells (De Biase et al., 2010). Beyond K^+ and Na^+ channels, NG2 cells also express voltage-gated Ca^{2+} channels, which constitute one of their various Ca^{2+} signaling pathways (Akopian et al., 1996; Haberlandt et al., 2011). Moreover, NG2 cells express ionotropic receptors for the major neurotransmitters of the CNS, glutamate and GABA (Bergles et al., 2010).

The most fascinating property of NG2 cells, however, is that they receive direct synaptic input from glutamatergic and GABAergic neurons (Bergles et al., 2000; Lin and Bergles, 2004; Jabs et al., 2005). Moreover, this finding is not limited to a subpopulation of NG2 cells or a specific brain region. Instead, it is a common feature of NG2 cells throughout the CNS (De Biase et al., 2010). Even in white matter regions that are mainly composed of axonal tracts and free of classical synapses, a new mode of vesicular release from unmyelinated axons onto NG2 cells was discovered (Ziskin et al., 2007; Kukley et al., 2007). These results challenged the long accepted concept of synaptic transmission being an exclusively neuronal

feature. However, the function of this synaptic input is largely unknown so far. What is known is that these synapses are transferred to daughter cells when NG2 cells proliferate (Kukley et al., 2008; Ge et al., 2009) and are disassembled as soon as the cells start to differentiate (Kukley et al., 2010; De Biase et al., 2010). Furthermore, glutamatergic as well as GABAergic innervation is excitatory in NG2 cells (Lin and Bergles, 2004; Tong et al., 2009). This is in contrast to neurons, where a switch from an excitatory to an inhibitory action of GABA was shown during development due to a change in the intracellular Cl^- concentration ($[Cl^-]_i$) (Cherubini et al., 1991). Generally, synaptic currents or potentials are small and occur at a low frequency compared to neurons (Bergles et al., 2010). A few studies tackled the question of the role of neurotransmitter receptors and synaptic innervation in NG2 cells. In earlier studies it was shown that glutamate increases migration speed but inhibits proliferation and differentiation of NG2 cells to oligodendrocytes (Gallo et al., 1996; Yuan et al., 1998; Gudz et al., 2006). A recent study uncovered that decreased glutamatergic synaptic innervation increased the proliferation of NG2 cells in the barrel cortex (Mangin et al., 2012). Although the final proof is still missing, these data strongly suggest that glutamatergic signaling influences NG2 cell proliferation and differentiation in an activity-dependent manner. Furthermore, GABA-induced chemotactic migration was observed in hippocampal NG2 cells (Tong et al., 2009), suggesting a role for $GABA_A$ receptor activation in migration of these cells.

Despite all these promising indications, the function of synaptic transmission onto NG2 cells is still enigmatic. Even the very existence of NG2 cells in the adult brain is obscure. A recent study showed *in vivo* that NG2 cells maintain unique territories by self-avoidance causing an even distribution throughout the brain. As soon as an NG2 cell is lost due to differentiation or ablation, it is immediately replaced by proliferation of a nearby cell (Hughes et al., 2013). There must be a good reason for such an effort to maintain a stable and uniform distribution of these cells throughout the CNS that we yet do not understand.

Due to the intermediate phenotype of NG2 cells with neuronal and glial properties, their differentiation potential and their electrophysiological properties, different names occurred for NG2 cells in the literature of the past decades such as complex cells, synantocytes, polydendrocytes or OPCs (Bergles et al., 2010). Most of the recent studies including the

present one consistently use the term NG2 cells as nowadays the NG2 cell population is more clearly defined.

1.4 Principles of synaptic transmission

Chemical synapses are functional connections between cells where an electrical signal is passed from a presynaptic to a postsynaptic cell by transient conversion to a chemical signal. The synapse is an asymmetric structure as the information is passed unidirectionally from the presynaptic to the postsynaptic side. The presynaptic terminal of an axon, also called bouton, contains a highly specialized release machinery for neurotransmitter-filled vesicles. The postsynaptic side of a dendrite, also called spine, is characterized by a postsynaptic density which contains besides neurotransmitter receptors several modulatory and scaffolding proteins important for the dense clustering and function of the receptors. Pre- and postsynaptic side are separated by a 20-40 nm wide gap, the synaptic cleft. In a simplistic view, synaptic transmission at a chemical synapse includes the following steps. An action potential depolarizes the presynaptic terminal inducing the opening of voltage-gated Ca^{2+} channels. Invading Ca^{2+} causes the release of neurotransmitter-filled vesicles into the synaptic cleft. Upon binding of the agonist, postsynaptic neurotransmitter receptors open thereby facilitating the influx of cations in the postsynaptic compartment. The hereby evoked depolarizations from many synapses are integrated at the soma where they potentially generate a new action potential if the threshold is reached (Kandel et al., 2013).

1.4.1 Properties of presynaptic vesicle release

The vesicles on the presynaptic side can be assigned to one of three vesicle pools, namely the readily releasable pool, the recycling pool and the reserve pool. Vesicles of the readily releasable pool are docked to the membrane at the active zone of the presynaptic terminal where the release takes place. They are primed for release waiting for an increase in Ca^{2+} concentration to induce vesicle fusion. The vesicles of the recycling pool are meant to refill the readily releasable pool and are therefore important to maintain neurotransmitter release in response to continuous activity. This pool is constantly recycling by delivering vesicles to the active zone while getting refilled by recycling vesicles. However, the majority

of vesicles are located in the reserve pool, a rather immobile fraction of vesicles bound to scaffolding proteins. Under experimental conditions these vesicles are only mobilized in response to enduring high frequency stimulation when the other two pools are completely depleted. The exact role and dynamics of the reserve pool under physiological conditions *in vivo* remain uncertain so far. However, recent studies suggest that a frequent exchange between the recycling and reserve pool exists (Rizzoli and Betz, 2005; Denker and Rizzoli, 2010).

The presynaptic site is the origin of a certain form of synaptic plasticity, namely short-term synaptic plasticity or paired-pulse plasticity. It describes the phenomenon that the amount of released transmitter may vary in response to two closely spaced stimuli. In this respect, the paired-pulse facilitation where more neurotransmitters are released on the second stimulus is distinguished from the paired-pulse depression showing reduced neuro-transmitter release on the second stimulus. Whether a synapse displays facilitation or depression is mainly determined by the release probability of the presynaptic bouton. This release probability is defined by the chance that a readily releasable vesicle will fuse upon an initial stimulus which is mainly determined by presynaptic Ca^{2+} dynamics and properties of the vesicle pools (Regehr, 2012).

In case the release probability is low, only a minority of readily releasable vesicles will fuse upon an initial stimulus. An immediate second stimulation leads to an additional increase of the presynaptic Ca^{2+} concentration as it is not yet back to baseline from the first stimulus. Due to the higher Ca^{2+} concentration and the fact that the majority of readily releasable vesicles is still available, more vesicles fuse and therefore more neurotransmitter is released into the synaptic cleft in response to the second stimulus. The resulting larger postsynaptic current (PSC) on the second compared to the first pulse is referred to as paired-pulse facilitation.

In case the release probability is high, the majority of vesicles will fuse on the first stimulus. Although the Ca^{2+} concentration is again increased on an immediate second stimulus, only a minority of readily releasable vesicles is left. Consequently, their fusion evokes a smaller amplitude on the second stimulus which is termed paired-pulse depression (Regehr, 2012).

Short-term synaptic plasticity was considered to be an entirely presynaptic feature due to its dependence on the release probability. However, postsynaptic properties such as receptor saturation and desensitization may alter the degree of facilitation or depression measured (Regehr, 2012). Furthermore, it was demonstrated that even postsynaptic proteins like the synaptic cell adhesion proteins neuroligins may influence presynaptic transmitter release (Futai et al., 2007).

Interestingly, vesicle fusion also occurs in the absence of action potential-driven Ca^{2+} elevations due to spontaneous fusion at the active zone. These currents are considered to be quantal, meaning that they arise from the fusion of a single vesicle. Assuming that in average the neurotransmitter content of a vesicle is constant, the amplitude of the corresponding PSC provides information about the receptor density in the postsynaptic membrane as the current amplitude largely depends on the amount of neurotransmitter available, at least at sub-saturating concentrations. These currents are referred to as miniature PSCs (mPSCs) and may be measured in the presence of tetrodotoxin (TTX), a well-characterized blocker of voltage-gated Na^{+} channels that effectively prevents action potential generation (Vautrin and Barker, 2003).

1.4.2 Properties of PSCs

Neurotransmitter released from the presynaptic terminal diffuse through the synaptic cleft to activate postsynaptic receptors. The kinetic properties of the PSCs are influenced by a variety of factors. A typical PSC is commonly characterized by two kinetic components, the rise time and the decay time. Neurotransmitter receptors are clustered at a high density in the postsynaptic membrane. Upon transmitter release, these receptors bind the ligand almost synchronously leading to a steep rising phase of the PSC which is referred to as the rise time. Regarding the closure or inactivation of the receptors, two receptor properties may be distinguished. Deactivation is the process of channel closure after removal of the agonist (Fig. 1.4A). Desensitization in contrast describes the channel closure in the maintained presence of the agonist (Fig. 1.4B). For most synapses an intermediate state between deactivation and desensitization applies which is considered as the decay time (Guzman and Jonas, 2010).

The relationship between deactivation and desensitization implies that the decay time critically depends on the time-course of neurotransmitter concentration in the synaptic cleft. This in turn depends on diffusion parameters, the microanatomical structure and the removal of neurotransmitter from the synaptic cleft (Jonas, 2000). The latter is also mediated by neurotransmitter transporters on astrocytic processes that tightly wrap the synapse. Astrocytes are thus able to actively modify the PSC response (Volterra, 2013).
Kinetic properties of PSCs also depend on the receptors themselves which are introduced in the following section. Detailed information about the experimental characterization of PSCs is given in section 4.1.9.

1.5 Neurotransmitter receptors

Two classes of neurotransmitter receptors are distinguished. Ionotropic receptors are ligand-gated ion channels that open a pore in the cell membrane permeable for certain ions in response to binding of the corresponding ligand. Metabotropic receptors in contrast are G protein-coupled receptors that activate intracellular signaling cascades upon binding of the specific ligand. Neurons as well as glial cells are equipped with a variety of neurotransmitter receptors (Kandel et al., 2013).
In the present study, ionotropic AMPA and $GABA_A$ receptors were investigated in NG2 cells and will be introduced in this section.

1.5.1 AMPA receptors

The endogenous agonist of AMPA receptors is glutamate, the most frequent neurotransmitter in the CNS. Glutamate primarily acts as an excitatory neurotransmitter, although it may also mediate inhibition depending on the type of receptor. AMPA receptors are composed of the four subunits GluA1-4 that assemble in different combinations to form a tetrameric receptor (Fig. 1.3). The four subunits display the same general structure including an extracellular N-terminal domain that modulates receptor assembly, one ligand-binding site, three transmembrane domains and a reentrant loop that lines the pore and a cytoplasmic C-terminal domain (Fig. 1.3A). The C-terminus of each subunit interacts with specific proteins that influence the trafficking and anchoring of the receptor in the

postsynaptic density. The subunits GluA2/3 for example specifically bind to the glutamate receptor-interacting protein (GRIP) which is important for the stabilization of the receptor in the postsynaptic density (Palmer et al., 2005; Traynelis et al., 2010).

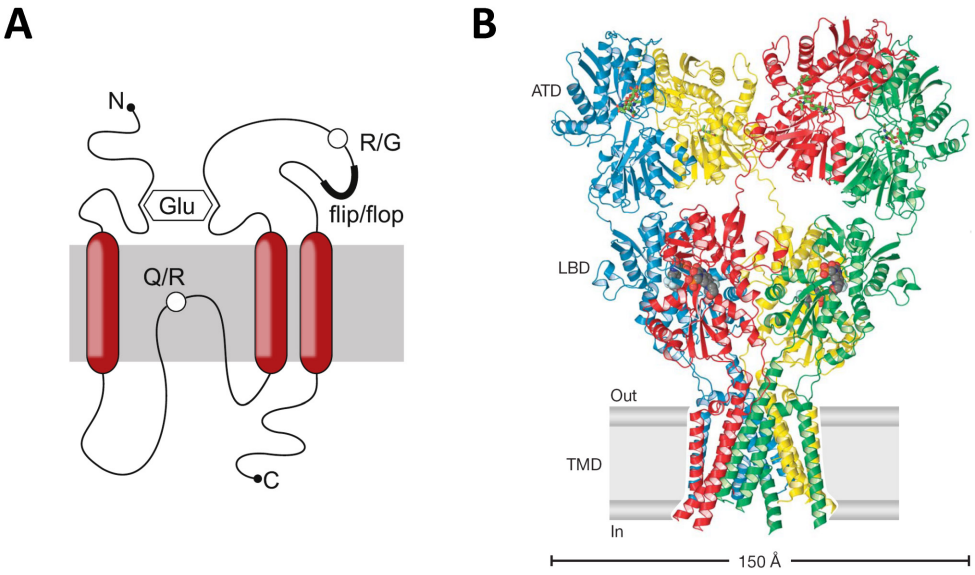

Fig. 1.3 Molecular structure of AMPA receptors. (A) Schematic of the general structure of an AMPA receptor subunit including extracellular N-terminal domain, glutamate (Glu) binding site, three transmembrane domains and a reentrant loop lining the pore, cytoplasmic C-terminal domain, flip/flop site of alternative splicing and Q/R and R/G site which are targets for RNA editing (Guzman and Jonas, 2010). **(B)** Crystal structure of a homomeric GluA2 AMPA receptor. The large amino-terminal domain (ATD) which is important for receptor assembly, the ligand-binding domain (LBD) and the transmembrane domain (TMD) are displayed. Each subunit is in different color (Sobolevsky et al., 2009).

All subunits are subject to intensive posttranscriptional modifications that influence the receptor assembly as well as the properties of the functional receptor. As a result of alternative splicing all subunits occur in a so called 'flip' or 'flop' form which display differences in receptor kinetics and trafficking (Fig. 1.3A). 'Flop' versions were shown to deactivate and desensitize more rapidly compared to the 'flip' versions (Fig. 1.4). Another important modification occurring mainly at the GluA2-subunit of AMPA receptors is the RNA editing. A single nucleotide exchange at the so called Q/R site located in the pore region of the GluA2-subunit causes the substitution of the uncharged amino acid glutamine (Q) to the positively-charged arginine (R) (Fig. 1.3A). Due to the change in charge and size, GluA2-containing AMPA receptors are impermeable for Ca^{2+} (Palmer et al., 2005; Traynelis et al.,

2010). Presence of the GluA2 subunit has therefore significant functional consequences since Ca^{2+} is an important signaling molecule implicated in synaptic plasticity (Derkach et al., 2007). Another target for RNA editing is the R/G site located in an extracellular loop (Fig. 1.3A). Substitution of the arginine (R) to glycine (G) at this position also influences receptor current kinetics. Moreover, AMPA receptor subunits are subject to posttranslational modifications such as phosphorylation, glycosylation or palmitoylation that alter the channel current kinetics as well as trafficking of the receptors (Palmer et al., 2005; Traynelis et al., 2010).

In addition to the various posttranscriptional modification mechanisms, the properties of AMPA receptors are also influenced by auxiliary AMPA receptor subunits. Different families of these proteins were identified to date, namely transmembrane AMPA receptor regulatory proteins (TARPs), cornichon related proteins (CNIH) and cysteine-knot AMPA receptor modulating protein 44 (CKAMP44). These proteins have profound effects on gating kinetics, surface expression and pharmacology of AMPA receptors (Guzman and Jonas, 2010). The majority of auxiliary AMPA receptor subunits causes prolonged deactivation as well as desensitization times (Fig. 1.4). Interestingly, TARPs are also expressed by NG2 cells where they similarly modify the functional properties of glial AMPA receptors (Zonouzi et al., 2011).

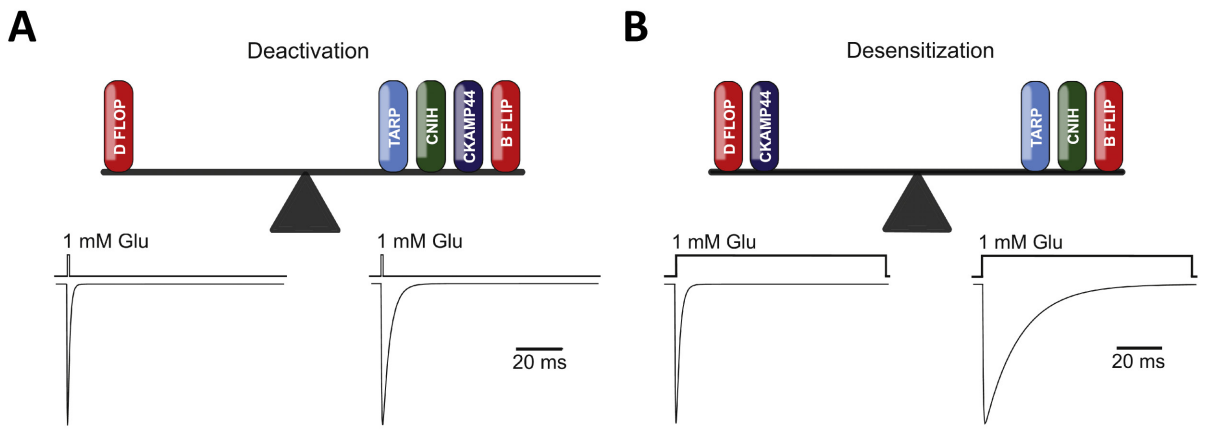

Fig. 1.4 Impact of alternative splicing and auxiliary subunits on AMPA receptor gating. (A) Deactivation was investigated using 1 ms glutamate pulses. Flip forms of AMPA receptor subunits as well as auxiliary AMPA receptor subunits prolong the deactivation time. **(B)** Desensitization was investigated using 100 ms glutamate pulses. Flip forms of AMPA receptor subunits as well as auxiliary AMPA receptor subunits except CKAMP44 prolong the desensitization time. Modified from Guzman and Jonas (2010).

The expression of AMPA receptor subunits varies depending on cell type and brain region. In the adult hippocampus, the majority of neuronal AMPA receptors are composed of the subunit combination GluA1/2 and to a lower extent of GluA2/3, thus displaying rather low Ca^{2+} permeability under baseline conditions (Lu et al., 2009). AMPA receptors are also expressed by all major classes of glial cells. Especially NG2 cells that are innervated by glutamatergic neurons display extensive AMPA receptor signaling (Bergles et al., 2010). For juvenile hippocampal NG2 cells an intermediate Ca^{2+} permeability of AMPA receptors was demonstrated while cells from older animals were more uniform with lower Ca^{2+} permeability. In the same study, single-cell RT-PCR expression analysis revealed that the majority of NG2 cells expressed the subunits GluA1, 2 and 4 during postnatal development (Seifert et al., 2003). Ca^{2+}-permeable AMPA receptors were also observed in NG2 cells of the cerebellum and corpus callosum (Lin et al., 2005; Ziskin et al., 2007). With respect to their function in NG2 cells, Ca^{2+}-permeable AMPA receptors were shown to mediate long-term potentiation of hippocampal neuron-NG2 cell synapses (Ge et al., 2006b).

During differentiation of NG2 cells to oligodendrocytes, glutamatergic synapses are disassembled. However, oligodendrocytes still express functional AMPA receptors albeit at lower levels (De Biase et al., 2010; Kukley et al., 2010). Furthermore, AMPA receptor-mediated currents were measured in astrocytes of various brain regions (Kettenmann and Zorec, 2013). Surprisingly, classical astrocytes of the hippocampus completely lack AMPA receptors (Matthias et al., 2003). The rich repertoire of neurotransmitter receptors expressed by microglia also includes AMPA receptors. All four subunits were detected in microglia *in vitro* (Noda and Verkhratsky, 2013).

1.5.2 $GABA_A$ receptors

$GABA_A$ receptors are ligand-gated ion channels mainly permeable for Cl^- ions when activated by their endogenous ligand GABA. Sixteen subunits have been identified in the mammalian CNS, namely α1-6, β1-3, γ1-3, δ, ε, θ and π, which assemble in different combinations to form a pentameric receptor. Another three subunits of ionotropic GABA receptors were identified (ρ1-3) that are sometimes referred to as $GABA_C$ receptors, as they do not co-assemble with the other $GABA_A$ receptor subunits. A more recent nomenclature, however, recommends designating them '$GABA_A$-ρ receptors'. However, they are mainly expressed in

the retina and not in focus of the present study (Olsen and Sieghart, 2009). All subunits share a common structure including an extracellular N-terminal domain, four transmembrane domains with a large intracellular loop between transmembrane domain three and four and a short extracellular C-terminal domain (Fig. 1.5A) (Hevers and Luddens, 1998; Jacob et al., 2008).

The minimum requirement for a functional $GABA_A$ receptor is the presence of α- and β-subunits that form the binding site for GABA. Further studies revealed that native $GABA_A$ receptors largely display the following stoichiometry of subunits: 2α, 2β and 1γ or 1δ (Olsen and Sieghart, 2009). As a consequence, each $GABA_A$ receptor contains two GABA binding sites, each located at the interface of α- and β-subunit. Beyond that, several allosteric binding sites for different modulators and blockers were identified on $GABA_A$ receptors which include but are not limited to benzodiazepines, barbiturates and Zn^{2+} (Fig. 1.5B). The occurrence of these binding sites and the degree of modulation by a certain substance critically depend on the subunit composition of the particular $GABA_A$ receptor. Pharmacological modulation with specific compounds therefore allows the functional identification of the $GABA_A$ receptor subunit composition. The best studied allosteric interaction site is the benzodiazepine binding site located at the interface of α- and γ-subunit. Several modulators have been characterized that specifically bind to this site including the benzodiazepine diazepam, the non-benzodiazepine zolpidem or the β-carboline methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM). They display differential modulatory effects depending on the particular α- and γ-subunits. The β-subunits contain the barbiturate binding site which is among others a target of the barbiturate pentobarbital. Another modulator acting on β-subunits is loreclezole although binding to another site as pentobarbital (Hevers and Luddens, 1998; Mehta and Ticku, 1999). Zn^{2+} is an endogenous inhibitor of $GABA_A$ receptors that recognizes three different binding sites. One of them is located in the receptor pore while the two others are at the interface between α- and β-subunit. The modulatory effect of Zn^{2+}, however, depends on the presence of a γ-subunit since this incorporation disrupts two of the binding sites (Hosie et al., 2003). More detailed information about the modulatory action of the various modulators is given in the corresponding results and discussion sections (section 5.1 and 6.1).

The most abundant $GABA_A$ receptor expressed in the mammalian CNS displays the subunit composition α1β2γ2 (Pirker et al., 2000; Olsen and Sieghart, 2009). However, the subunit composition varies between different brain regions, types of neurons and individual cells and was shown to effect the subcellular localization of $GABA_A$ receptors. They may either be targeted to postsynaptic densities to mediate phasic currents or to extrasynaptic regions to mediate tonic currents (Farrant and Nusser, 2005). In this respect the γ2-subunit is of particular interest as it was shown to be crucial for clustering and anchoring of $GABA_A$ receptors in the postsynaptic density (Essrich et al., 1998; Schweizer et al., 2003). Consequently, the γ2-subunit is a common part of synaptic $GABA_A$ receptors while being less frequent in extrasynaptic ones.

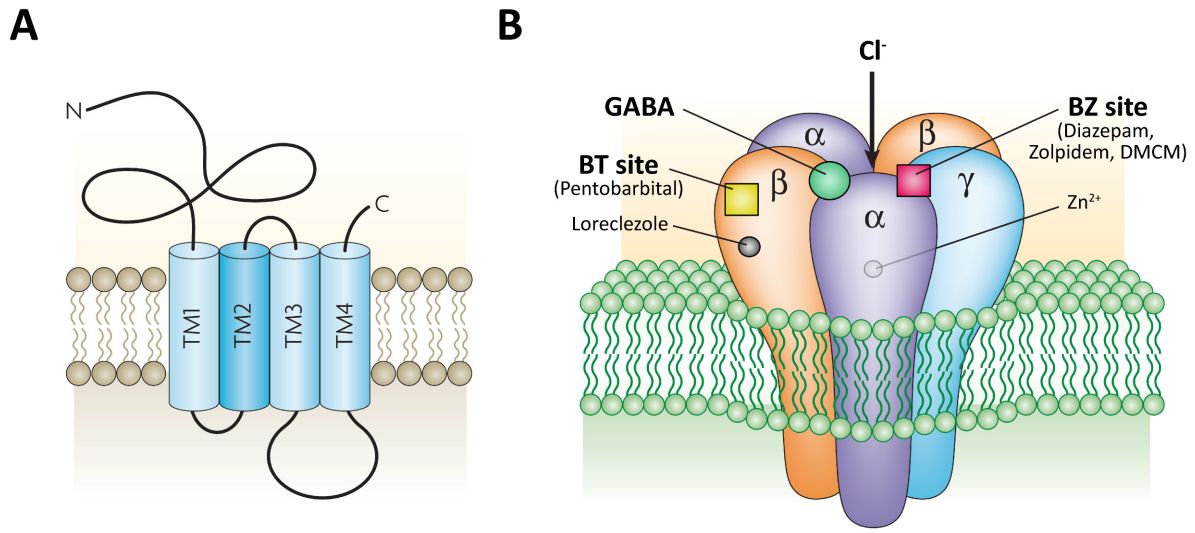

Fig. 1.5 Molecular structure and modulatory binding sites of $GABA_A$ receptors. (A) Schematic of the general structure of a $GABA_A$ receptor subunit including extracellular N-terminal domain, four transmembrane domains (TM) with a large intracellular loop between TM3 and 4 and a short extracellular C-terminal domain. TM2 of each subunit lines the ion pore (Jacob et al., 2008). **(B)** Structure of a pentameric $GABA_A$ receptor displaying the frequent subunit combination: 2α-, 2β- and 1γ-subunit. The GABA-binding sites are located at the interface of α- and β-subunits, the benzodiazepine (BZ)-binding site at the interface of α- and γ-subunit and the barbiturate (BT)-binding site on the β-subunits. Loreclezole binds to β-subunits, Zn^{2+} inside the pore and at the interface of α- and β-subunits. Modified from Belelli and Lambert (2005).

Apart from neurons, functional $GABA_A$ receptors were also identified in glial cells. Several studies demonstrated GABA-evoked currents in NG2 cells of different brain regions (von Blankenfeld et al., 1991; Steinhäuser et al., 1994a; Williamson et al., 1998). Since the

discovery of GABAergic innervation of NG2 cells it is known that $GABA_A$ receptors are also located in synaptic clusters in these cells (Lin and Bergles, 2004; Jabs et al., 2005; Velez-Fort et al., 2010). After maturation of NG2 cells to oligodendrocytes, $GABA_A$ receptor currents are still detectable although to a lower extent (Gallo and Mangin, 2013). Astrocytes of different brain regions were also shown to express functional $GABA_A$ receptors (Kettenmann and Zorec, 2013), which is in contrast to microglia where $GABA_A$ receptor-mediated currents have never been detected (Noda and Verkhratsky, 2013).

GABA is commonly considered to be the most frequent inhibitory neurotransmitter in the CNS. Although this is not entirely wrong, it is misleading in a way as it critically depends on the $[Cl^-]_i$. In fact, several studies demonstrated that GABA is excitatory in developing neurons (Cherubini et al., 1991). The switch from an excitatory/depolarizing to an inhibitory/ hyperpolarizing action during the first two postnatal weeks is due to a change in $[Cl^-]_i$. The upregulation of the K^+-Cl^- cotransporter 2 (KCC2) that transports Cl^- out of the cell and the downregulation of the Na^+-K^+-Cl^- cotransporter 1 (NKCC1) that transports Cl^- into the cell causes a reduction in $[Cl^-]_i$ and thereby changes the driving force of Cl^- (Rivera et al., 1999; Stein et al., 2004; Yamada et al., 2004; Khirug et al., 2008).

A depolarizing action of GABA also applies to NG2 cells. Furthermore, unlike neurons, GABA remains excitatory in NG2 cells during development (Lin and Bergles, 2004; Tong et al., 2009; Tanaka et al., 2009).

1.6 Synaptic cell adhesion molecules – the neuroligin-neurexin complex

The coordinated assembly and maintenance of the synaptic microarchitecture is critical for efficient synaptic transmission. Synaptic cell adhesion molecules are transmembrane proteins in pre- and postsynaptic specializations. They directly interact with each other thereby bridging the synaptic cleft. However, this connection is far more than a mechanical link. Synaptic cell adhesion molecules were shown to modulate formation, maturation, specificity, function and plasticity of synapses, making them important components of the highly specialized synaptic junction. Several different synaptic cell adhesion molecules were identified such as neurexins and neuroligins, ephrin-B receptors and ephrin-Bs, synaptic cell

adhesion molecule (SynCAM), neural cell adhesion molecule (NCAM) and cadherins (Dalva et al., 2007; Missler et al., 2012). This section will introduce properties and functions of neurexins and neuroligins.

Presynaptic neurexins and their postsynaptic binding partners neuroligins display the best characterized group of synaptic cell adhesion molecules. Although only three neurexin genes exist in mammals they display an incredible diversity. All three genes exhibit two promotors each giving rise to a long form called α-neurexin and a short form named β-neurexin. Alternative splicing at five sites as well as N- and O-glycosylation additionally increase the diversity. Similarly, neuroligins are transcribed from five different genes and are equally subject to alternative splicing and posttranslational modifications. Both, neurexins and neuroligins, are characterized by a large extracellular N-terminal domain important for the interaction with one another, a single transmembrane region and a relatively short cytoplasmic C-terminus that terminates in postsynaptic density 95/discs large/zonula-occludens-1 (PDZ) domain-binding sites that mediate the interaction with a variety of intracellular communication partners. α-Neurexins contain extracellularly six laminin G/neurexin/sex hormone binding globulin (LNS) domains which are common protein interaction domains that mediate the transsynaptic binding of neurexins to neuroligins and other binding partners (Fig. 1.6A, B) (Craig and Kang, 2007; Missler et al., 2012).

Several studies suggest that neuroligins as well as neurexins are critical determinants of synaptogenesis as both are able to induce synapse formation when expressed in non-neuronal cells (Scheiffele et al., 2000; Graf et al., 2004; Nam and Chen, 2005). Also, overexpression of neuroligins in cultured neurons increased the number of functional synapses, an effect that was blocked when soluble neurexin was added to the culture (Prange et al., 2004; Levinson et al., 2005). Surprisingly, studies investigating knockout animals of all neuroligins or neurexins, respectively, revealed that synapse number was unchanged but synaptic transmission was disturbed due to a dysfunction of presynaptic Ca^{2+} channels causing early postnatal lethality (Missler et al., 2003; Varoqueaux et al., 2006). These results suggest that the neuroligin-neurexin complex is necessary for normal synapse function but not for the formation of synapses. However, this idea was reconsidered by a recent quite elegant study. By selectively reducing or increasing the expression of neuroligin-

1 in single cells *in vivo*, the authors were able to show that neuroligin-1 does influence the number of synapses. However, this was not dependent on the absolute but instead on the relative expression level of neuroligin-1 compared to neighboring cells revealing a transcellular competitive mechanism of synapse formation for neuroligin-1 (Kwon et al., 2012). Other studies demonstrated that neuroligins are involved in synapse specificity. Neuroligin-1 is almost exclusively present in glutamatergic synapses (Song et al., 1999), neuroligin-2 is mainly located in GABAergic synapses (Fig. 1.6B) (Varoqueaux et al., 2004), while neuroligin-3 was found to be present in both, glutamatergic and GABAergic synapses (Budreck and Scheiffele, 2007). Neuroligin-4 is considered to be specific for glycinergic synapses throughout the brain (Hoon et al., 2011). Besides contributing to the specificity of synapses, neuroligins were also implicated in the maturation of these synapses (Varoqueaux et al., 2006; Wittenmayer et al., 2009). Furthermore, it was shown that presynaptic neurexins are able to regulate the postsynaptic AMPA receptor density (Aoto et al., 2013), while postsynaptic neuroligins may regulate the presynaptic release probability (Futai et al., 2007). This demonstrates that pre- and postsynapse are functionally connected via the neuroligin-neurexin complex which allows transsynaptic bidirectional communication.

1.7 The NG2 protein

The initial description of the molecular structure of the NG2 protein revealed that it belongs to the group of chondroitin sulfate proteoglycans (CSPGs) (Nishiyama et al., 1991). Proteoglycans are generally characterized by a core protein that is heavily glycosylated via the attachment of glycosaminoglycans (GAGs). In case these GAGs are sulfated, the corresponding proteins are referred to as CSPGs. After its initial discovery in rats, NG2 homologs were detected among others in mice (AN2) (Niehaus et al., 1999; Schneider et al., 2001) and in humans (melanoma chondroitin sulfate proteoglycan, MCSP) (Pluschke et al., 1996). The mammalian NG2 protein is encoded by a single gene (*CSPG-4*). The resulting 330 kDa protein consists of 2327 amino acids. Due to the lack of alternative splicing, the NG2 protein presumable occurs in one isoform only (Trotter et al., 2010).

Structurally, the NG2 protein is composed of a large extracellular N-terminal domain, a single transmembrane region and a short C-terminal cytoplasmic tail (Fig. 1.6C). Several structural

characteristics of these domains were predicted based on the amino acid sequence. The extreme N-terminal region is stabilized by intramolecular disulfide bridges. More importantly this region contains two LNS domains which represent common protein interaction domains. However, explicit binding partners for this domain in NG2 cells have not been determined so far. The central part of the NG2 protein contains a collagen binding domain as well as the single chondroitin sulfate chain. The lower third of the extracellular domain contains N-linked oligosaccharides as well as sites for proteolysis of the ectodomain. The regulation and role of this proteolysis is unknown so far but might exhibit some important physiological or pathophysiological function. It also causes the deposition of NG2 on other cell types which might have led to false identification of NG2-positive cells in earlier studies. The cytoplasmic C-terminal region contains two functionally relevant structures, a PDZ-binding motif and several phosphorylation sites. The PDZ domain of the NG2 protein was shown to be recognized by the scaffolding proteins MUPP1, syntenin-1 and GRIP. The phosphorylation sites are targeted by protein kinase C α and extracellular-signal-regulated kinase that were both shown to influence the physiology of NG2 cells (Fig. 1.6C) (Makagiansar et al., 2007; Stallcup and Huang, 2008).

The NG2 protein was initially discovered on the surface of cells with neuronal (N) and glial (G) properties, cells today known as NG2 cells (section 1.3.4) (Wilson et al., 1981; Stallcup, 1981). Further studies revealed that the NG2 protein is frequently expressed by different types of immature cells throughout the body, including chondrocytes, osteoblasts, myoblasts and epidermal stem cells. In the brain, the NG2 protein is expressed by NG2 cells and vascular pericytes (Nishiyama et al., 2009). However, under pathological conditions a massive upregulation of NG2 expression was observed, e.g. in many gliomas, suggesting a role for NG2 in glioma progression (Stallcup and Huang, 2008).

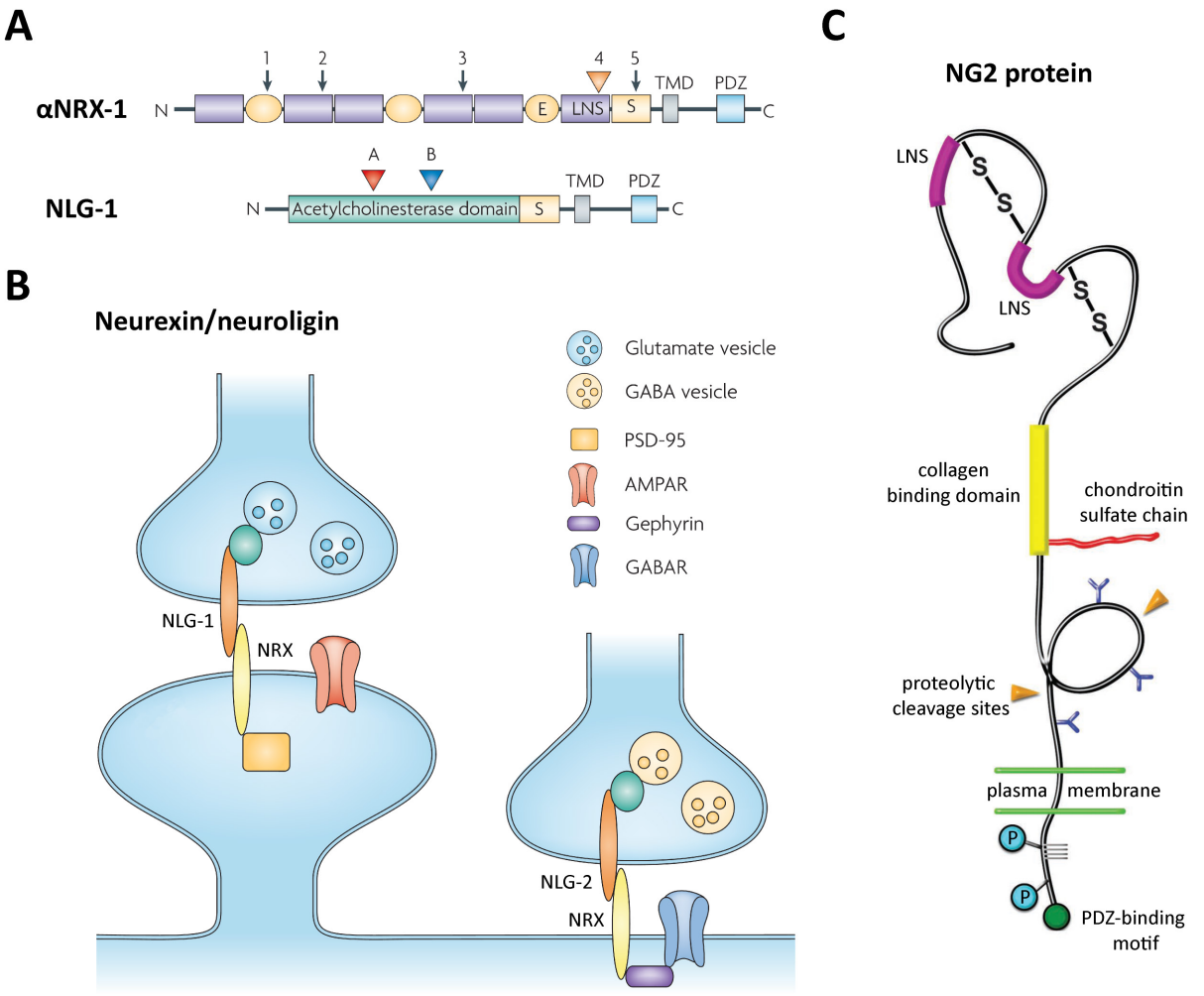

Fig. 1.6 Structure and function of neurexins, neuroligins and the NG2 protein. (A) Structure of α-neurexin-1 (αNRX-1, top) showing the large extracellular N-terminal domain containing six LNS domains and several sites of alternative splicing (arrows). The single transmembrane domain (TMD) is followed by the cytoplasmic C-terminal region containing a PDZ-binding motif. Bottom panel depicts structure of neuroligin-1 (NLG-1) including the two splice regions A and B. E, epidermal growth factor-like sequence; S, carbohydrate attachment site. **(B)** Localization of neuroligins (NLG) and neurexins (NRX) at excitatory (left) and inhibitory (right) synapses. (A) and (B) modified from Dalva et al. (2007). **(C)** Structure of the NG2 protein. The extracellular N-terminal domain exhibits two LNS domains, a central collagen-binding domain, a single chondroitin sulfate chain and proteolytic cleavage sites. The intracellular C-terminal domain contains several phosphorylation sites (P) and a single PDZ-binding motif. Modified from Stallcup and Huang (2008).

Several functions have been assigned to the NG2 protein. Due to its binding to components of the extracellular matrix (Burg et al., 1996; Tillet et al., 2002) and its intracellular interaction via scaffolding proteins with the cytoskeleton, many studies were able to demonstrate a role for NG2 in cytoskeletal reorganization, polarization and directional migration (Niehaus et al., 1999; Fang et al., 1999; Makagiansar et al., 2004; Biname et al.,

2013). Furthermore, it was shown that the NG2 protein is necessary for the correct function of the platelet-derived growth factor (PDGF) α receptor making it an important regulator of differentiation and proliferation of NG2 cells (Nishiyama et al., 1996; Goretzki et al., 1999; Fruttiger et al., 1999; Makagiansar et al., 2007). However, the intriguing finding that the NG2 protein interacts intracellularly with GRIP which is a known binding partner of AMPA receptors attracted much less attention so far (Stegmüller et al., 2003). This observation in the context of the synaptic innervation of NG2 cells by neurons gave rise to the hypothesis that the NG2 protein might be important for the clustering of glial AMPA receptors in the postsynaptic density. Furthermore, the extracellular LNS domains of the NG2 protein are equally present in the synaptic cell adhesion proteins neurexins where they mediate the transsynaptic binding to presynaptic neuroligins (Fig. 1.6A, C) (section 1.6). Likewise, the NG2 protein might serve as a synaptic cell adhesion protein in NG2 cells by binding to a yet unknown neuronal binding partner (Trotter et al., 2010). The investigation of this hypothesis was a central part of the present study (section 5.3 and 6.2).

2 Aim of the study

NG2 cells are equipped with a variety of ion channels and receptors (Bergles, 2013). Intriguingly, these cells receive direct synaptic input from glutamatergic and GABAergic neurons. However, in contrast to neuron-neuron synapses, our knowledge of the properties and physiological relevance of this unique type of neuron-glia communication is basal at best (Bergles et al., 2010). The present study aimed at acquiring a more detailed insight into elementary principles of glial neurotransmitter receptor and synapse physiology. Three main aspects ought to be investigated in detail.

(i) *Functional characterization of synaptic and extrasynaptic $GABA_A$ receptors in NG2 cells.*
In contrast to AMPA receptors and the glutamatergic innervation, much less in known about $GABA_A$ receptors and the GABAergic innervation (Bergles et al., 2010). The subunit composition of $GABA_A$ receptors determines critical aspects of their physiology. Therefore, the first part of the study aimed at identifying the subunit composition of $GABA_A$ receptors functionally expressed by hippocampal NG2 cells. From neurons it is known that $GABA_A$ receptors may mediate phasic synaptic currents as well as tonic extrasynaptic currents (Farrant and Nusser, 2005). Whether a similar differential subcellular distribution of $GABA_A$ receptors occurs in NG2 cells and how these receptors might vary in subunit composition was another target of this part of the study. Answers to these questions would provide an important basis to understand the function that these receptors serve in NG2 cells.

(ii) *Analysis of the $GABA_A$ receptor subunit expression pattern during postnatal development.*
In neocortical NG2 cells synaptic GABAergic innervation is replaced by a form of extrasynaptic GABAergic signaling during postnatal development (Velez-Fort et al., 2010). Since the molecular basis of this transition is unclear, the second part of the present study aimed at investigating the $GABA_A$ receptor subunit expression pattern during postnatal development. Potential alterations that accompany the loss of direct synaptic innervation would provide important information about the characteristics of GABAergic synapses in NG2 cells.

(iii) *Is the NG2 protein a critical component of neuron-NG2 cell synapses?*

Several critical aspects of synaptic transmission such as synapse formation, function and plasticity are mediated by transsynaptic cell adhesion proteins at neuron-neuron synapses (Dalva et al., 2007; Missler et al., 2012). How these fundamental processes are regulated at neuron-NG2 cell synapses is completely unknown. The NG2 protein itself was identified as a potential synaptic cell adhesion protein at glutamatergic neuron-NG2 cell synapses as it

(a) displays structural similarities to the synaptic cell adhesion proteins neurexins (section 1.6 and 1.7) (Trotter et al., 2010; Missler et al., 2012)

(b) was shown to form a complex with AMPA receptors via the synaptic protein GRIP (Stegmüller et al., 2003).

Based on these findings the hypothesis was raised that the NG2 protein might be an important regulator of neuron-NG2 cell synapse formation and clustering of AMPA receptors in the glial postsynaptic density (Trotter et al., 2010). This hypothesis ought to be tested in the third part of the present study by investigating glutamatergic synaptic transmission between neurons and NG2 cells in NG2 knockout compared to control mice.

3 Mouse models and materials

3.1 Mouse models

Within the framework of the present study three different transgenic mouse lines were used. Maintenance and handling of animals was according to European and local government regulations. Animals were kept and bred in-house at the "Haus für experimentelle Therapie" of the university hospital Bonn. All measures were taken to minimize the number of animals used.

3.1.1 hGFAP-EGFP mice

In this transgenic mouse line, the coding region of the enhanced green fluorescent protein (EGFP) is under control of a 2.2 kb fragment of the human glial fibrillary acidic protein (hGFAP) promotor (Nolte et al., 2001). Mice were generated from oocytes with the genetic FVB/N background. Expression of EGFP exhibits an overlap with GFAP immunoreactivity in the majority of cells while no colocalization with neuronal or oligodendroglial markers is detectable. However, the density of EGFP-positive cells varies drastically with only a subset of astrocytes being labeled in adult animals and different brain regions (Nolte et al., 2001).
Initially, this mouse line was generated to specifically visualize astrocytes in living brain slices. Detailed analysis, however, revealed that two separate populations of glial cells are labeled by EGFP fluorescence in these animals. Cells with intense EGFP fluorescence display irregularly shaped cell bodies with highly branched processes, exhibit a passive current pattern with time- and voltage-independent conductances and express glutamate transporters but no receptors (Fig. 3.1A2). Accordingly, these cells have been named GluT cells. Another population of cells expresses lower levels of EGFP, features round cell bodies with fewer processes and displays a complex current pattern with time- and voltage-dependent Na^+ and K^+ conductances (Fig. 3.1A1). As this population expresses glutamate receptors but no transporters, they have been termed GluR cells (Matthias et al., 2003). Another study demonstrated that GluT cells exhibit gap junctional coupling characteristic for astrocytes while GluR cells completely lack these intracellular connections (Wallraff et al., 2004). Furthermore, it has been shown that GluR cells express the NG2 protein but not the

GFAP protein as opposed to GluT cells that are GFAP-positive but NG2 negative (Fig. 3.1) (Matthias et al., 2003; Jabs et al., 2005). These findings suggest that GluT cells represent classical astrocytes while GluR cells may be considered as NG2 cells. This assumption is further strengthened by the observation that GluR cells are innervated by glutamatergic and GABAergic neurons, which is unique for NG2 cells among all glial cell types (Jabs et al., 2005).

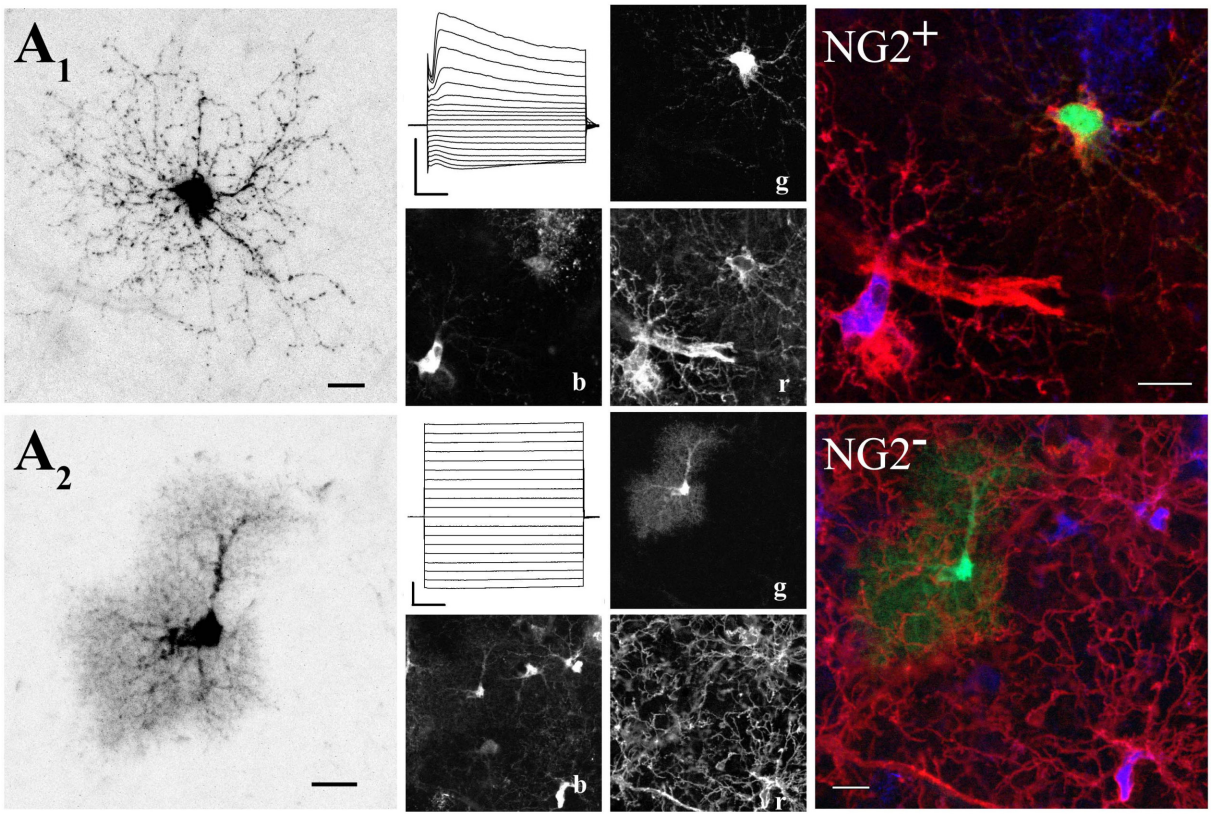

Fig. 3.1 EGFP fluorescence labels two distinct populations of glial cells in hGFAP-EGFP mice. A weakly EGFP-positive GluR cell/NG2 cell with round cell body and few thin processes (A1) and a strongly EGFP-positive GluT cell/astrocyte with highly branched fine processes (A2) were filled with Texas Red-dextran during a whole-cell recording. The GluR cell/NG2 cell displayed a complex current pattern while the GluT cell/astrocyte exhibited a passive current pattern in response to de- and hyperpolarizing voltage steps between +20 and -160 mV (holding potential -80 mV) (A1 and A2 middle panel, respectively). GluR cells/NG2 cells show immunoreactivity for NG2 (A1 middle and right panel) while GluT cells/astrocytes are NG2 negative (A2 middle and right panel). (r) NG2 signal in red, (g) Texas Red-dextran signal in green and (b) EGFP signal in blue (Jabs et al., 2005).

hGFAP-EGFP mice were used for all experiments of section 6.1. In these cases, NG2 cells were unambiguously identified by morphology, low EGFP-expression and characteristic complex whole-cell current pattern. Homozygous mice were used for all experiments.

3.1.2 NG2-DsRed mice

In this transgenic mouse line, the red fluorescent protein DsRed T1 is expressed under control of the NG2 promotor (Zhu et al., 2008). To achieve NG2 cell-specific expression without disrupting the endogenous NG2 gene, the bacterial artificial chromosome (BAC) modification technique was employed. BACs are DNA vectors that may carry large inserts including all regulatory elements necessary for cell type-specific expression. For the generation of the NG2-DsRed mouse line, a BAC containing the entire NG2 gene flanked by additional sequences on both sides of the gene was used. The DsRed T1 coding sequence was inserted into the first exon of the NG2 gene by homologous recombination in bacteria. The correctly modified BAC was then injected into oocytes with C57BL/6 genetic background that were subsequently transferred into pseudopregnant foster mothers. Analysis of transgenic NG2-DsRed mice revealed colocalization of DsRed and NG2 throughout the CNS, while no overlap with markers for other cell types was observed. However, as expected NG2-expressing pericytes were also DsRed-positive, but were easily distinguishable from NG2 cells by their close association to blood vessels and different morphology (Zhu et al., 2008).
This mouse line therefore facilitates the faithful identification of NG2 cells without interfering with the endogenous expression of the NG2 protein. Heterozygous and homozygous mice were used in the present study (section 5.2). NG2 cells were identified by DsRed fluorescence and characteristic complex whole-cell current pattern.

3.1.3 NG2-EYFP mice

In order to obtain faithful fluorescent labeling of NG2 cells in mice, Karram et al. (2008) generated the NG2-EYFP knockin mouse line. They inserted the coding region of the enhanced yellow fluorescent protein (EYFP) into exon 1 of the NG2 gene by homologous recombination in mouse embryonic stem cells. Correctly modified embryonic stem cells were injected into blastocysts with C57BL/6 genetic background which were then transferred into pseudopregnant foster mothers. By inserting the EYFP sequence into exon 1 of the NG2 gene, EYFP is under control of all regulatory elements facilitating highly specific expression of the fluorescent protein in NG2-expressing cells. However, at the same time expression of NG2 itself from the corresponding allele is disrupted. A heterozygous NG2-

EYFP knockin therefore corresponds to a heterozygous NG2 knockout while a homozygous NG2-EYFP knockin results in a complete loss of NG2-expression.

In heterozygous NG2-EYFP mice, all fluorescently labeled cells expressed the NG2 proteoglycan as determined by immunostaining. These mice also showed reduced levels of NG2 protein in western blots of whole brain lysates. Homozygous NG2-EYFP mice obviously displayed a complete lack of NG2 protein in western blot and immunostaining. Colocalization of EYFP-positive cells with neuronal, astrocytic or microglial markers has never been observed. Electrophysiological whole-cell recordings also revealed that fluorescent cells displayed the characteristic complex current pattern of NG2 cells. Nevertheless, as described for the NG2-DsRed mouse, pericytes were also EYFP-positive but clearly distinguishable from NG2 cells by their close association to blood vessels and different morphology (Karram et al., 2008).

In the present study, mice were usually bred in heterozygous matings. The genotype was determined by genotyping PCR of tailtip biopsies as described in Karram et al. (2008) (performed by Gerald Seifert and staff members). NG2-EYFP mice of the three possible genotypes were investigated: wild type NG2 on both alleles (wt), heterozygous NG2 knockout (+/-) and homozygous NG2 knockout (-/-) (section 5.3). In electrophysiological recordings, NG2 cells were unambiguously identified by their expression of EYFP and by the characteristic complex whole-cell current pattern in +/- and -/- mice. In wt mice, NG2 cells were initially selected by morphology as they typically display a relatively small and round cell soma. After establishing the whole-cell configuration, the characteristic complex current pattern was elicited to confirm cell identity. Additionally, the cytoplasm was harvested after the recording and a post-hoc single-cell RT-PCR for expression of the PDGFα receptor was performed as described in section 4.2.

3.2 Devices and software

3.2.1 Devices

A/D converter	ITC-16 (HEKA Elektronik, Lambrecht/Pfalz, Germany)
Bath chamber/shifting table	Base plate 500B, shifting table V240, bridge 500 with control units SM-5 and -6 (Luigs & Neumann, Ratingen, Germany)
CCD camera	VX45 (Optronis, Kehl, Germany)
Differential amplifier	DPA-2FS (npi electronic, Tamm, Germany)
Fluorescence system	Polychrome II, 75W xenon lamp (Till Photonics, Martinsried, Germany)
Glass pipettes	Borosilicate glass GB150F-10 (Science Products, Hofheim, Germany)
Micromanipulators	Micromanipulator 6540 R094 with control unit 5171 (Eppendorf, Hamburg, Germany) LN mini 25 (Luigs & Neumann, Ratingen, Germany)
Micropipette Puller	P-87 (Sutter Instrument, Novato, USA)
Microscope	Axioskop FS2 (Zeiss, Oberkochen, Germany)
Objectives	CP-Achromat (5x; NA: 0.12) (Zeiss, Oberkochen, Germany) LUMPlan FI/IR (60x; NA: 0.90) (Olympus, Tokio, Japan)
Oscilloscope	HM 507 (Hameg, Mainhausen, Germany)
Patch-clamp amplifier	EPC-7 and -800 (HEKA Elektronik, Lambrecht/Pfalz, Germany)
PCR-Cycler	PTC-200 (Biozym, Hessisch Oldendorf, Germany)
Pressure application system	Octaflow (ALA Scientific Instruments, Farmingdale, USA)

Pulse stimulator	Isolated Pulse Stimulator Model 2100 (A-M Systems, Carlsborg, USA)
Tubing pump	ISM 930C (Ismatec/Idex, Wertheim, Germany)
Vibration isolation platform	Vision Isostation VIS-3036 (Newport, Irvine, USA)
Vibratome	VT1000 S (Leica Microsystems, Wetzlar, Germany)

3.2.2 Software

IGOR Pro 6 -> data analysis, figures	WaveMetrics, Lake Oswego, USA
Office 2010 -> data analysis, figures	Microsoft, Redmond, USA
Origin Pro 9 -> statistical analysis	OriginLab, Northampton, USA
pClamp 10 -> mPSC detection	Molecular Devices, Union City, USA
TIDA 5 -> electrophysiological recordings	HEKA Elektronik, Lambrecht/Pfalz, Germany

3.3 Chemicals

Standard chemicals were purchased from AppliChem (Darmstadt, Germany), Roth (Karlsruhe, Germany) und Sigma-Aldrich (St. Louis, USA).

Other chemicals and materials

4-AP -> a-type K^+ channel blocker	Sigma Aldrich, St. Louis, USA
*Ban*I -> restriction endonuclease	New England BioLabs, Ipswich, USA
Bicuculline -> $GABA_A$ receptor antagonist	Ascent (now: Abcam), Cambridge, UK
CGP 55845 -> $GABA_B$ receptor antagonist	Tocris Bioscience, Bristol, UK

CNQX -> AMPA/kainate receptor antagonist	Ascent (now: Abcam), Cambridge, UK
Diazepam -> $GABA_A$ receptor modulator	Sigma Aldrich, St. Louis, USA
DMCM -> $GABA_A$ receptor modulator	Sigma Aldrich, St. Louis, USA
dNTPs -> for RT-PCR	Applied Biosystems, Darmstadt, Germany
DTT -> for RT-PCR	Life Technologies, Karlsbad, USA
First-Strand Buffer -> reaction buffer for RT	Life Technologies, Karlsbad, USA
GABA -> GABA receptor agonist	Ascent (now: Abcam), Cambridge, UK
Gabazine (SR-95531) -> $GABA_A$ receptor antagonist	Ascent (now: Abcam), Cambridge, UK
Gramicidin-A -> antibiotic for perforated patch recordings	Sigma Aldrich, St. Louis, USA
Ionomycin -> Ca^{2+} ionophore	Ascent (now: Abcam), Cambridge, UK
Loreclezole -> $GABA_A$ receptor modulator	Sigma Aldrich, St. Louis, USA
Low Molecular Weight DNA Ladder -> size marker for gel-electrophoresis	New England BioLabs, Ipswich, USA
MinElute PCR Purification Kit -> purification of PCR products	Qiagen, Hilden, Germany
Muscimol -> $GABA_A$ receptor agonist	Ascent (now: Abcam), Cambridge, UK
NBQX -> AMPA/kainite receptor antagonist	Tocris Bioscience, Bristol, UK
Nipecotic acid -> GABA transporter inhibitor	Sigma Aldrich, St. Louis, USA
PCR Buffer -> reaction buffer for PCR	Life Technologies, Karlsbad, USA
Pentobarbital -> $GABA_A$ receptor modulator	Sigma Aldrich, St. Louis, USA

Phaclofen -> $GABA_B$ receptor antagonist	Sigma Aldrich, St. Louis, USA
ϕX174 *Hinc*II digest -> size marker for gel-electrophoresis	Eurogentec, Liège, Belgium
Picrotoxin -> $GABA_A$ receptor blocker	Ascent (now: Abcam), Cambridge, UK
Platinum® Taq Polymerase -> for RT-PCR	Life Technologies, Karlsbad, USA
*Pst*I -> restriction endonuclease	New England BioLabs, Ipswich, USA
Random Hexamer Primer -> for RT-PCR	Roche Applied Science, Mannheim, Germany
RNasin® RNase Inhibitor -> for RT-PCR	Promega, Madison, USA
Superscript® III Reverse Transcriptase -> for RT-PCR	Life Technologies, Karlsbad, USA
Taq DNA Polymerase -> for RT-PCR	Life Technologies, Karlsbad, USA
TTX -> Na^+ channel blocker	Ascent (now: Abcam), Cambridge, UK
Zolpidem -> $GABA_A$ receptor modulator	Sigma Aldrich, St. Louis, USA

3.4 Solutions

3.4.1 Preparation solutions

Preparation solution 1 (hippocampus)

NaCl	87 mM
KCl	2.5 mM
NaH_2PO_4	1.25 mM
$MgCl_2$	7 mM
$CaCl_2$	0.5 mM
$NaHCO_3$	25 mM
Glucose	25 mM
Sucrose	75 mM

pH 7.0 at 5°C and 7.6 at 35°C with 5% CO_2/95% O_2

Preparation solution 2 (barrel cortex)	KCl	2.5 mM
	NaH_2PO_4	1.25 mM
	$MgCl_2$	7 mM
	$CaCl_2$	1 mM
	$NaHCO_3$	26 mM
	Glucose	20 mM
	Sucrose	215 mM
	Pyruvate	5 mM
	pH 7.0 at 5°C and 7.6 at 35°C with 5% CO_2/95% O_2	

3.4.2 Extracellular solutions

Artificial cerebrospinal fluid (ACSF)	NaCl	126 mM
	KCl	3 mM
	NaH_2PO_4	1.25 mM
	$MgSO_4$	2 mM
	$CaCl_2$	2 mM
	$NaHCO_3$	26 mM
	Glucose	10 mM
	pH 7.38 at 25°C with 5% CO_2/95% O_2	
HEPES solution	NaCl	150 mM
	KCl	5 mM
	$MgSO_4$	2 mM
	$CaCl_2$	2 mM
	HEPES	10 mM
	Glucose	10 mM
	pH 7.38 at 25°C with HCl/NaOH	
Blocking solution	NaCl	135 mM
	KCl	5 mM
	$MgSO_4$	2 mM
	$CaCl_2$	2 mM
	HEPES	10 mM
	Glucose	10 mM
	$BaCl_2$	10 mM
	4-AP	4 mM
	$CdCl_2$	30 µM
	TTX	1 µM
	pH 7.38 at 25°C with HCl/NaOH	

3.4.3 Intracellular solutions

Pipette solution 1	KCl	130 mM
	$MgCl_2$	2 mM
	$CaCl_2$	0.5 mM
	BAPTA	5 mM
	HEPES	10 mM
	Na_2-ATP	3 mM
	pH 7.28 at 25°C with HCl/KOH	
Pipette solution 2	K-gluconate	125 mM
	KCl	20 mM
	$MgCl_2$	2 mM
	NaCl	3 mM
	EGTA	0.5 mM
	HEPES	10 mM
	Na_2-ATP	2 mM
	pH 7.28 at 25°C with HCl/KOH	
Pipette solution 3	CsCl	130 mM
	$MgCl_2$	2 mM
	$CaCl_2$	0.5 mM
	BAPTA	5 mM
	HEPES	10 mM
	Na_2-ATP	3 mM
	pH 7.28 at 25°C with HCl/CsOH	
Pipette solution 4	K-gluconate	125 mM
	KCl	20 mM
	$MgCl_2$	2 mM
	NaCl	3 mM
	EGTA	0.5 mM
	HEPES	10 mM
	pH 7.28 at 25°C with HCl/KOH	

4 Methods and statistics

4.1 Electrophysiology

4.1.1 Preparation of acute brain slices

For the preparation of acute brain slices, mice were anaesthetized by a gas mixture of 50% CO_2 and 50% O_2 and killed by decapitation. The brain was rapidly removed and transferred to ice-cold preparation solution. Depending on the respective experiment, 200 or 300 µm sections were cut in different orientations as indicated in Tab. 4.1.

Frontal/coronal sections (hippocampus): The cerebellum and the frontal third of the brain were cut off. The brain was then glued frontally onto a specimen holder. Preparation solution 1 was used.

Horizontal sections (hippocampus): The cerebellum and small part of the dorsal brain were cut off. The brain was then glued dorsally onto a specimen holder. Preparation solution 1 was used.

Parasagittal sections (barrel cortex): The cerebellum was cut off and the hemispheres were separated. Each half was glued with the medial cutting edge on a specimen holder equipped with a plastic block with an angle of 10°. Preparation solution 2 was used.

In each case, the holder was fixed in the buffer tray of the vibratome containing ice-cold preparation solution constantly gassed with 5% CO_2/95% O_2. After cutting, slices were incubated for 15-20 min in preparation solution at 35°C. The slices were then transferred to gassed (5% CO_2/95% O_2) ACSF (preheated to 35°C) cooled down to and stored at room temperature until further use.

Tab. 4.1 Types of brain sections used in the present study.

brain region	orientation	thickness	used in section
hippocampus	frontal/coronal	200 µm	5.1 (except ePSCs)
hippocampus	horizontal	200 µm	5.3 (mPSCs)
hippocampus	horizontal	300 µm	5.1, 5.3 (ePSCs)
barrel cortex	parasagittal	200 µm	5.2

4.1.2 Electrophysiological setup and recording conditions

The electrophysiological setup used in the present study was composed of the following components. The microscope was mounted on a vibration isolation platform in a Faraday cage to reduce movements and electrical noise. A recording chamber was fixed under the objectives of the microscope. The microscope was equipped with a motorized focus and positioned on an electrical shifting table enabling its movement without touching the setup. Two separate electrically-driven micromanipulators were available to move the recording pipette and, if necessary, to position a stimulation electrode. Glass pipettes for patch-clamp recordings were pulled with a horizontal puller and had resistances of 3-6 MΩ when filled with pipette solution. The microscope was further equipped with differential interference contrast (DIC) optics to enhance the contrast and improve the recognition of cellular structures in the brain slice. To visualize fluorescent proteins expressed by living cells of transgenic mice, the setup exhibited a fluorescence system. The optical signals were detected by a CCD-camera and displayed on a monitor. Electrical signals were recorded by a Teflon-coated silver electrode with a chlorinated tip connected via the preamplifier (head stage) to the patch-clamp amplifier. The reference electrode in the recording chamber similarly consisted of a silver/silver chloride pellet.

The patch-clamp amplifier may operate in two different modes, the voltage-clamp mode and the current-clamp mode. In the voltage-clamp mode, the amplifier compares the resting potential of a patched cell with the selected holding potential. Whenever a difference occurs, current is injected via the pipette electrode to compensate for this difference and to keep the cell at the desired holding potential. Consequently, this current represents the current flowing over the membrane of the cell and is recorded by the amplifier. In the current-clamp mode, the patched cell is at its physiological resting potential and no current is injected. Therefore, in this mode physiological activity patterns may be recorded as changes in the resting potential.

Signals were filtered at 1, 3 or 10 kHz and sampled at 6 or 20 kHz. The exact technical devices and software used in the present study are listed in section 3.1.3.

4.1.3 Patch-clamp technique

Electrophysiological measurements were obtained by applying the patch-clamp technique developed by Erwin Neher and Bert Sackmann (Neher and Sakmann, 1976). Experiments were performed in the conventional whole-cell configuration (Edwards et al., 1989) if not stated otherwise.

To do so, a brain section was transferred to the recording chamber of the electrophysiological setup and fixed in place with a u-shaped platinum wire stringed with nylon threads. The chamber was constantly perfused (1-2 ml/min) with gassed extracellular solution at room temperature. The recording pipette was filled with pipette solution and a constant overpressure was applied via a tubing system. This caused a permanent outflow of pipette solution that prevented plugging of the pipette tip by tissue fragments. In the voltage-clamp mode, a depolarizing 10 mV step was applied via the pipette electrode to monitor changes in resistance. The pipette was then moved under optical control close to the cell membrane. Subsequently, the overpressure was released and mild negative pressure applied. This resulted in the formation of the cell-attached configuration characterized by a tight connection between cell membrane and pipette tip causing a strong increase in resistance ideally reaching more than 1 GΩ. During this process the cell was clamped to the holding potential of -80 mV (ACSF) or -70 mV (HEPES solution) if not stated otherwise. Capacitive artifacts caused by the glass pipette were compensated at this point. By applying brief pulses of negative pressure the small membrane patch under the pipette tip was ruptured resulting in a sudden drop in resistance. In this so called whole-cell configuration, currents over the entire cell membrane were measured in the voltage-clamp mode. By switching to the current-clamp mode, the resting potential was determined. Whole-cell current patterns in response to de- and hyperpolarizing voltage steps were offline compensated for capacitive artefacts by using IGOR Pro macros custom-written by Ronald Jabs.

4.1.4 Calculation of passive membrane properties

When performing electrophysiological recordings in the whole-cell configuration, the electrical properties of the cell may be calculated by considering the following assumptions. The cell membrane consists of a lipid bilayer with embedded proteins which separates extracellular and intracellular solutions containing different ion concentrations. Therefore, the cell membrane constitutes a capacitor that stores charge. As the cell membrane also contains ion channels which facilitate the transfer of charged ions over the membrane, it also possesses properties of a resistor. Consequently, the cell represents an electrical circuit where a capacitor (with the membrane capacitance C_m) is connected in parallel with a resistor (with the membrane resistance R_m). In the voltage-clamp whole-cell configuration, this electrical circuit is in series with the pipette electrode via which the cell is clamped to a certain holding potential. In this case further resistances occur at the transition between pipette tip and cell interior that depend on the access to the cell interior, differences in composition of pipette solution and cytoplasm and diffusion parameters. These resistances are summarized as the series resistance (R_s), as they are in series with the electrical circuit of the cell (Pusch and Neher, 1988). Thus, a whole-cell patch-clamp recording is equivalent to an electrical circuit as depicted in Fig. 4.1.

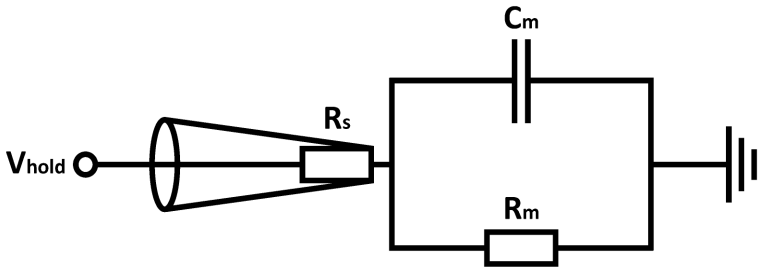

Fig. 4.1 Electrical circuit representing the whole-cell configuration. The R_s occurs at the transition between pipette and cell and is in series with C_m and R_m of the cell. V_{hold} = holding potential.

The amount of charge (Q) stored by a capacitor increases with its size. Accordingly, C_m is proportional to the membrane surface area and may be considered as an indirect measure of cell size. The R_m is also characteristic for a cell as it directly depends on the type and amount of open ion channels present in the cell membrane at rest. The fact that the R_s is in series with R_m and C_m implicates that R_s induces voltage errors as part of the voltage drops at

this resistance. Therefore, the R_s was constantly monitored and cells were discarded when R_s exceeded 40 MΩ.

To calculate the R_s and the passive membrane properties (R_m, C_m) of a patched cell, the following procedure was performed. In the voltage-clamp whole-cell configuration, 10 de-polarizing steps of 10 mV each were elicited (ΔV = 10 mV) for 50 ms and averaged (Fig. 4.2). Neglecting the very first rising phase of the current that mainly depends on the sampling rate, at the onset of ΔV at t_0, the measured current (I_{t0}) is only limited by R_s since C_m short-circuited R_m. Accordingly, R_s may be calculated by Ohm's law (equation 1) (Fig. 4.2).

$$R_s = \frac{\Delta V}{I_{t0}} \qquad (1)$$

Each change in voltage (ΔV) causes a recharging of the capacitor. During this recharging the current I_{t0} decreases and finally reaches a steady state I_{t1}. At this time point, the capacitor is fully recharged and has an infinite ohmic resistance. Therefore, the remaining current I_{t1} only depends on R_s + R_m. By using the R_s determined by equation 1, the R_m may be calculated (equation 2) (Fig. 4.2).

$$R_m = \frac{\Delta V}{I_{t1}} - R_s \qquad (2)$$

The current response to ΔV between t0 and t1 may be described by the function I(t) given in equation 3. As mentioned before, this current response is composed of the capacitive current due to the recharging of the capacitor and the steady-state ionic current determined by R_s + R_m (I_{t1}). The area under the function I(t) between t0 and t1, subtracted by the area I_{t1} * dt represents the change of charge (ΔQ, gray area in Fig. 4.2) of the capacitor in response to ΔV. As ΔQ is directly proportional to ΔV, C_m is defined by equation 4. Considering these assumptions, ΔQ was determined by calculating the integral of I(t) minus the integral of I_{t1} * dt between t0 and t1 according to equation 5 (gray area in Fig. 4.2). ΔQ was then entered in equation 4 to determine C_m.

$$I(t) = I * e^{-\frac{t}{\tau}} + I_{t1} \quad (3)$$

$$C_m = \frac{\Delta Q}{\Delta \mathrm{V}} \quad (4)$$

$$\Delta Q = \int_{t0}^{t1} I(t)dt - \int_{t0}^{t1} I_{t1}dt \quad (5)$$

The protocol applied to calculate the passive membrane properties (10 x ΔV(10 mV) for 50 ms each) was recorded in the cell-attached and the whole-cell configuration. The capacitive transients measured in the cell-attached configuration almost exclusively arise from properties of the pipette. To exclude these during the calculation, the protocol measured in cell-attached configuration was subtracted from the one measured in whole-cell configuration. The resulting trace was used for the above mentioned analysis (Fig. 4.2). All calculations were performed by using IGOR Pro macros custom-written by Ronald Jabs.

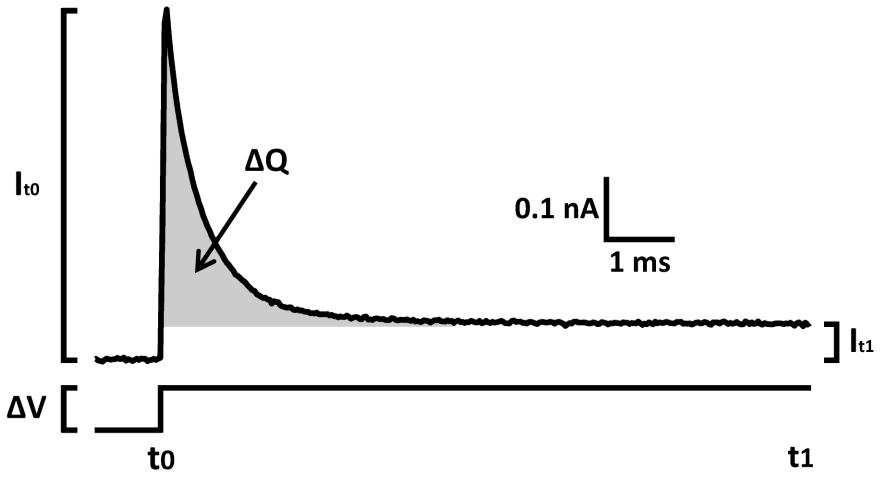

Fig. 4.2 Determination of R_s and passive membrane properties. Example current response of an NG2 cell to a depolarizing voltage step (ΔV = 10 mV, holding potential = -80 mV). Average of 10 successive steps in the whole-cell configuration is shown. The same protocol in cell-attached configuration was subtracted to exclude capacitive currents arising from the pipette. R_s is determined from the current at t0 (I_{t0}; equation 1). R_m is determined from the current at t1 (I_{t1}; equation 2). C_m is determined by calculating ΔQ, the integral of the capacitive current (gray area; equation 3, 4). For the present example the following values were calculated: R_s = 18.2 MΩ, R_m = 166 MΩ, C_m = 36.3 pF.

4.1.5 Liquid junction potential

A liquid junction potential occurs at the interface of two solutions with different ion concentrations. In electrophysiological experiments the extracellular bath solution and the intracellular pipette solution usually differ in their ingredients. This results in a liquid junction potential at the pipette tip when the pipette is lowered into the bath solution. Before establishing the cell-attached configuration, any potential occurring between the electrodes including the liquid junction potential is compensated for. However, in the whole-cell configuration the pipette content diffuses into the cell and the liquid junction potential disappears as no direct interface between the two solutions exists anymore. The voltage, however, is still compensated for the liquid junction potential resulting in a shift of the holding potential by the value of the liquid junction potential which needs to be adequately corrected.

The magnitude of the liquid junction potential is determined by the mobility of the different ions which depends on their weight and charge. In the present study the liquid junction potential for the various solutions was calculated by using the liquid junction potential calculator in the Patcher's Power Tools of IGOR Pro.

A considerable liquid junction potential occurred in experiments using ACSF with pipette solution 2 (6 mV; section 5.3.1, 5.3.4, 5.3.5, 5.3.6) and in experiments using blocking solution and pipette solution 4 (13 mV; section 5.1.4). The liquid junction potential was offline compensated for.

4.1.6 Perforated patch configuration

In the whole-cell configuration the direct connection between pipette and cytoplasm results in an exchange of the cell interior with the pipette solution. Therefore, no information about the intrinsic ion concentration is available in this patch-clamp configuration. To circumvent this problem, the perforated patch configuration is utilized. In this case, a substance is added to the pipette solution that forms pores in the cell membrane that are selectively permeable only for certain ions besides the one of interest. In this way currents may be measured in the cell-attached configuration without interfering with the intracellular ion concentration. In the present study, the intrinsic Cl^- concentration of NG2 cells was investigated (section 5.1.4). Therefore, the antibiotic gramicidin-A was added to the pipette solution which has

been shown to form pores selectively permeable for cations only, leaving the intrinsic Cl^- concentration intact (Kyrozis and Reichling, 1995).

To enable formation of a high resistance cell-attached configuration, the pipette tip was filled with gramicidin-A-free pipette solution 4. It was then filled up with pipette solution 4 containing 50 µg/ml gramicidin-A yielding a final gramicidin-A concentration of 20-40 µg/ml. Gramicidin-A containing pipette solution was freshly prepared on the day of experimentation.

The cell-attached configuration was established as described for the whole-cell configuration (section 4.1.3). The incorporation of gramicidin-A is a slow process that may be monitored by a decrease of the series resistance (Fig. 4.3). To do so, depolarizing 10 mV steps were elicited and the series resistance was calculated as described in section 4.1.4. The data were imported to the program IGOR Pro and automatically analyzed with custom-written macros by Ronald Jabs. When the series resistance reached a stable plateau around 100 MΩ, the experiment was started (Fig. 4.3).

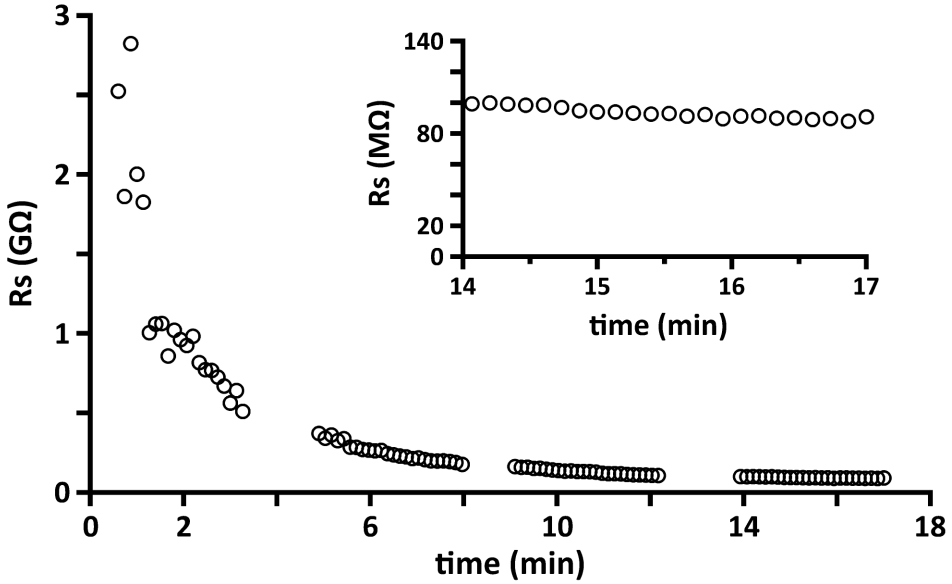

Fig. 4.3 Monitoring of the series resistance while establishing the perforated patch configuration. After formation of the cell-attached configuration, depolarizing 10 mV steps were elicited to calculate the series resistance (R_s). The experiment was started when the series resistance reached stable values around 100 MΩ (inset).

4.1.7 Application techniques

In the present study two different application techniques were used. These differ particularly in speed of application which is a critical parameter especially when studying receptor currents.

4.1.7.1 Bath application

In case speed of application is negligible, substances were added to the extracellular solution and applied via the bath perfusion system. This allows a uniform distribution of a defined concentration of the desired substance throughout the brain slice. However, spreading of the substance is a variable process especially depending on the flowing properties inside the recording chamber that may vary between recordings. Therefore, bath application is not suitable to compare receptor properties in response to a defined agonist concentration. For this purpose a faster application technique was used.

4.1.7.2 Focal pressure application

To facilitate a fast and local application of substances, the OctaFlow pressure application system was used. This system is composed of 8-12 pressure controlled application channels that lead via a Teflon tubing system to a combined small reservoir with a fine quartz pipette output (diameter 100 µm). The reservoir is further connected to a gravitational-driven flush channel that constantly rinses extracellular solution into the reservoir. In this way leaking of substances from the application channels is prevented. Whenever application from a certain channel is induced, the flush channel is stopped enabling the fast and local application of a defined concentration of the desired substance. Different application protocols as well as the application pressure can be adjusted via the OctaFlow Software. Application protocols were controlled by a trigger set by the electrophysiology recording software thus enabling time correlated recordings of substance application and cellular response. To keep the pH in the application channels constant, a 5% CO_2/95% O_2 gas mixture was used as a pressure source for experiments using ACSF-based extracellular solution. In case HEPES-based solutions were used, pure oxygen was employed.

After selecting a cell for the recording, the OctaFlow application pipette was positioned close to the cell immediately above the slice. Before applying a certain substance, normal bath

solution was applied from another channel using the same pressure settings to test for potential pressure artifacts.

4.1.8 Extracellular stimulation of presynaptic neurons

In order to record evoked PSCs (ePSCs) in NG2 cells, extracellular stimulation of glutamatergic neuronal fiber tracts (Schaffer collaterals) (section 5.3.2, 5.3.3) or GABAergic local interneurons (section 5.1.3) was performed. A chlorinated silver electrode in a glass pipette filled with ACSF served as a monopolar stimulation electrode. Pipettes used for stimulation generally had resistances below 1 MΩ. Biphasic stimulation pulses with a duration of 100-150 µs were generated by an isolated pulse stimulator in the constant voltage mode.

Experiments were performed as follows. An NG2 cell of the hippocampal CA1 region (stratum radiatum) was held in the voltage-clamp whole-cell configuration and the current was monitored. The stimulation pipette was moved under optical control while test pulses were applied. When an innervating fiber was detected the stimulation intensity was adjusted to obtain monosynaptic minimal stimulation characterized by ePSCs with a short delay (few ms) after stimulation as well as so called 'failures' where stimulation did not elicit transmitter release. Single stimulation pulses were applied with an inter-stimulus interval of 15 s.

In some experiments investigating short-term synaptic plasticity, paired-pulse stimulation was employed. In this case two immediate pulses with an inter-stimulus interval of 20 ms were applied every 15 s and the PSC responses were recorded.

4.1.9 Analysis of PSCs

4.1.9.1 GABAergic PSCs

For the analysis of GABAergic ePSCs in hippocampal NG2 cells (section 5.1.3), near-field stimulation of local interneurons was performed as described in section 4.1.8. Experiments were conducted in ACSF containing the AMPA/kainate receptor antagonist NBQX (5 µM) to isolate $GABA_A$ receptor-mediated PSCs. To investigate the effect of modulatory substances on ePSC amplitudes, the modulators were locally applied via focal pressure application

(section 4.1.7.2) after recording control responses in the absence of the modulator. Each stimulation pulse was visually inspected and classified as failure or ePSC. All ePSCs were then averaged and the amplitude (I_{amp}) was determined as depicted in Fig. 4.4. Modulatory effects of the different substances on ePSCs were normalized to the control amplitude before applying the modulator.

Electrophysiological data was imported to and analyzed with IGOR Pro software.

4.1.9.2 Glutamatergic PSCs

For the analysis of glutamatergic ePSCs in hippocampal NG2 cells (section 5.3.2, 5.3.3), stimulation of Schaffer collaterals was performed as described in section 4.1.8. Moreover, miniature PSCs (mPSCs) were analyzed in the presence of TTX (1 µM) to block action potential-driven transmitter release. To increase the presynaptic transmitter release, the Ca^{2+} ionophore ionomycin (3 µM) was locally applied by focal pressure application (section 4.1.7.2) in some experiments. Ionomycin has been shown to increase the mPSC frequency without affecting the amplitude (Capogna et al., 1996). All experiments were performed in ACSF containing the $GABA_A$ receptor blocker picrotoxin (150 µM) to isolate AMPA receptor-mediated PSCs.

For the analysis of ePSCs, each stimulation pulse was visually inspected and classified as failure or ePSC. Kinetic properties were calculated for each single event and averaged per cell. mPSCs were identified by using the template search of pClamp 10. After transferring the data to IGOR Pro, each detected event was visually inspected and discarded in case of any doubt. Furthermore, all events with an amplitude < 5 pA were rejected. All mPSCs were averaged per cell to determine kinetic properties and average amplitudes. Amplitudes of all events per cell were used for cumulative probability distributions. All single distributions per genotype were averaged and used for comparison between genotypes.

The amplitude (I_{amp}) was calculated as the difference between the maximal peak of the PSC and the average baseline current directly preceding the PSC (Fig. 4.4). To compare the kinetic properties of PSCs, rise time and decay time were determined. For the rise time the 20-to-80% value was analyzed, which corresponds to the time between 20 and 80% of the maximal amplitude of the PSC. The 20-to-80% rise time was used to exclude the non-linear

rising phases at the beginning and at the maximum amplitude of the PSC. The decay time is given as the decay time constant (τ) of a monoexponential fit of the PSC decay (Fig. 4.4).

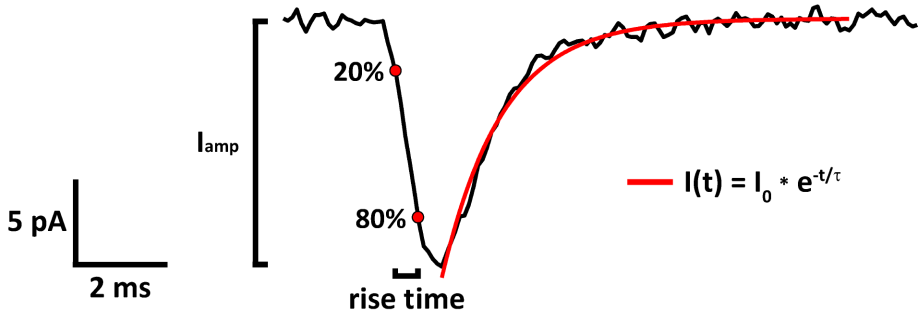

Fig. 4.4 Analysis of the amplitude and kinetic properties of PSCs. An exemplary averaged glutamatergic PSC is shown. The amplitude (I_{amp}) is given as the maximal peak of the PSC. The rise time was defined as the time between 20% and 80% of I_{amp} of the PSC. The decay time is given as the time constant (τ) of a monoexponential fit of the PSC decay (red). For this example the following values were calculated: rise time = 0.55 ms, decay time = 1.33 ms.

In experiments investigating short-term synaptic plasticity, paired-pulse stimulation was performed as described in section 4.1.8. All pairs of pulses including failures were averaged and the paired-pulse ratio (PPR) was calculated by dividing the average amplitude on the second pulse (I_{amp2}) by the one on the first pulse (I_{amp1}) (equation 6).

$$PPR = \frac{I_{amp2}}{I_{amp1}} \qquad (6)$$

A PPR > 1 indicates paired-pulse facilitation while a PPR < 1 is considered as paired-pulse depression.

Analysis of amplitudes, kinetic properties and PPRs of PSCs was carried out with IGOR Pro macros custom-written by Annika Wefers and Ronald Jabs.

4.2 Single-cell RT-PCR

4.2.1 Cell harvesting

Single cells were electrophysiologically characterized in the whole-cell configuration (section 4.1.3). Subsequently, the cell was carefully lifted above the slice and soaked into the pipette. It was then transferred to an eppendorf tube filled with 3 µl diethyl pyrocarbonate (DEPC)-treated water and rapidly frozen in liquid nitrogen. The samples were then stored at -20°C or -80°C until further use.

4.2.2 RT-PCR

RT-PCR was performed by Gerald Seifert and staff members.

As a first step, the reverse transcription (RT) was conducted. The eppendorf tube containing 3 µl DEPC-treated water and ~1.5 µl pipette solution including the harvested cell was thawed on ice. The RT reaction mixture (Tab. 4.2) was added to the sample and RT was executed for 1 h at 37°C. As a positive control, the same reaction was performed with 2 ng RNA isolated from whole mouse brain. Omission of the RT enzyme and substitution of template by bath solution served as negative controls.

Tab. 4.2 Reaction mixture for RT.

quantity	substance	final concentration
~4.5 µl	DEPC water + cytoplasm	
2 µl	5 x First-Strand Buffer	50 mM Tris-Cl, 75 mM KCl, 3 mM $MgCl_2$
1 µl	DTT	10 mM
1 µl	dNTPs	4 x 250 µM
0.5 µl	Random Hexamer Primer	50 µM
0.5 µl	RNasin® RNase Inhibitor	20 U
0.5 µl	Superscript® III Reverse Transcriptase	100 U

Following RT, a multiplex two-round PCR was performed. In the first round, primers were used that recognize all subunits of the respective $GABA_A$ receptor family (α, β, γ, and δ; section 5.1.1.1 and 5.2) or primers specific for the different neuroligins (1-3; section 5.3.7), respectively. In addition, primers for the NG2 cell-specific PDGFα receptor mRNA were

added to the reaction. They served as a positive control for successful cell harvesting and as a cell type-specific marker. All primer sequences are given in Tab. 4.7.

The total RT reaction (Tab. 4.2) served as a template for the first PCR. The exact composition of the reaction mixture for the first PCR is given in Tab. 4.3, the PCR program is given in Tab. 4.4.

Tab. 4.3 Reaction mixture for first PCR.

quantity	substance	final concentration
10 µl	RT reaction (Tab. 4.2)	
5 µl	PCR-Buffer	20 mM Tris-Cl, 50 mM KCl
2.5 µl	$MgCl_2$	2.5 mM
1 µl	sense (se) Primer	200 nmol
1 µl	antisense (as) Primer	200 nmol
0.5 µl	se PDGFα receptor Primer	100 nmol
0.5 µl	as PDGFα receptor Primer	100 nmol
0.75 µl	Taq DNA Polymerase	3.5 U
Add 40 µl	dH_2O	

Tab. 4.4 PCR program for first PCR.

cycles	temperature	time
1	94°C	4 min
5	94°C	25 s
	49°C	2 min
	72°C	25 s
40	94°C	25 s
	49°C	45 s
	72°C	25 s
1	72°C	7 min

In the second round, nested primers specific for the different $GABA_A$ receptor subunits of each family or for the different neuroligins were employed, respectively. 2 µl of the first PCR reaction served as a template for the second reaction. The exact composition of the reaction mixture for the second PCR is given in Tab. 4.5, the PCR program is given in Tab. 4.6.

Tab. 4.5 Reaction mixture for second PCR.

quantity	substance	final concentration
2 µl	first PCR reaction (Tab. 4.3)	
5 µl	PCR-Buffer	20 mM Tris-Cl, 50 mM KCl
2.5 µl	$MgCl_2$	2.5 mM
1 µl	dNTPs	4 x 50 µM
1 µl	se primer (nested)	200 nmol
1 µl	as primer (nested)	200 nmol
0.5 µl	Platinum® Taq DNA Polymerase	2.5 U
Add 40 µl	dH_2O	

Tab. 4.6 PCR program for second PCR.

cycles	temperature	time
1	94°C	4 min
5	94°C	25 s
	54°C	2 min
	72°C	25 s
30	94°C	25 s
	54°C	45 s
	72°C	25 s
1	72°C	7 min

The $GABA_A$ receptor subunits β2 and β3 display a high degree of sequence identity and may not be distinguished by specific primers. Therefore, the corresponding PCR product from the second PCR using the nested primer β2/3 was purified and an endonuclease restriction analysis was performed to unambiguously identify the subunits β2 and β3. The purified PCR product was digested with the restriction endonuclease *Pst*I which cuts the subunit β2 in a 202 bp and a 105 bp fragment and with *Ban*I which cuts the β3 subunit in a 169 bp and a 138 bp fragment. The reaction mixture contained 7 µl of the purified PCR product and 10 U of the respective enzyme (final volume: 15 µl). Reaction was carried out for 6 h at 37°C.

PCR products and digested PCR products (for subunits β2/3) were analyzed by agarose gel-electrophoresis. 10 µl from each reaction were mixed with 2 µl loading buffer and loaded onto a 1.5% agarose gel containing 0.013% ethidium bromide. ϕX174 *Hinc*II digest or Low Molecular Weight DNA Ladder served as size markers.

The primers for each transcript are located on different exons and span introns to prevent amplification of genomic DNA. To test for specificity of the primers and to optimize the reaction conditions, a two-round RT-PCR was performed with each primer set with 2 ng of whole mouse brain RNA as a template.

Tab. 4.7 Primers for single-cell RT-PCR. The sequence, position and product size of each primer pair is indicated. Position 1 was defined as the first nucleotide of the translation initiation codon for each transcript. The primer pairs for alpha, beta and gamma recognize all subunits of the respective $GABA_A$ receptor family. For the subunits α2, α3 and α4 a common 'as' primer was used, for γ2 and γ3 a common 'se' primer was used. Neuroligin-1 primer flanked splice site A resulting in two discrete PCR-products.

gene	primer sequence	position	product size
Alpha (Berger et al., 1998)	se 5'-TGGACTCCTGATACNTTYTT as 5'-GCHATRAACCARTCCATGGC	361, 364, 439, 382, 385 931, 934, 1009, 952, 955	590 bp
Alpha	se 5'-AACATGACMAYGCCMAAYAAGCT as 5'-GCATARCAGACAGCWATRAACCA	409, 412, 487, 430, 433 940, 943, 1018, 961, 964	554 bp
a1 (nested)	se 5'-CCAGCCCGTTCAGTGGTTGTA as 5'-GCACGGCAGATATGTTTGAAT	601 760	180 bp
a2 (nested)	se 5'-TTACAATGCTTCTGACTCCGTTCA as 5'-CGRGCACTGATRCRWARGGT	597 883	306 bp
a3 (nested)	se 5'-CTTGGGAAGAACAAATCTGTGGA as 5'-CGRGCACTGATRCRWARGGT	673 958	305 bp
a4 (nested)	se 5'-ACCAAAGGCCCTGAGAAGTCA as 5'-CGRGCACTGATRCRWARGGT	613 901	308 bp
a5 (nested)	se 5'-GCTGGAGGATGATGGCACACTTCT as 5'-GTTGAGCCTGGAGCCATCTTCTG	462 647	208 bp
Beta (Berger et al., 1998)	se 5'-CTGGATGARCAAAACTGYAC as 5'-ACAAAGACAAARCAWCCCAT	508, 505, 508 931, 928, 931	443 bp
b1 (nested)	se 5'-ATGGAGGAGAGGGAGCAGTAACT as 5'-CAGCCCATGAGATAGATGTCAATC	581 915	358 bp
b2/3 (nested)	se 5'-GGCGYGGCGRTGACAAKGC as 5'-TCCCGRAGGTGRGTGTTGAT	575, 578 862, 865	307 bp

Gamma (Berger et al., 1998)	se 5'-TAGACAGCAAYATGGTGGG as 5'-TTGATCCAAAADGACACCCAGG	404, 407, 353 863, 866, 812	481 bp
Gamma	se 5'-ATTTGGATTCCAGACACYWTCTT as 5'-AAGTAGCCCATTCTTCKRCTCAG	427, 430, 376 796, 799, 745	392 bp
g1 (nested)	se 5'-CGCCTGCTGCGGATTTG as 5'-CACAGAGGGCTTTTTCCACTTGT	496 653	180 bp
g2 (nested)	se 5'-AAAAMRGCTGAGGCTCACTGGAT as 5'-AACTGCGCTTCCATTGATAAACA	463 651	211 bp
g3 (nested)	se 5'-AAAAMRGCTGAGGCTCACTGGAT as 5'-CTGAGGCCCATGAAGTCAAACTGA	409 657	272 bp
Delta	se 5′-ATGGCGCCAGGGCAATGAATG as 5′-GTGGAGGTGATGCGGATGCTGTAT	59 459	424 bp
δ (nested)	se 5′-TATGCCCGAAACTTCCGACCAG as 5′-AAAATCACCCCATCAGGCTGTAGG	151 435	308 bp
Neuroligin-1	se 5'-GACATCCGGAACGCCACTCA as 5'-TTGCCAAGACACTCCCATCATACA	316 632, 572	340 bp 280 bp
Neuroligin-1 (nested)	se 5'-CAGAATATCATTGATGGCAGATTG as 5'-ACTCCCATCATACAGATTTCCAGT	355 622, 562	291 bp 231 bp
Neuroligin-2	se 5'-ACCGCCAAGCTGCATGCCGACTAC as 5'-GCACGCGTGGTTTCAAGCCTATGT	1399 1745	370 bp
Neuroligin-2 (nested)	se 5'-GCAGAGGGCCGGCCAGAGTG as 5'-AGCGGTTGGGCTTGGTGTGGAT	1465 1672	229 bp
Neuroligin-3	se 5'-AAGATGGATCCGGCGCTAAGAAAC as 5'-CCGATGCCAGAGCCAAAGACAGTA	443 777	358 bp
Neuroligin-3 (nested)	se 5'-AATGACGGGGATGAAGATGAAGAC as 5'-GCGAAGGGCCTGGATTTGA	487 711	243 bp
PDGFa receptor	se 5'-TCAAAGGGAGGACGTTCAAGACC as 5'-GACGGGCAGCACATTCATACTC	578 859	303 bp
PDGFa receptor (nested)	se 5'-TGTGTATAAGGCAGGAGAAACGAT as 5'-TGGGGACGGTCAAAGTGTA	669 817	167 bp

4.3 Statistics

In section 5.1, differences between data sets were tested for significance by using the Student's t-test or the paired Student's t-test. The Pearson's χ^2-test was used to test for correlation between data sets (section 5.1.2). Data in bar graphs are represented as mean ± SD except for PCR results where the relative frequency is given.

In section 5.3, the following statistical analysis was performed. Each data set was tested for normal (Gaussian) distribution by using the Shapiro-Wilk-test. For clarity, Gaussian distributed data in this section is depicted as bar graphs and non-Gaussian distributed data as box plots. In case of Gaussian distribution, any data point outside two times the SD was excluded. Gaussian distributed data were tested for equal variances by applying the Levene-test that was followed by ANOVA and post-hoc Tukey-test without (test #1) or with Welch-correction (test #2) for equal or diverse variances, respectively. Non-Gaussian distributed data were tested either with Kruskal-Wallis-ANOVA with sequential Bonferroni's correction for more than two groups (test #3), or with the Mann-Whitney-U-test in case of two groups (test #4). Cumulative probability density functions were tested with the Kolmogorov-Smirnov-test (test #5). The Pearson's χ^2-test was used to compare relative frequencies (test #6). Data in bar graphs are represented as mean ± SD except for PCR results where the relative frequency is given. Box plots indicate median with lower quartile (25) and upper quartile (75), whiskers denote quartile 5 and 95. The number of the test applied is given in the respective figure legends.

The significance level was always set to $p < 0.05$. Statistical analysis was carried out with IGOR Pro, Origin Pro and Excel 2010.

5 Results

5.1 Functional characterization of $GABA_A$ receptors in hippocampal NG2 cells

NG2 cells express functional AMPA and $GABA_A$ receptors and receive direct synaptic input from neurons in different brain regions. While most studies focused on the investigation of AMPA receptors, much less is known about the physiology of $GABA_A$ receptors in NG2 cells (Bergles et al., 2010).

In this part of present study, molecular and functional techniques were combined to elucidate the subunit composition and functional properties of $GABA_A$ receptors in NG2 cells. For all experiments NG2 cells of the hippocampal CA1 region from juvenile (p8-12) transgenic hGFAP-EGFP mice (Nolte et al., 2001) were analyzed. In this mouse line, NG2 cells are labeled by weak fluorescence as distinct from the brightly fluorescent astrocytes (section 3.1.1) (Matthias et al., 2003).

5.1.1 Molecular and functional analysis of the $GABA_A$ receptor subunit composition in NG2 cells

The subunit composition of $GABA_A$ receptors in NG2 cells was investigated by two independent approaches. Initially, the expression was analyzed at the mRNA level using single-cell RT-PCR. This data was then complemented by pharmacological modulation of functional $GABA_A$ receptors *in situ*. The experiments focused on the identification of the main $GABA_A$ receptor subunits expressed in the hippocampus at the age of p8-12, i.e. α1-5, β1-3 and γ1-3 (Laurie et al., 1992).

5.1.1.1 Molecular analysis of $GABA_A$ receptor subunit expression by single-cell RT-PCR

In order to identify the $GABA_A$ receptor subunits expressed by individual NG2 cells, single-cell RT-PCR was performed. To do so, whole-cell patch-clamp recordings of weakly EGFP-positive presumable NG2 cells were conducted (Matthias et al., 2003). Membrane currents in response to de- and hyperpolarizing voltage steps were elicited to confirm cell identity (Fig. 5.1), as NG2 cells display the characteristic complex current pattern clearly distinct from the passive current pattern of astrocytes (section 3.1.1) (Steinhäuser et al., 1994a; Steinhäuser et al., 1994b; Matthias et al., 2003). Subsequently, the cytoplasm was harvested and a two-round RT-PCR was performed. In the first round, primer sets were used that recognize all subunits of the respective $GABA_A$ receptor family (α, β, and γ) together with PDGFα receptor primers. Subunit-specific nested primers utilized in the second round allowed precise determination of the subunits expressed. To distinguish β2- from β3-transcripts, restriction analysis of purified PCR-products was performed. PDGFα receptor expression served as a positive control and cell type-specific marker. Cells negative for PDGFα receptor mRNA were excluded (detailed procedure in section 4.2).

Analysis of the α-subunits revealed that α1 and α2 were most abundant (70 and 78%, respectively; Fig. 5.1A, C), while α3, α4 and α5 were less frequent (30, 35 and 15%, respectively, n = 46; Fig. 5.1A, C). Among the β-subunits, β3 was expressed by virtually all NG2 cells (95%), while β1 and β2 were found in 50 and 64%, respectively (n = 26; Fig. 5.1C). All three β-subunits were expressed by 32% of all cells (n = 26). Regarding γ-subunits, γ1 and γ2 were frequently expressed (77 and 64%, respectively, n = 47; Fig. 5.1B, C). γ3 mRNA was detected in 53% of all NG2 cells tested (n = 47; Fig. 5.1B, C). Coexpression of γ1 and γ2 was found in 45% of cells while 28% expressed all three γ-subunits (n = 47).

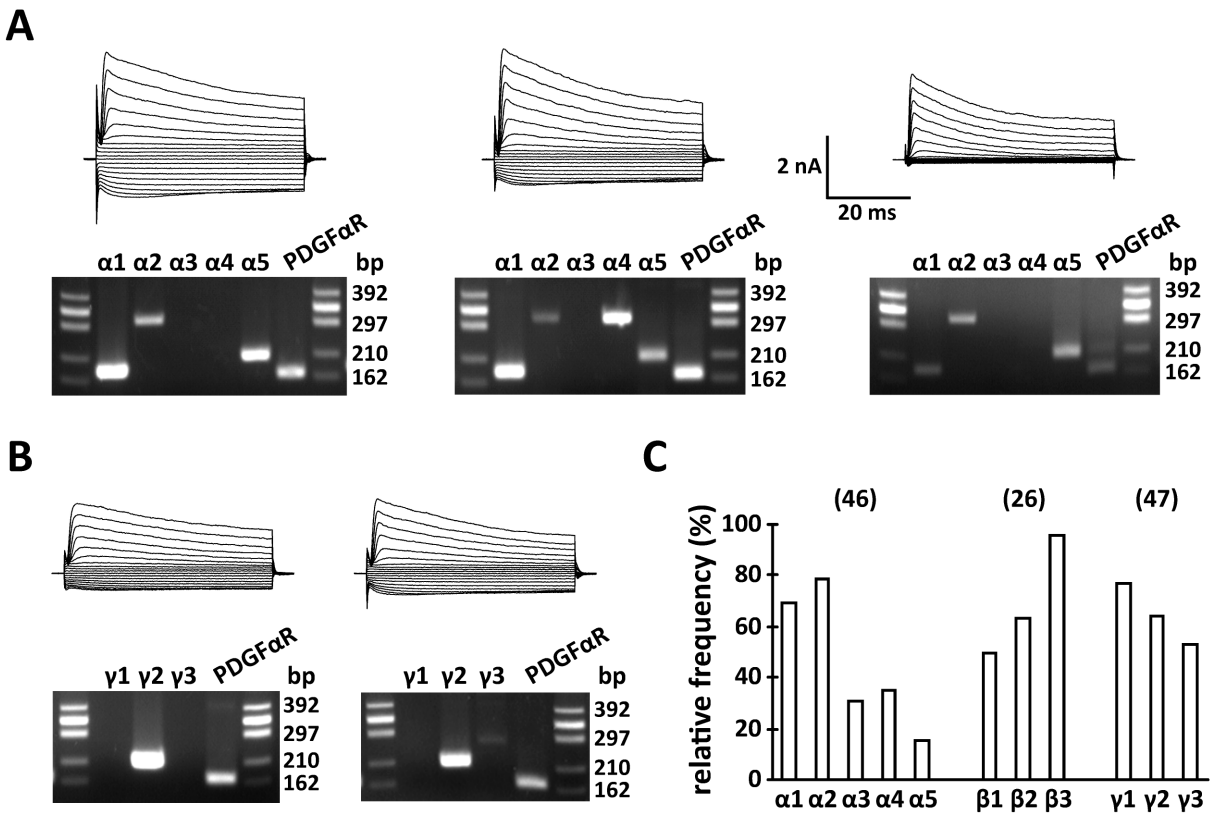

Fig. 5.1 Expression analysis of $GABA_A$ receptor subunits in NG2 cells by single-cell RT-PCR. (A, B) Agarose gels of α- and γ-subunit PCR-products of individual NG2 cells together with their respective current responses to de- and hyperpolarization between -160 and +20 mV (holding potential -80 mV). Expression of PDGFα receptor (PDGFαR) mRNA served as a positive control and cell type-specific marker. ϕX174 *Hinc*II digest served as size marker. Recordings were performed using extracellular HEPES solution (section 3.4.2) and pipette solution 1 (section 3.4.3). **(C)** Summary bar graph of the relative frequency of $GABA_A$ receptor subunit expression of all NG2 cells tested. Cell numbers are given in parentheses. See also Passlick et al. (2013).

During early development NG2 cells display increased proliferation and differentiation compared to later developmental stages (Kang et al., 2010). As GFP is a stable protein with a half-life of approximately 26 hours (Corish and Tyler-Smith, 1999), GFP fluorescence might still be present when NG2 cells already initiated differentiation. As a consequence, the variability in expression of $GABA_A$ receptor subunits found in this study might reflect NG2 cells at different stages of differentiation. It is known that during differentiation to oligodendrocytes, NG2 cells considerably change their membrane properties, such as membrane resistance and membrane capacitance (De Biase et al., 2010). To ensure that the results were not influenced by differentiation, subunit expression was compared with the membrane properties of the corresponding cells. Membrane resistance and membrane

capacitance were indistinguishable between subunit-expressing and -non-expressing cells indicating no contamination of the results by different stages of differentiation of the cells investigated (Tab. 5.1).

Tab. 5.1 Correlation between $GABA_A$ receptor subunit expression and membrane properties. No differences between subunit-expressing and -non-expressing cells regarding membrane resistance (R_m) or membrane capacitance (C_m) were detected. Data is expressed as mean +/- SD.

subunit	expression	R_m (MΩ)	C_m (pF)
α1	+	271.3 ± 313.1	27.7 ± 7.3
	-	215.5 ± 138	29.1 ± 7.8
α2	+	277.9 ± 329.9	29.8 ± 8
	-	229.1 ± 196.4	27.6 ± 7.2
γ2	+	197.8 ± 120.4	28.8 ± 8.3
	-	231.5 ± 251.3	29.7 ± 7.3

Taken together, the molecular analysis suggested that juvenile hippocampal NG2 cells predominantly express the $GABA_A$ receptor subunits α1, α2, β2, β3, γ1 and γ2. However, a considerable heterogeneity was detected among the juvenile NG2 cell population.

5.1.1.2 Functional analysis of $GABA_A$ receptor subunit composition by pharmacological modulation

Besides the two active binding sites for its endogenous ligand GABA, $GABA_A$ receptors contain several allosteric binding sites for different modulators and blockers, e.g. benzodiazepines, barbiturates and Zn^{2+}. As their occurrence depends on the subunit composition of the prevailing $GABA_A$ receptor, pharmacological modulation allows the functional identification of the $GABA_A$ receptor subunits expressed by individual cells (overview in section 1.5.2) (Hevers and Luddens, 1998; Mehta and Ticku, 1999).

In this study, focal pressure application of GABA with different modulators was used to investigate the subunit composition of functional $GABA_A$ receptors in hippocampal NG2 cells. The experiments were performed in a solution containing blockers for Na^+ and K^+ channels (blocking solution, section 3.4.2) to minimize secondary effects by the activation of other conductances that might influence the GABA-evoked responses. To estimate the modulatory effect of a certain compound, the following protocol was employed. Initially, 50 µM GABA

were applied for 2 s successively until a stable current response was reached. Next, the respective modulator was pre-applied for 30 s alone before coapplication of GABA with the modulator (2 s). Subsequently, GABA was applied without the modulator again to check for reversibility of the modulatory effect.

The barbiturate pentobarbital is a well characterized modulator of $GABA_A$ receptors that facilitates stronger binding of GABA to the receptor thereby causing longer opening of the receptor pore (Twyman et al., 1989; Hevers and Luddens, 1998). It binds to the β-subunit of $GABA_A$ receptors and potentiates currents of receptors containing any of the three existing β-subunits with the same efficiency (Hadingham et al., 1993). As the β-subunit is present in all functional $GABA_A$ receptors (Mehta and Ticku, 1999), modulation was expected in all cells tested. Indeed, application of 50 µM GABA with 50 µM pentobarbital reversibly enhanced the control GABA response in all cells tested (179 ± 46%, n = 9; Fig. 5.2), confirming ubiquitous expression of β-subunit-containing $GABA_A$ receptors in NG2 cells. It was shown that besides a modulatory action, pentobarbital might also act as an agonist at $GABA_A$ receptors and induce currents in the absence of GABA when applied at high concentrations (Muroi et al., 2009). However, pre-application of 50 µM pentobarbital alone never induced a current in NG2 cells (data not shown).

The binding site for benzodiazepines is located at the interface of α- and γ-subunits. Several modulators have been characterized that specifically bind to this site and modulate the receptor response depending on the respective α- and γ-subunits (Hevers and Luddens, 1998). The benzodiazepine diazepam positively modulates $GABA_A$ receptors containing the α-subunits 1-3 and 5 and the γ-subunits 1-3 (Olsen and Sieghart, 2009). In the present study coapplication of GABA (50 µM) with diazepam (20 µM) potentiated the GABA response to 156 ± 35% (n = 12; Fig. 5.2) indicating frequent expression of the $GABA_A$ receptor subunits α1-3 and 5 and γ1-3. This effect was completely reversible.

Another modulator acting at the benzodiazepine binding site is the non-benzodiazepine zolpidem whose binding increases the receptor affinity for GABA (Biggio et al., 1989). Zolpidem displays a higher specificity for α- and γ-subunits compared to diazepam, as it only potentiates $GABA_A$ receptors containing the subunits α1-3 and γ2 (Pritchett and Seeburg, 1990; McKernan et al., 1991; Puia et al., 1991; Sanna et al., 2002). Especially regarding the

γ-subunits, zolpidem is highly specific for γ2 as γ1- and γ3-containing receptors were entirely insensitive (Wingrove et al., 1997). Coapplication of 50 µM GABA with 10 µM zolpidem increased the GABA response to 167 ± 49% (n = 10; Fig. 5.2). It was shown that reducing the concentration of zolpidem increases the specificity for the α1-subunit compared to α2 and α3 (Pritchett and Seeburg, 1990; McKernan et al., 1991). To test whether lower concentrations of zolpidem were still sufficient to modulate NG2 cell $GABA_A$ receptors, 50 µM GABA were coapplied with 1 µM zolpidem. At this concentration the GABA response was still potentiated to 145 ± 35% in 8/11 cells (Fig. 5.2) indicating expression of α1- and γ2-containing $GABA_A$ receptors in the majority of hippocampal NG2 cells.

The benzodiazepine binding site is also a target of the β-carboline DMCM. DMCM is an inverse agonist whose modulatory effect may be positive or negative depending on the subunit composition (Hevers and Luddens, 1998). GABA-evoked currents have been shown to increase when γ1- and/or β2/3-subunits are present, for all other combinations DMCM is inhibitory (Puia et al., 1991; Wafford et al., 1993; Stevenson et al., 1995). NG2 cells displayed a variable effect in response to coapplication of GABA (50 µM) with DMCM (10 µM). While in 7/14 cells an inhibition of the GABA-induced current to 81 ± 6% was observed (Fig. 5.2), 4/14 cells showed a potentiation to 113 ± 8% (Fig. 5.2). The remaining three cells were not influenced by DMCM. In contrast to the other modulators, the effect of DMCM was irreversible in the majority of cells tested. These data indicate that the expression of functional $GABA_A$ receptors containing γ1- and/or β2/3-subunits is variable among hippocampal NG2 cells.

In order to verify functional expression of specific β-subunits, the allosteric modulator loreclezole was applied. Loreclezole has been shown to specifically enhance $GABA_A$ receptor-mediated currents if the subunits β2/3 are present (Wafford et al., 1994; Wingrove et al., 1994). Coapplication of GABA (50 µM) with loreclezole (10 µM) increased the control GABA (50 µM)-evoked current in all cells tested to 145 ± 13% (n = 8; Fig. 5.2), indicating frequent expression of β2/3-subunit-containing $GABA_A$ receptors.

21 of the total 64 measurements included in this part of the study were measured by M. Grauer (Passlick et al., 2013).

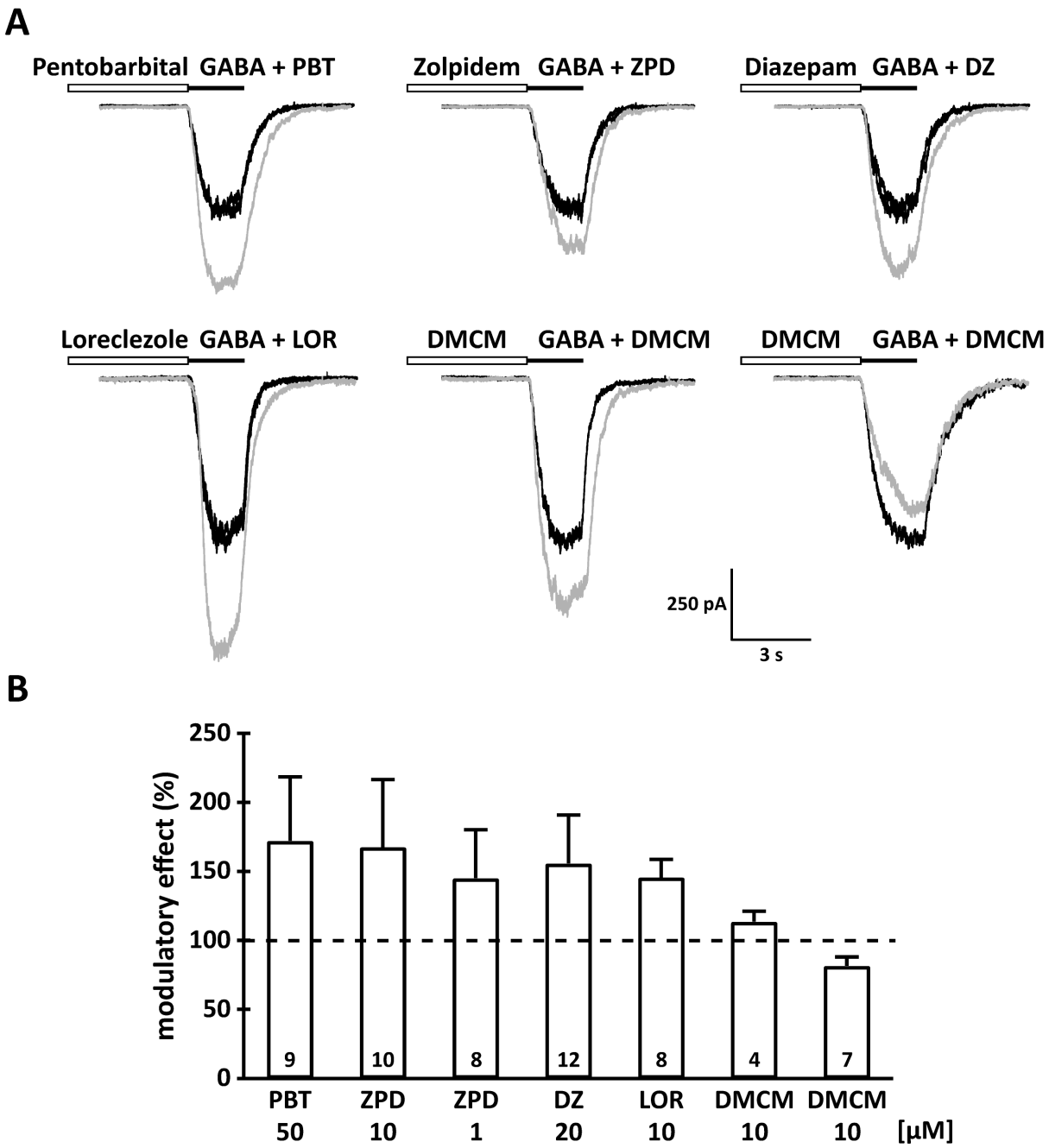

Fig. 5.2 Pharmacological modulation of $GABA_A$ receptor-mediated currents in NG2 cells. (A) Pre-application of receptor modulators (30 s) was followed by coapplication of GABA (50 µM) with the modulator (2 s). Gray traces represent responses upon coapplication, black traces give GABA responses before and after application of the modulator. The following concentrations were used: pentobarbital (PBT; 50 µM), zolpidem (ZPD; 1 µM), diazepam (DZ; 20 µM), loreclezole (LOR; 10 µM), DMCM (10 µM). Traces showing PBT, ZPD, DZ and LOR, DMCM (bottom center) were obtained from the same cell, respectively. Recordings were performed using extracellular blocking solution (section 3.4.2) and pipette solution 1 (section 3.4.3) **(B)** Summary bar graph of the modulatory effect of all modulators applied. Data were normalized to the GABA control response before application of the modulator. All modulators significantly affected the GABA responses. Cell numbers are indicated at the base of each bar. See also Passlick et al. (2013).

It was shown that metabotropic $GABA_B$ receptors may activate K^+ conductances (Sodickson and Bean, 1996). Furthermore, a recent study revealed that $GABA_B$ receptors are able to influence $GABA_A$ receptor-mediated currents in neurons in some brain regions (Tao et al., 2013). To ensure that the GABA-evoked currents and modulatory effects measured in this study are exclusively mediated by $GABA_A$ receptors, it was tested whether coapplication of $GABA_B$ receptor antagonists influences GABA-evoked currents in NG2 cells. Neither CGP 55845 (10 µM, n = 10) nor phaclofen (200 µM, n = 7) affected the current amplitude activated by GABA alone (to 94 ± 17% and 101 ± 10%, respectively; Fig. 5.3), indicating that GABA-evoked currents are probably not influenced by $GABA_B$ receptors in NG2 cells. Experiments were performed in HEPES solution (section 3.4.2) containing TTX (1 µM) to inhibit synaptic activity.

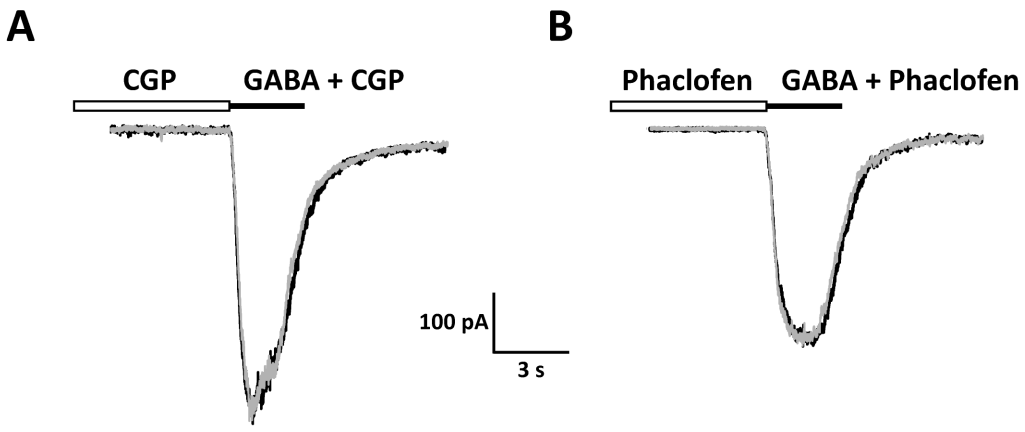

Fig. 5.3 GABA-evoked currents are not influenced by $GABA_B$ receptor activation. (A, B) Pre-application (30 s) of the $GABA_B$ receptor antagonists CGP 55845 (10 µM, A) or phaclofen (200 µM, B) was followed by coapplication of GABA (50 µM) with the antagonist (2 s). Gray traces represent responses upon coapplication, black traces depict GABA responses before application of the modulator. Recordings were performed using extracellular HEPES solution (section 3.4.2) containing 1 µM TTX and pipette solution 1 (section 3.4.3).

The pharmacological analysis revealed that the majority of juvenile hippocampal NG2 cells exhibit functional $GABA_A$ receptors containing the subunits α1, β2, β3 and γ2.

5.1.2 Correlation of $GABA_A$ receptor Zn^{2+} sensitivity and subunit expression in NG2 cells

To further characterize the subunit composition of $GABA_A$ receptors in NG2 cells, the influence of the endogenous $GABA_A$ receptor inhibitor Zn^{2+} was directly correlated with subunit expression of individual cells. It has been shown that the inhibitory action of Zn^{2+} strongly depends on the particular α- and γ-subunits of the $GABA_A$ receptor. Especially the subunit combination α1/γ2 was found to be much less Zn^{2+}-sensitive compared to $GABA_A$ receptors lacking these subunits (Draguhn et al., 1990; Smart et al., 1991; White and Gurley, 1995; Hosie et al., 2003).

To directly correlate Zn^{2+} sensitivity with subunit expression in individual NG2 cells, the following experiment was conducted. Initially, 50 µM GABA (2 s) were applied until a stable current response was reached. Next, 50 µM Zn^{2+} were pre-applied for 30 s alone before coapplication of GABA with Zn^{2+} (2 s). Subsequently, the cytoplasm of the analyzed cell was harvested and RT-PCR for $GABA_A$ receptor subunits α1-5 and γ1-3 was performed as described before (section 4.2 and 5.1.1.1). Experiments were performed in a solution containing blockers for Na^+ and K^+ channels (blocking solution, section 3.4.2). Furthermore, the AMPA receptor antagonist CNQX (25 µM) was added, as it was shown that Zn^{2+} might influence AMPA receptors (Paoletti et al., 2009).

The electrophysiological data revealed a large heterogeneity regarding Zn^{2+} sensitivity of NG2 cell $GABA_A$ receptors with values ranging from strong inhibition (to 23%) to weak inhibition (to 90%) of the GABA-evoked current. Correlation of Zn^{2+} sensitivity of individual NG2 cells with their respective subunit expression pattern showed that cells lacking the γ2-subunit displayed strong inhibition by Zn^{2+} (to 58.6 ± 18.8%, n = 14; Fig. 5.4A, C). Interestingly, cells expressing γ2 were less sensitive to Zn^{2+} (to 72.5 ± 10.6%, n = 8; Fig. 5.4B, C). Moreover, in NG2 cells negative for γ2, the subunits α1 and α5 were also less prevalent (21 and 43%, respectively, n = 14) as compared to cells expressing γ2 (75 and 88%, respectively, n = 8; Fig. 5.4D). The α-subunits 2, 3 and 4 did not show a correlation with γ2-expression (γ2-expressing cells: 88, 50 and 38%, respectively, n = 8; γ2-lacking cells: 57, 21 and 29 %, respectively, n = 14; Fig. 5.4D). Similar results were obtained for the remaining γ-subunits 1 and 3, that were not preferentially coexpressed with γ2 (γ2-expressing cells: 75 and 63%, respectively, n = 8; γ2-lacking cells: 64 and 50%, respectively, n = 14; Fig. 5.4D).

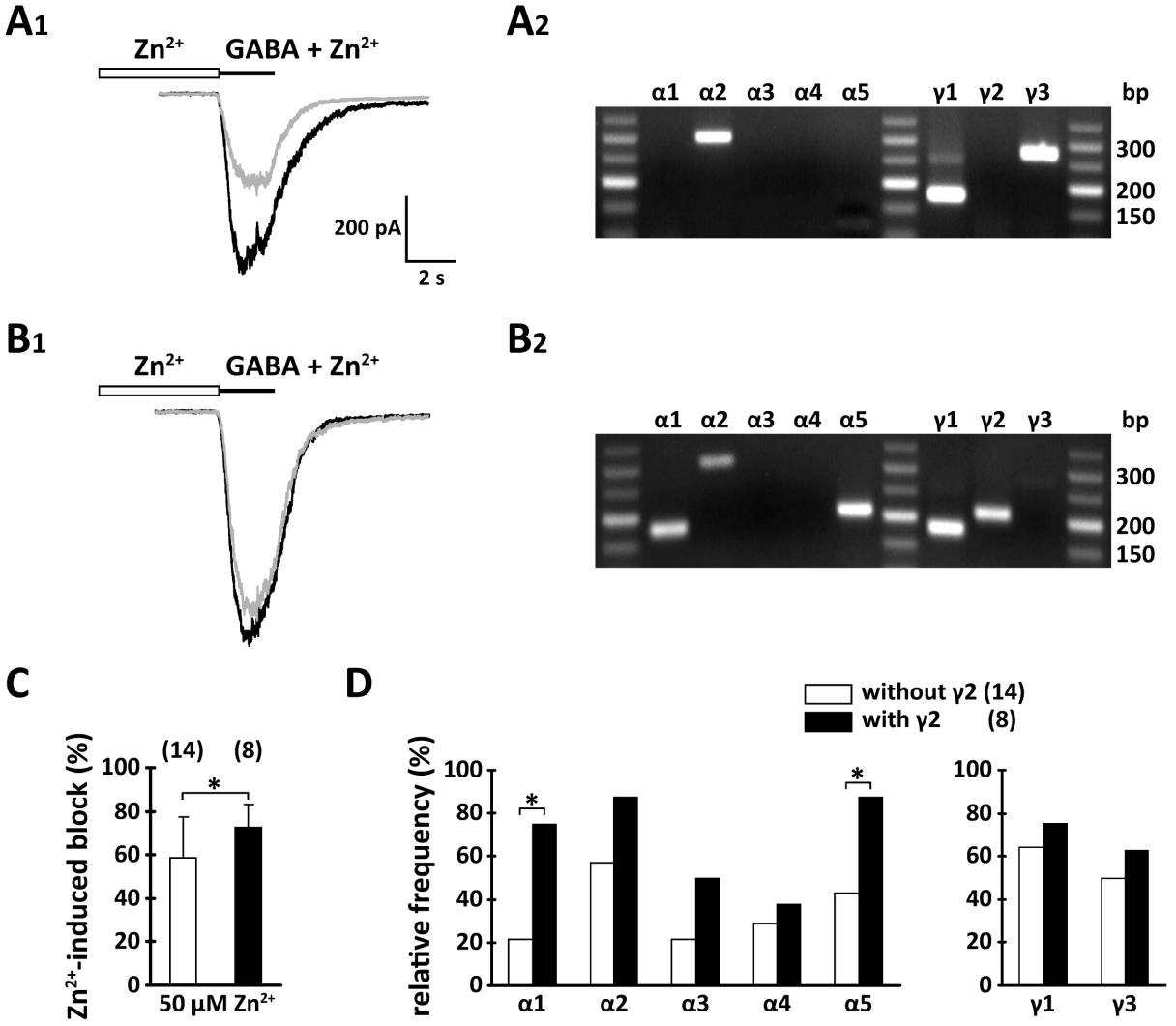

Fig. 5.4 Zn^{2+} sensitivity of $GABA_A$ receptors correlates with γ2-subunit expression in NG2 cells. (A1, B1) Pre-application of Zn^{2+} (50 µM, 30 s) was followed by coapplication of Zn^{2+} with GABA (50 µM, 2s). Gray traces represent responses upon coapplication, black traces show GABA responses before coapplication with Zn^{2+}. The GABA-induced current was substantially reduced in one NG2 cell (A1) while another cell was much less sensitive to Zn^{2+} (B1). **(A2, B2)** Agarose gels showing the α- and γ-subunit composition of the cells in A1 and B1, respectively. Low Molecular Weight DNA Ladder served as size marker. Recordings were performed using extracellular blocking solution (section 3.4.2) containing CNQX (25 µM) and pipette solution 1 (section 3.4.3). **(C)** Summary bar graph of Zn^{2+}-induced inhibition of GABA-evoked currents as a function of γ2-subunit expression (white bars: cells lacking γ2; black bars: cells expressing γ2). **(D)** Comparison of α- and γ-subunit expression of cells lacking γ2 (white bars) and those expressing γ2 (black bars). Asterisks indicate statistically significant differences. Cell numbers are given in parentheses (C, D). See also Passlick et al. (2013).

These results indicate heterogeneous expression of α1- and γ2-containing $GABA_A$ receptors in hippocampal NG2 cells and demonstrate a direct correlation between expression of the γ2-subunit and Zn^{2+} sensitivity in these cells.

5.1.3 Modulation of phasic and tonic $GABA_A$ receptor currents in NG2 cells

GABA released from presynaptic terminals activates postsynaptic $GABA_A$ receptors that mediate the so called phasic $GABA_A$ receptor currents. Furthermore, extrasynaptically located $GABA_A$ receptors may be activated by ambient GABA mediating the tonic $GABA_A$ receptor currents as shown for neurons in different brain regions (Farrant and Nusser, 2005). In this context, the γ2-subunit of $GABA_A$ receptors has a special role as it is important for clustering of synaptic $GABA_A$ receptors while being less relevant for extrasynaptic ones (Essrich et al., 1998; Lüscher and Keller, 2004). Synaptic innervation of NG2 cells by GABAergic neurons has been demonstrated in different brain regions by several groups (Lin and Bergles, 2004; Jabs et al., 2005; Velez-Fort et al., 2010). However, the presence of tonic currents in NG2 cells is controversial so far. While a study by Tong et al. (2009) suggested the presence of a tonic $GABA_A$ receptor current in hippocampal NG2 cells, others did not detect a tonic current in hippocampal (Lin and Bergles, 2004; Mangin et al., 2008) or neocortical NG2 cells (Velez-Fort et al., 2010).

The aim of this part of the present study was to investigate whether hippocampal NG2 cells are able to sense ambient GABA via extrasynaptic $GABA_A$ receptors and, if so, to determine whether the γ2-subunit is preferentially present in synaptic compared to extrasynaptic $GABA_A$ receptors as shown for neurons.

To test for the presence of a tonic $GABA_A$ receptor current in NG2 cells the $GABA_A$ receptor antagonist bicuculline was applied via the bath solution and the effect on the holding current was analyzed. A Cs^+-based pipette solution was used for these experiments to decrease K^+ currents (pipette solution 3, section 3.4.3). In all cells tested application of bicuculline (10 µM) induced a small positive shift in holding current by 5.3 ± 3.3 pA (n = 7; Fig. 5.5). Surprisingly, when repeating this experiment using the $GABA_A$ receptor antagonist gabazine (10 µM), only 4/6 NG2 cells displayed a positive shift in holding current that was smaller (2.5 ± 1.1 pA; Fig. 5.5) compared to the bicuculline-sensitive current.

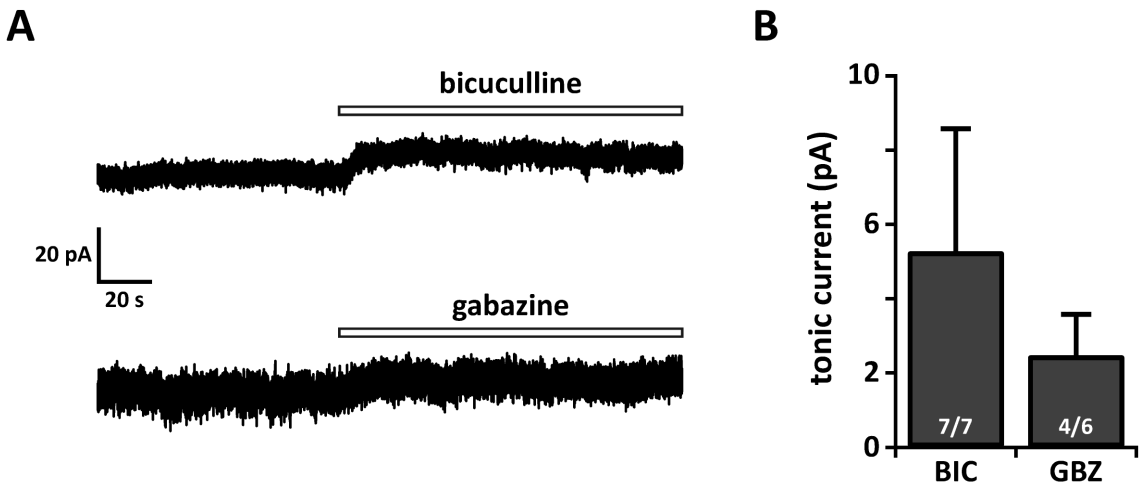

Fig. 5.5 Tonic $GABA_A$ receptor currents in NG2 cells. (A) Application of bicuculline (10 µM; top) or gabazine (10 µM; bottom) unmasked a tonic $GABA_A$ receptor current in two NG2 cells. Recordings were performed using extracellular ACSF solution (section 3.4.2) and pipette solution 3 (section 3.4.3). **(B)** Summary bar graph of tonic currents uncovered by bicuculline (BIC) and gabazine (GBZ). Cell numbers are indicated at the base of each bar. See also Passlick et al. (2013).

Next, pharmacological modulation of synaptic vs. extrasynaptic $GABA_A$ receptors was performed to investigate the subcellular localization of γ2-containing receptors.

To record ePSCs in hippocampal NG2 cells near-field stimulation was performed while holding the cell in the voltage-clamp configuration. The AMPA/kainate receptor antagonist NBQX (5 µM) was added to the bath solution to block AMPA receptor currents. Initially, the effect of the benzodiazepine diazepam on NG2 cell ePSCs was tested. Diazepam positively modulates $GABA_A$ receptors containing the α-subunits 1-3 and 5 and the γ-subunits 1-3 (Olsen and Sieghart, 2009).

In the presence of diazepam (10 µM) averaged control ePSCs were potentiated to 126 ± 9% in 5/8 cells, while three other cells exhibited no modulation (Fig. 5.6). To specifically verify presence of the γ2-subunit, the modulatory effect of zolpidem on ePSCs was tested, as zolpidem particularly modulates α1-/γ2-containing $GABA_A$ receptors at low concentrations (Pritchett and Seeburg, 1990; McKernan et al., 1991; Wingrove et al., 1997). In 6/9 cells zolpidem (1 µM) potentiated the control current to 128 ± 5% while three cells exhibited no modulation (Fig. 5.6).

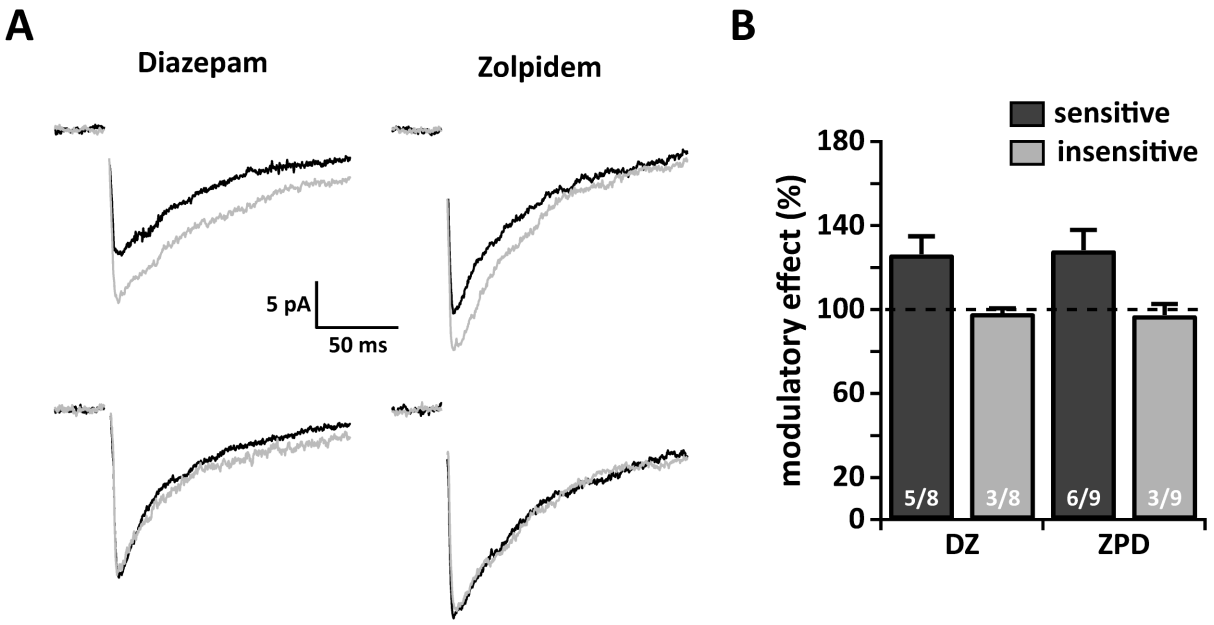

Fig. 5.6 Pharmacological modulation of synaptic $GABA_A$ receptor currents in NG2 cells. (A) Example responses of four NG2 cells to near-field stimulation in the presence (gray traces) and absence (black traces) of diazepam (10 µM, left) and zolpidem (1 µM, right). Sensitive (top) and insensitive (bottom) examples are shown. Stimulus artifacts were blanked for visibility. Black arrowheads indicate time of stimulation. Recordings were performed using extracellular ACSF solution (section 3.4.2) containing 5 µM NBQX and pipette solution 1 (section 3.4.3). **(B)** Summary bar graph of the modulatory effect of diazepam (DZ; 10 µM) and zolpidem (ZPD; 1 µM) on ePSCs. Data were normalized to the response before application of the modulator. Cell numbers are indicated at the base of each bar. See also Passlick et al. (2013).

These results indicate that the majority of NG2 cells contain postsynaptic $GABA_A$ receptors carrying the subunits α1 and γ2.

To facilitate a detailed analysis of the pharmacological properties of tonic currents in NG2 cells, the ambient GABA concentration was enhanced by wash-in of nipecotic acid (1 mM), an inhibitor of the mouse GABA transporter mGAT-1 and mGAT-4. In the presence of nipecotic acid, tonic currents increased to 18.7 ± 7.2 pA (n = 6). Coapplication of nipecotic acid (1 mM) with diazepam (10 µM) potentiated the tonic current to 140 ± 32% in 4/6 NG2 cells tested, while two other cells remained unaffected (Fig. 5.7). Interestingly, zolpidem (1 µM) has never modulated tonic currents of NG2 cells in the presence of nipecotic acid (to 103 ± 2.9%, n = 5; Fig. 5.7). These data imply that extrasynaptic $GABA_A$ receptors of NG2 cells are composed of other α- and γ-subunits than α1 and γ2.

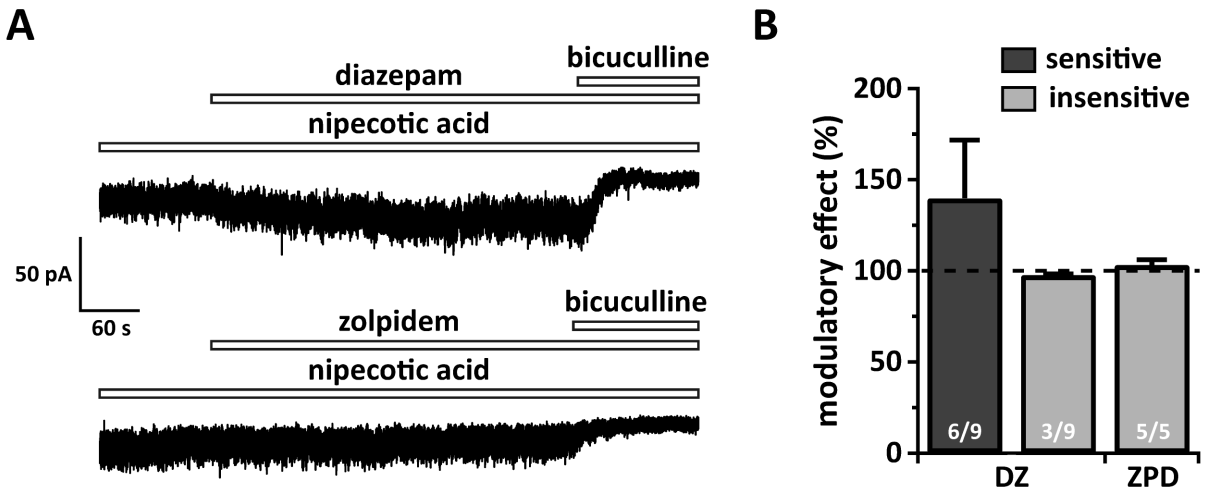

Fig. 5.7 Pharmacological modulation of tonic $GABA_A$ receptor currents in NG2 cells. (A) The bicuculline (20 µM)-sensitive tonic $GABA_A$ receptor current was potentiated by diazepam (10 µM) in an NG2 cell (top). In contrast, application of zolpidem (1 µM) did not modulate the bicuculline-sensitive current in another NG2 cell (bottom). Recordings were performed using extracellular ACSF solution (section 3.4.2) and pipette solution 3 (section 3.4.3). 1 mM nipecotic acid was washed in to increase the ambient GABA concentration. **(B)** Summary bar graph of the modulatory effect of diazepam (DZ; 10 µM) and zolpidem (ZPD; 1 µM) on tonic $GABA_A$ receptor currents. Data were normalized to the tonic current before application of the modulator. Cell numbers are indicated at the base of each bar. See also Passlick et al. (2013).

Taken together, these results suggest that the γ2-subunit is preferentially localized in synaptic $GABA_A$ receptors in the majority of NG2 cells while being absent in extrasynaptic ones mediating tonic currents in these cells.

5.1.4 Reversal potential analysis of $GABA_A$ receptors in NG2 cells

The $[Cl^-]_i$ determines whether the activation of $GABA_A$ receptors causes depolarization or hyperpolarization of the membrane potential of a cell. In neurons, a developmental change in $[Cl^-]_i$ due to upregulation of KCC2 and downregulation of NKCC1 leads to a switch from a depolarizing to a hyperpolarizing action of GABA (Cherubini et al., 1991; Rivera et al., 1999; Stein et al., 2004; Yamada et al., 2004; Khirug et al., 2008).

In NG2 cells, GABA was shown to be depolarizing throughout development as revealed by voltage-clamp-based reversal potential analysis (Lin and Bergles, 2004; Tanaka et al., 2009; Tong et al., 2009). However, in a similar experiment an increasing negative shift in the reversal potential during application of GABA was observed when holding the cell in the voltage-clamp configuration (Passlick et al., 2013). These results indicated a depletion of $[Cl^-]_i$ as previously reported for neurons (DeFazio and Hablitz, 2001; Karlsson et al., 2011). To circumvent this effect, a modified approach was employed in the present study.

To determine the reversal potential of $GABA_A$ receptors in NG2 cells perforated patch recordings were performed. To do so, the antibiotic gramicidin-A was included in the pipette solution (pipette solution 4, section 3.4.3) that forms cation-selective pores in the cell membrane leaving the $[Cl^-]_i$ unaffected (Kyrozis and Reichling, 1995). The incorporation of gramicidin-A is a slow process that was monitored by a decrease in series resistance while holding the cell in the cell-attached configuration. Reversal potential analysis was started when series resistance reached ~100 MΩ (detailed procedure in section 4.1.6). Experiments were performed in a solution containing blockers for Na^+ and K^+ channels to inhibit other conductances that might influence the Cl^- reversal potential (blocking solution, section 3.4.2).

To reduce potential redistribution of Cl^- to a minimum the following experiment was conducted. Brief pulses of the $GABA_A$ receptor agonist muscimol (400 µM, 2 s) were focally applied. Immediately before application, the cell was clamped in 10 mV steps to a certain membrane potential (between -70 and +20 mV) and reset to the holding potential of -70 mV shortly after the application pulse. After each application the cell was held at -70 mV for 15 s to re-equilibrate the $[Cl^-]_i$.

Using this approach an average reversal potential of -56.42 ± 7.6 mV was measured (n = 9; Fig. 5.8A, C). As a control, the experiment was repeated in the whole-cell configuration using the same pipette and bath solutions. In this case, muscimol-evoked currents reversed close to the calculated reversal potential for Cl^- of -43 mV (-43.4 ± 0.7 mV, n = 6; Fig. 5.8B, C). Potentials were corrected for a liquid junction potential of 13 mV.

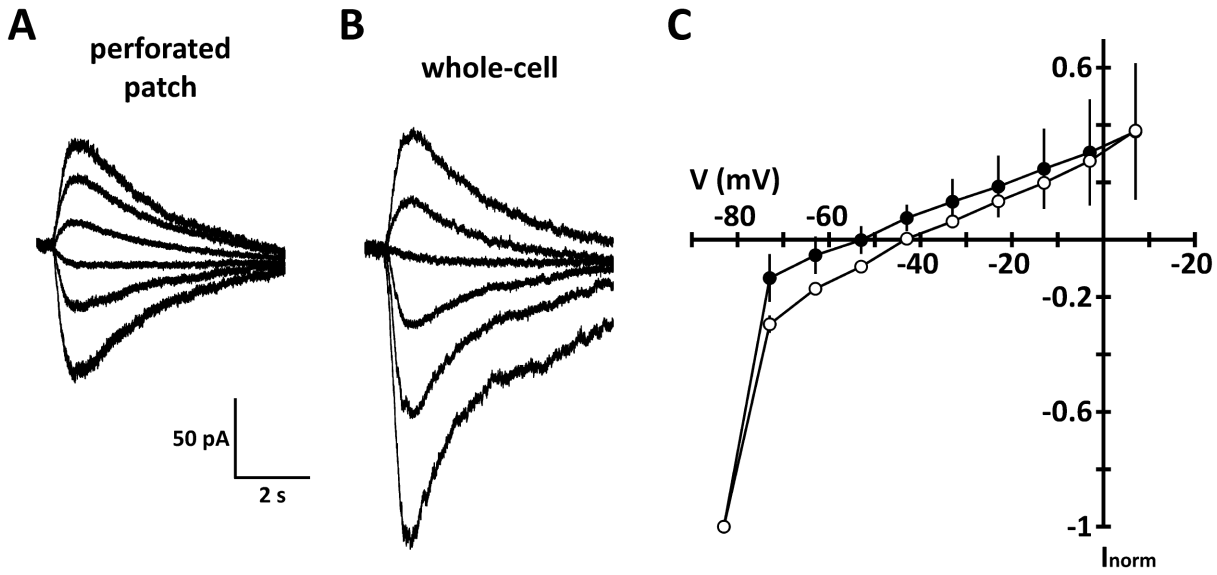

Fig. 5.8 Reversal potential analysis of NG2 cell $GABA_A$ receptors. (A) Muscimol (400 µM)-evoked responses of an NG2 cell in the perforated patch configuration (pressure application, 2 s). The cell was clamped between -70 mV and +20 mV (here only -60 mV to -10 mV are shown). Between each muscimol application the cell was held at -70 mV for 15 s. Recordings were performed using extracellular blocking solution (section 3.4.2) and pipette solution 4 containing 20-40 µg gramicidin-A (section 3.4.3 and 4.1.6). Recordings were offline compensated for liquid junction potential (13 mV). **(B)** In the whole-cell configuration using the same solutions and protocols as in (A), currents reversed close to the calculated reversal potential for Cl^- of -43 mV in an NG2 cell. **(C)** Average I/V curves of the muscimol-evoked responses at different voltages in perforated patch (black circles, n = 9) and whole-cell (white circles, n = 6) configuration. Data were normalized to the muscimol response at -83 mV.

The reversal potential measured in the present study is close to the earlier mentioned shifted one, indicating that a similar Cl^- redistribution occurred. Therefore, current-clamp-based reversal potential experiments reducing the risk of Cl^- redistribution probably provide more reliable information about the $[Cl^-]_i$ of hippocampal NG2 cells (Passlick et al., 2013).

5.2 Molecular analysis of $GABA_A$ receptor subunit expression in neocortical NG2 cells during development

For neocortical NG2 cells, a developmental switch from synaptic to extrasynaptic GABAergic transmission was demonstrated. In this process, the synaptic communication between interneurons and NG2 cells in juvenile mice is replaced by a form of GABA spillover activating extrasynaptic $GABA_A$ receptors in adult NG2 cells (Velez-Fort et al., 2010). Data from the present study revealed that synaptic and extrasynaptic $GABA_A$ receptors of hippocampal NG2 cells differ in expression of the γ2-subunit (section 5.1.3), which is essential for clustering of postsynaptic $GABA_A$ receptors in immature and mature neurons (Essrich et al., 1998; Schweizer et al., 2003). Therefore, it was tested whether the developmental transition from synaptic to extrasynaptic communication in neocortical NG2 cells is accompanied by alterations of the $GABA_A$ receptor subunit expression.

To investigate the $GABA_A$ receptor subunit composition of neocortical NG2 cells during postnatal development, single-cell RT-PCR experiments were performed. For this part of the study transgenic NG2-DsRed mice of the two age groups p7-11 and p21-29 were used, since these are the time periods where the switch from synaptic to extrasynaptic transmission was observed (Velez-Fort et al., 2010). Fluorescent NG2 cells of layer V of the barrel cortex were analyzed in the whole-cell configuration. Membrane currents in response to de- and hyperpolarizing voltage steps were elicited to unambiguously identify the cell. In both age groups, NG2 cells displayed the characteristic complex current-voltage relationship exhibiting time- and voltage-dependent conductances (Steinhäuser et al., 1994a; Steinhäuser et al., 1994b). Furthermore, neocortical NG2 cells displayed the known developmental increase of K_{ir} conductances (Fig. 5.9A, B) that was also observed for hippocampal NG2 cells in the present study (section 5.3.1) (Kressin et al., 1995; Bordey and Sontheimer, 1997; Maldonado et al., 2013). Subsequent to functional characterization, the cytoplasm was harvested for transcript analysis. NG2 cells were tested for presence of the subunits α1-5, β1-3, γ1-3 and δ, which are the main $GABA_A$ receptor subunits expressed in the neocortex (Laurie et al., 1992; Golshani et al., 1997). A two-round RT-PCR was performed as described before (section 4.2 and 5.1.1.1).

At p7-11, the subunits α1, α2 and α5 were most frequently expressed (62, 56 and 44%, respectively, n = 16) while α3 and α4 were less often detected (25 and 19%, respectively, n = 16; Fig. 5.9A, C). An age-dependent decrease in expression frequency was observed for α2 (37%) and α5 (16%, n = 19). In contrast, α3 and α4 expression was increased (52 and 47%, respectively) while α1 expression was similar (53%, n = 19) at p21-29 (Fig. 5.9A, C). Regarding β-subunits, β2 and β3 were most abundant in both age groups (79 and 63% at p7-11, n = 19; 71 and 57% at p21-29, respectively, n = 14; Fig. 5.9D). β1-subunit expression showed a slight decline from p7-11 (21%, n = 19) to p21-29 (7%, n = 14; Fig. 5.9D). Analysis of the γ-subunits revealed no age-dependency of γ3 (67% at p7-11, n = 21, and 75% at p21-29, n = 20) and γ1 (48% at p7-11, n = 21, and 55% at p21-29, n = 20; Fig. 5.9B, E). Interestingly, γ2 displayed a strong age-dependent drop in expression frequency from 43% at p7-11 (n = 21) to 5% at p21-29 (n = 20; Fig. 5.9). The δ-subunit which is a major component of extrasynaptic $GABA_A$ receptors in neurons (Farrant and Nusser, 2005), was completely absent at p7-11 (n = 19) and rarely detected at p21-29 (18%, n = 11; Fig. 5.9E).

These data indicate that neocortical NG2 cells experience a complex developmental regulation of their $GABA_A$ receptor subunit expression. Interestingly, the developmental switch from synaptic to extrasynaptic transmission is accompanied by a decline in the expression frequency of γ2, an important subunit of synaptic $GABA_A$ receptors.

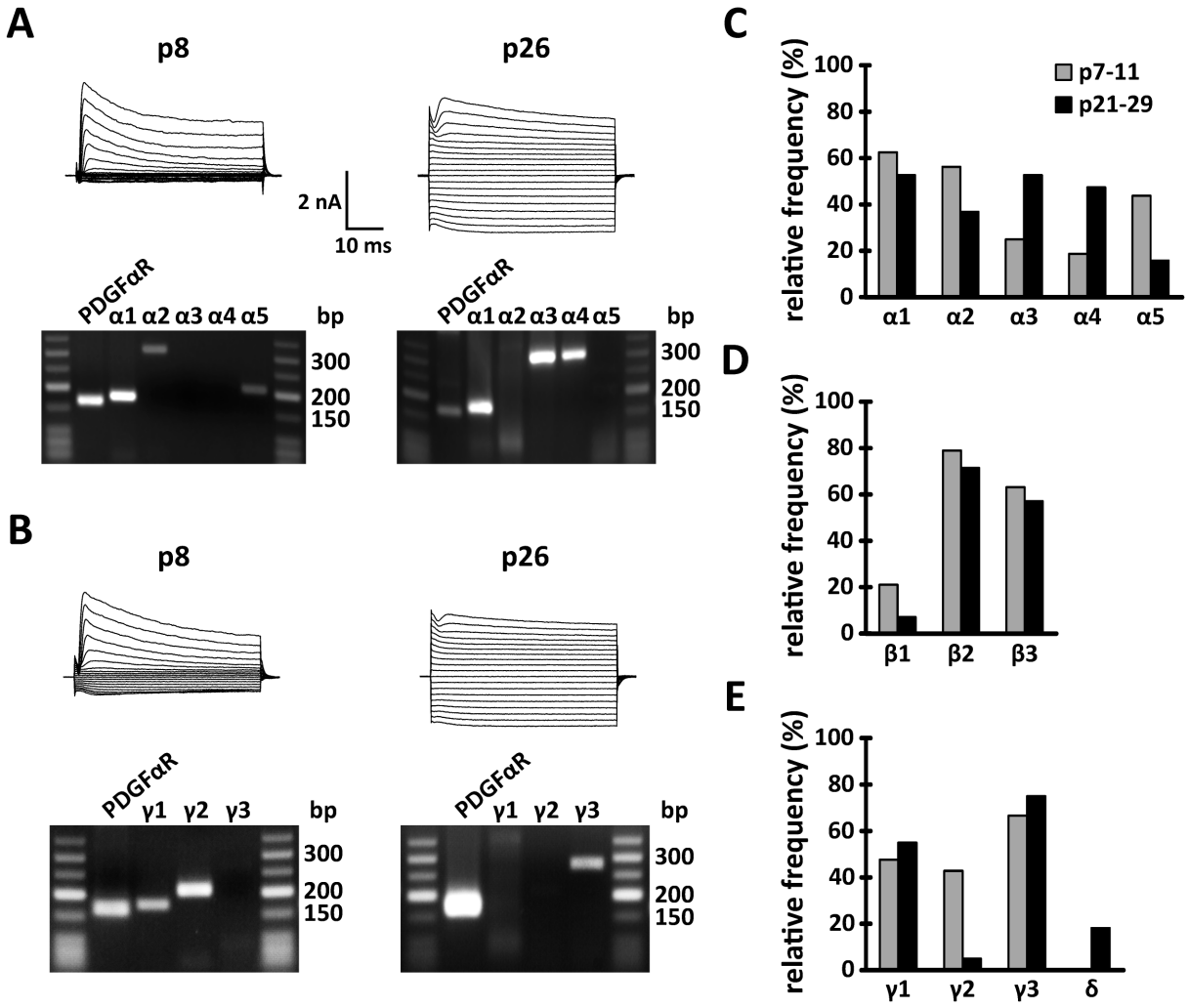

Fig. 5.9 Expression analysis of $GABA_A$ receptor subunits in juvenile and adult neocortical NG2 cells by single-cell RT-PCR. (A, B) Agarose gels of α- and γ-subunit PCR-products of individual NG2 cells at p8 and p26 together with their respective current responses to de- and hyperpolarization between -160 and +20 mV (holding potential -80 mV). Expression of PDGFα receptor (PDGFαR) mRNA was used as a positive control and cell type-specific marker. Low Molecular Weight DNA Ladder served as size marker. Recordings were performed using extracellular ACSF solution (section 3.4.2) and pipette solution 1 (section 3.4.3). **(C-E)** Summary bar graphs of the relative frequency of α-, β-, γ- and δ-subunit expression of all NG2 cells tested at p7-11 and p21-29. The number of tested cells is 16, 19, 21 and 19 for α-, β-, γ- and δ-subunits at p7-11, respectively, and 19, 14, 20 and 11 for α-, β-, γ- and δ-subunits at p21-29, respectively. See also Balia et al. (2013).

5.3 Role of the NG2 protein in neuron-NG2 cell glutamatergic synaptic signaling

NG2 cells are the only non-neuronal cells in the CNS that receive synaptic input from neurons. The cellular mechanisms underlying formation and maintenance of these neuron-glia synapses and their functional impact are still unclear (Bergles et al., 2010). Several studies have suggested that the NG2 protein itself is involved in neuron-NG2 cell synaptic transmission, as it was shown that

(i) NG2 contains two extracellular LNS domains that are important for transsynaptic binding of the neuroligin-neurexin complex in neurons (section 1.6 and 1.7) (Trotter et al., 2010; Missler et al., 2012)

(ii) NG2 is complexed via GRIP with GluA2/3-subunit containing AMPA receptors, thereby possibly mediating receptor clustering in the glial postsynaptic density (Stegmüller et al., 2003).

However, this hypothesis has never been tested. To elucidate a potential contribution of NG2 in this process, neuron-NG2 cell glutamatergic synaptic transmission was investigated in NG2-EYFP knockin mice of the three different genotypes: wild type NG2 (wt), heterozygous NG2 knockout with reduced NG2 expression (+/-) and homozygous NG2 knockout (-/-) (section 3.1.3) (Karram et al., 2008). To consider besides genotype- also age-dependent effects, experiments were performed in juvenile (p8-12) and aged (> 9 months) animals. For this study, the hippocampal CA1 region was chosen as a well characterized system of synaptic transmission (section 1.1) (Malenka and Nicoll, 1999). All recordings of this section were performed using extracellular ACSF solution (section 3.4.2) containing 150 µM of the $GABA_A$ receptor blocker picrotoxin and pipette solution 1 (section 3.4.3) if not stated otherwise.

5.3.1 Influence of NG2 on passive membrane properties of NG2 cells

In the first set of experiments, passive membrane properties of NG2 cells were compared. NG2 cells of juvenile and aged NG2-EYFP mice of all genotypes displayed the characteristic complex current pattern with time- and voltage-dependent currents in response to de- and hyperpolarizing voltage steps (Fig. 5.10) (Steinhäuser et al., 1994a; Steinhäuser et al., 1994b). As reported previously, the current patterns of juvenile NG2 cells were more variable with respect to the expression of K_{ir} conductances (Fig. 5.10A), while those of aged NG2 cells were more uniform with clear upregulation of K_{ir} channel expression (Fig. 5.10B) (Kressin et al., 1995; Bordey and Sontheimer, 1997; Maldonado et al., 2013). Similar results were also observed for neocortical NG2 cells in the present study (section 5.2).

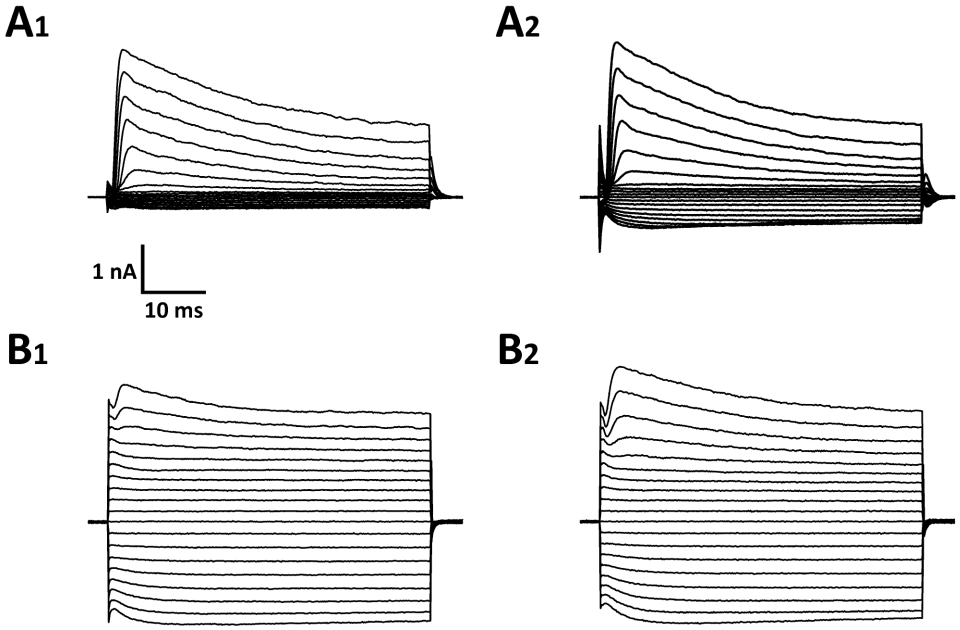

Fig. 5.10 NG2 cells upregulate K_{ir} conductances during development. (A) Current responses of two juvenile NG2 cells (A_1: p9, A_2: p11) to de- and hyperpolarization between -160 and +20 mV (holding potential -80 mV). Juvenile NG2 cells exhibited variable degrees of K_{ir} conductances. **(B)** Current responses of two aged NG2 cells (B_1: p337, B_2: p350) to de- and hyperpolarization between -160 and +20 mV (holding potential -80 mV). Aged NG2 cells consistently displayed increased K_{ir} conductances. Scale bar in A_1 applies to A and B.

The observed increase in K_{ir} conductances from juvenile to aged NG2 cells was also reflected by the membrane resistance. Aged NG2 cells of all genotypes showed a decrease in membrane resistance compared to juvenile cells (Tab. 5.2, Fig. 5.11A). Moreover, the observed variability regarding the amount of K_{ir} conductances in juvenile NG2 cells was mirrored by a larger interquartile range of the membrane resistance compared to aged NG2 cells (Tab. 5.2, Fig. 5.11A). Interestingly, aged NG2 cells with reduced levels of NG2 expression (+/- and -/-) showed an increase in membrane resistance compared to wt cells.

The resting potential of NG2 cells was not influenced by NG2 expression (Tab. 5.2, Fig. 5.11B). However, a small decrease in resting potential was observed between juvenile and aged wt animals.

Analysis of the membrane capacitance of NG2 cells revealed genotype- and age-dependent changes (Tab. 5.2, Fig. 5.11C). Juvenile NG2 cells displayed larger membrane capacitances when NG2 expression was reduced or lacking. Conversely, this effect was absent in aged NG2 cells. During development no alterations of membrane capacitance were observed for wt cells. However, due to the increased membrane capacitances in juvenile +/- and -/- cells, a decrease from juvenile to aged in +/- and -/- animals was observed.

Passive membrane properties were determined as described in section 4.1.4.

Tab. 5.2 Passive membrane properties of NG2 cells. Gaussian data are given as mean ± SD, non-Gaussian data as median and interquartile range (quartile 25 – quartile 75). Cell numbers are given in square brackets. R_m = membrane resistance, V_{rest} = resting potential, C_m = membrane capacitance.

	juvenile			aged		
	wt	**+/-**	**-/-**	**wt**	**+/-**	**-/-**
R_m (MΩ)	396.0 (198-620) [19]	196.5 (117-455) [40]	274.9 (136-715) [25]	28 (21-30) [11]	34.8 (29-44) [20]	40.2 (27-58) [31]
V_{rest} (mV)	-91 (-93 - -88) [19]	-90 (-91 - -88) [40]	-89 (-91 - -86) [25]	-87 (-89 - -86) [11]	-89 (-90 - -88) [20]	-89 (-90 - -87) [31]
C_m (pF)	27.3 ± 4.9 [18]	32 ± 6.8 [38]	35.5 ± 6.3 [23]	28.3 ± 3.5 [11]	27.2 ± 4.7 [19]	24.9 ± 5.1 [29]

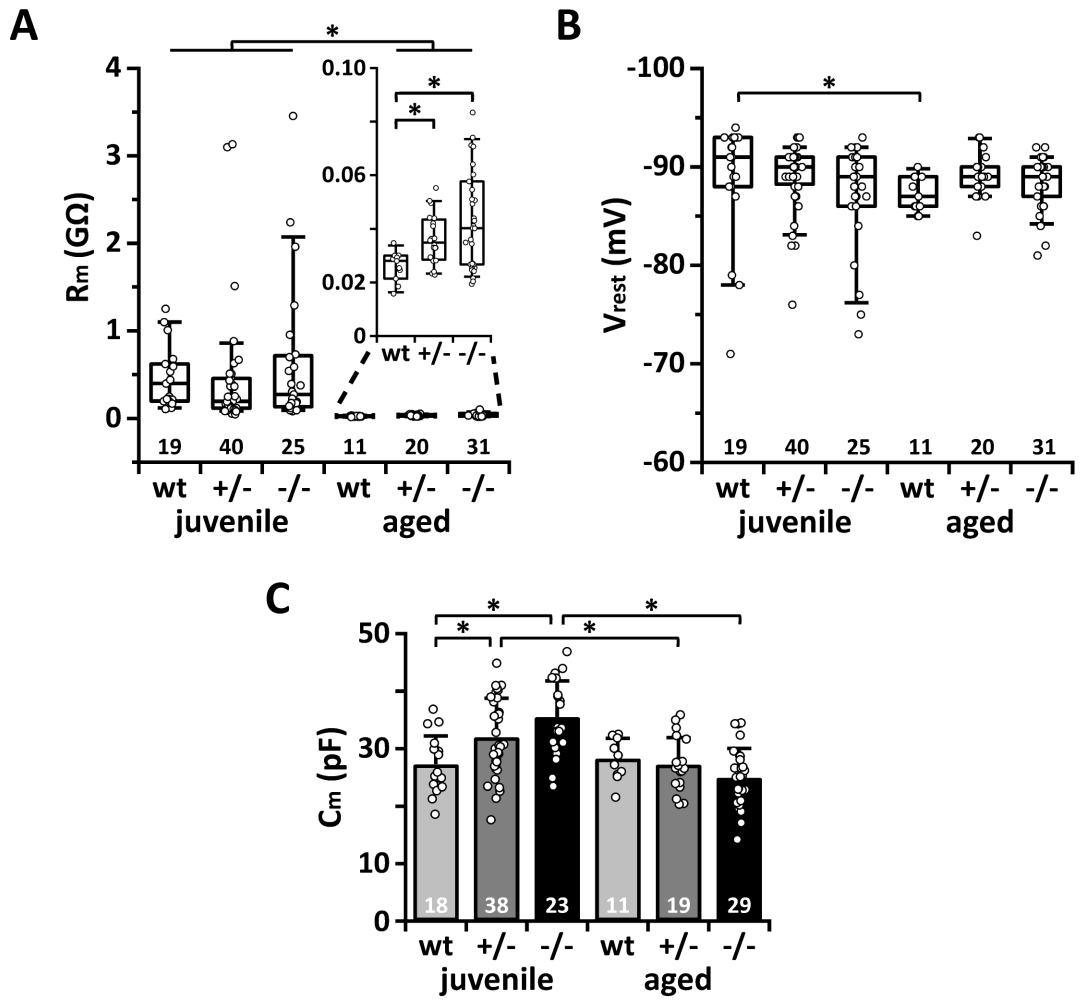

Fig. 5.11 Membrane resistance and membrane capacitance are influenced by NG2. (A-C) Membrane resistance (R_m), resting potential (V_{rest}) and membrane capacitance (C_m) of NG2 cells in juvenile and aged wt, +/- and -/- mice. **(A)** The membrane resistance decreased from juvenile to aged NG2 cells in all genotypes (test #4). Furthermore, aged NG2 cells exhibited an NG2-dependent increase in membrane resistance (test #3). **(B)** Resting potential was slightly depolarized in aged compared to juvenile wt cells (test #4), but showed no NG2-dependent changes. **(C)** Membrane capacitance was increased in +/- and -/- cells compared to wt cells in juvenile animals (test #1) while this effect was absent in aged NG2 cells. In addition, membrane capacitance decreased in +/- and -/- cells during development (test #1) while being stable in wt cells. Asterisks indicate statistically significant differences. Cell numbers for each group indicated at the base of each bar.

Taken together, these results indicate that NG2 expression may have an impact on membrane resistance and membrane capacitance of NG2 cells. Moreover, the current patterns of NG2 cells change during development, including a pronounced increase in K_{ir} channel expression.

5.3.2 Influence of NG2 on kinetic properties of ePSCs in NG2 cells

To investigate the role of the NG2 protein in synaptic transmission, Schaffer collaterals were stimulated to record ePSCs in NG2 cells of the hippocampal CA1 region. In the presence of the $GABA_A$ receptor blocker picrotoxin (150 µM), stimulation elicited glutamatergic ePSCs in NG2 cells of all genotypes and age groups tested (Fig. 5.12A). These currents were completely abolished by bath application of the AMPA/kainate receptor antagonist NBQX (10 µM) indicating that they are exclusively mediated by AMPA receptors (Fig. 5.12B).

The kinetic properties of synaptic AMPA receptor-mediated currents vary depending on subunit composition of the receptor, association with auxiliary subunits and time course of glutamate concentration in the synaptic cleft (section 1.4.2) (Jonas, 2000; Cathala et al., 2005; Jackson and Nicoll, 2011). As NG2 has been found in a complex with GluA2/3 containing AMPA receptors (Stegmüller et al., 2003), it might influence the subunit composition of AMPA receptors in the postsynaptic density. Furthermore, assuming NG2 binds to a presynaptic protein via its LNS domains, it might affect synaptic structure and thereby the time course of glutamate concentrations in the synaptic cleft. To test this, kinetic properties of ePSCs were compared between the genotypes and age groups investigated (detailed procedure in section 4.1.9.2).

Rise times and decay times of ePSCs were indistinguishable between genotypes within each age group (Tab. 5.3, Fig. 5.12C). Nevertheless, an acceleration of rise times and decay times in +/- and -/- NG2 cells became evident when comparing the age groups.

Tab. 5.3 Kinetic properties of ePSCs in NG2 cells. Data are given as mean ± SD. Cell numbers are given in square brackets.

	juvenile			aged		
	wt	**+/-**	**-/-**	**wt**	**+/-**	**-/-**
rise time (ms)	0.52 ± 0.1 [7]	0.55 ± 0.2 [15]	0.53 ± 0.1 [10]	0.43 ± 0.1 [6]	0.37 ± 0.03 [8]	0.4 ± 0.04 [8]
decay time (ms)	1.36 ± 0.49 [7]	1.38 ± 0.46 [14]	1.5 ± 0.49 [11]	1.0 ± 0.31 [6]	0.7 ± 0.14 [8]	0.89 ± 0.19 [8]

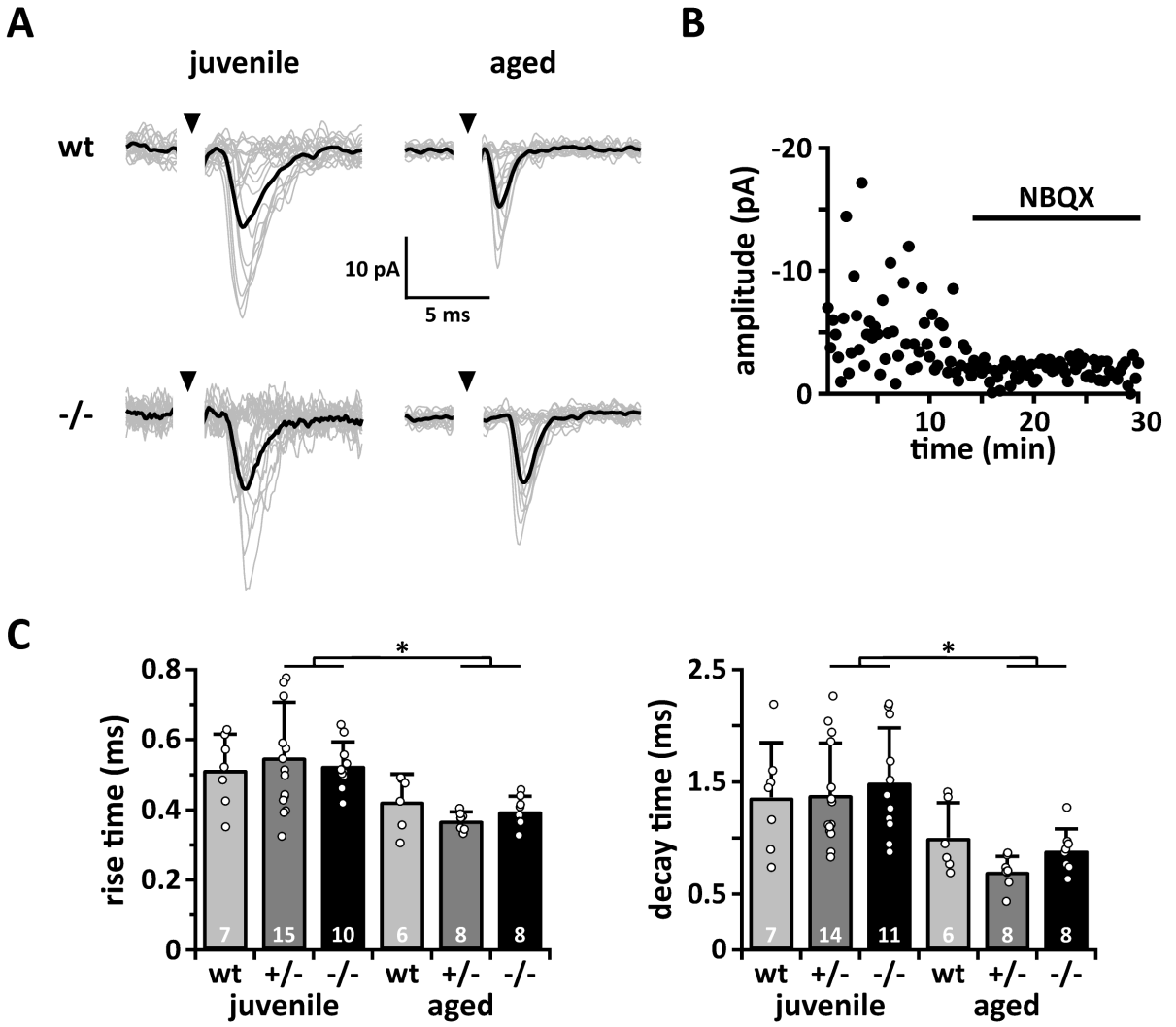

Fig. 5.12 NG2 cells form functional glutamatergic synapses exhibiting unchanged AMPA receptor current kinetics in the absence of NG2. (A) Representative responses of four NG2 cells to Schaffer collateral stimulation in juvenile (p9, both) and aged (top: p301, bottom: p300) wt and -/- mice. Single traces (gray) and average of ePSCs (black) are shown. **(B)** ePSCs were completely blocked by wash-in of NBQX (10 µM) in a juvenile (p9) +/- NG2 cell. **(C)** Summary bar graphs of ePSC rise times and decay times of juvenile and aged wt, +/- and -/- NG2 cells. No differences were detected among genotypes within each age group. However, rise times and decay times decreased from juvenile to aged in +/- and -/- cells (test #2). Asterisks indicate statistically significant differences. Cell numbers for each group indicated at the base of each bar.

These findings indicate that functional neuron-NG2 cell synapses form in the absence of the NG2 protein. Furthermore, these synapses exhibit AMPA receptors displaying current kinetics indistinguishable from controls.

5.3.3 Influence of NG2 on short-term synaptic plasticity in NG2 cells

Short-term synaptic plasticity describes a temporary change in synaptic efficacy that mainly depends on the presynaptic release probability (section 1.4.1). Although it is considered to be a primarily presynaptic feature, a study by Futai et al. (2007) showed that retrograde signaling from the postsynaptic cell via neurexin-neuroligin interaction can also regulate the presynaptic release probability and thereby short-term synaptic plasticity. Furthermore, triple-knockout studies of all α-neurexins revealed dysfunction of presynaptic transmitter release due to non-functional Ca^{2+} channels (Missler et al., 2003). Assuming that NG2 binds to a presynaptic protein via its LNS domains, it might also regulate these features at neuron-NG2 cell synapses. To test this, paired-pulse stimulation experiments with an interstimulus interval of 20 ms were performed. NG2 cells of all genotypes and age groups displayed strong paired-pulse facilitation as indicated by the PPR (Tab. 5.4, Fig. 5.13) (detailed procedure in section 4.1.8 and 4.1.9.2). Comparison of the different genotypes revealed no NG2-dependent alteration in PPR. A decrease was only observed between juvenile and aged wt cells (Fig. 5.13).

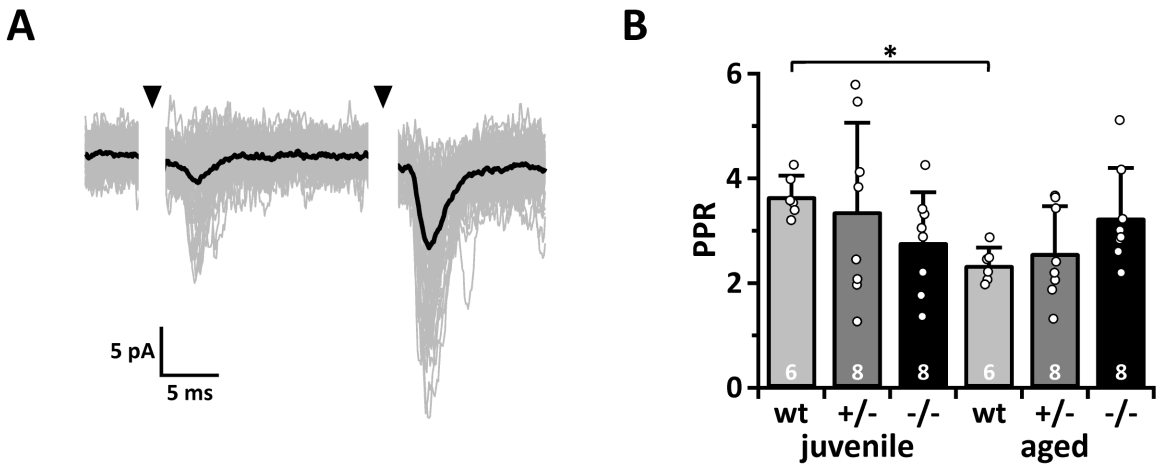

Fig. 5.13 Expression of NG2 does not influence short-term synaptic plasticity of neuron-NG2 cell synapses. (A) Paired-pulse stimulation (interstimulus interval 20 ms) of Schaffer collaterals revealed facilitation in a juvenile (p9) -/- NG2 cell. Average of single traces (gray) is given in black. **(B)** Summary bar graph of the PPR of juvenile and aged wt, +/- and -/- NG2 cells. PPR was unchanged between the genotypes and age groups except for wt juvenile vs. aged (test #2). Asterisks indicate statistically significant differences. Cell numbers for each group indicated at the base of each bar.

Tab. 5.4 PPR of ePSCs in NG2 cells. Data are given as mean ± SD. Cell numbers are given in square brackets.

	juvenile			Aged		
	wt	**+/-**	**-/-**	**wt**	**+/-**	**-/-**
PPR	3.7 ± 0.4 [6]	3.4 ± 1.7 [8]	2.8 ± 1 [8]	2.4 ± 0.3 [6]	2.6 ± 0.9 [8]	3.3 ± 0.9 [8]

These data suggest that presynaptic release probability and short-term synaptic plasticity are not influenced by NG2 expression.

5.3.4 Influence of NG2 on synaptic connectivity of NG2 cells

Contradictory studies involve the NG2 protein in neurite outgrowth. While some studies indicate that NG2 is inhibiting neurite outgrowth (Ughrin et al., 2003; Tan et al., 2005; Tan et al., 2006), others suggest that it constitutes a permissive substrate for neurites (Jones et al., 2003; de Castro et al., 2005). Furthermore, it was shown that axons actively contact NG2 cells but avoid oligodendrocytes (Yang et al., 2006). Assuming that NG2 has either a permissive or inhibiting effect on neuronal processes and considering that it may bind to a presynaptic partner via its LNS domains, reduced levels of NG2 may influence the number of functional synapses. To estimate whether NG2 has an influence on synaptic connectivity, the frequency of AMPA receptor-mediated mPSCs was measured in the presence of 150 µM picrotoxin and 1 µM TTX in juvenile and aged wt, +/- and -/- mice (detailed procedure in section 4.1.9.2).

The analysis revealed a relatively low mPSC frequency in juvenile NG2 cells that decreased even further during development (Tab. 5.5, Fig. 5.14). Importantly, no NG2-dependent alterations were detected in both age groups, while the developmental decrease in mPSC frequency was evident in all genotypes tested.

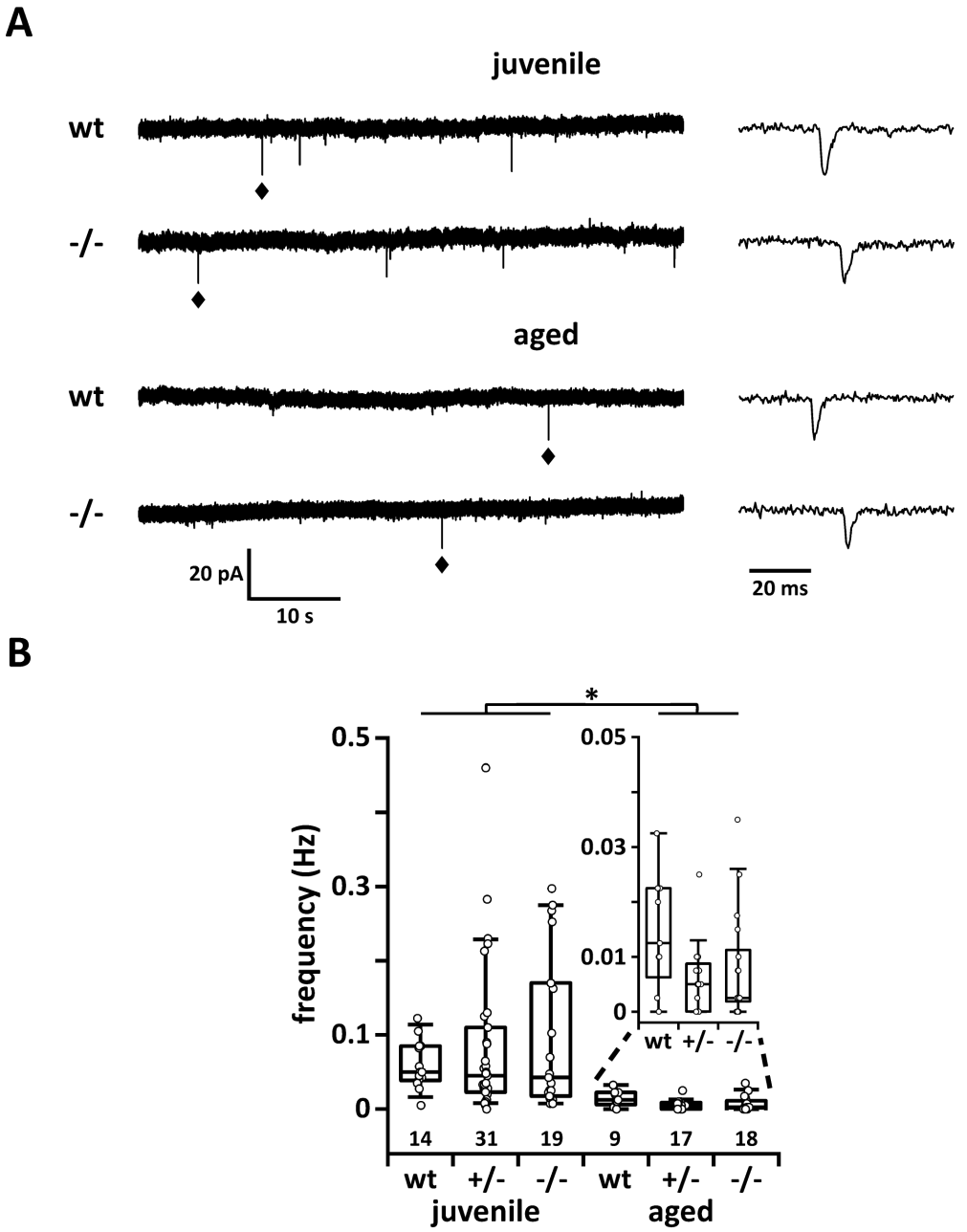

Fig. 5.14 Synaptic connectivity is preserved in absence of NG2. (A) Example traces of mPSC recordings from juvenile (top: p8, bottom: p11) and aged (top: p407, bottom: p372) wt and -/- NG2 cells. Recordings were performed in the presence of 150 µM picrotoxin and 1 µM TTX using pipette solution 2 (section 3.4.3). Diamonds indicate regions shown at an expanded timescale (right). **(B)** Summary box plot of mPSC frequency in juvenile and aged wt, +/- and -/- NG2 cells. Different levels of NG2 expression did not influence mPSC frequency. A decrease from juvenile to aged animals was detected in all genotypes tested (test #4). Asterisks indicate statistically significant differences. Cell numbers for each group indicated at the base of each bar.

Tab. 5.5 Frequency of mPSCs in NG2 cells. Data are given as median and interquartile range (quartile 25 – quartile 75). Cell numbers are given in square brackets.

	juvenile			aged		
	wt	**+/-**	**-/-**	**wt**	**+/-**	**-/-**
frequency (Hz)	0.05 (0.04-0.09) [14]	0.045 (0.02-0.11) [31]	0.043 (0.02-0.17) [19]	0.013 (0.006-0.022) [9]	0.005 (0-0.009) [17]	0.003 (0.002-0.011) [18]

These findings suggest that the synaptic connectivity of NG2 cells is preserved and unchanged in the absence of NG2.

5.3.5 Influence of NG2 on postsynaptic AMPA receptor density in NG2 cells

The NG2 protein is complexed with GRIP and GluA2/3 containing AMPA receptors (Stegmüller et al., 2003). It may thus be important for AMPA receptor trafficking into the synapse and clustering of AMPA receptors in the glial postsynaptic density. To assess whether NG2-lacking cells exhibit changes in postsynaptic AMPA receptor density, mPSC amplitudes were compared among the genotypes and age groups tested (detailed procedure in section 4.1.9.2). Since the frequency of mPSCs was too low for a quantitative analysis (section 5.3.4), the presynaptic transmitter release was increased by locally applying 3 µM ionomycin, a Ca^{2+} ionophore that has been shown to increase mPSC frequency without affecting the amplitude (Capogna et al., 1996). In the presence of 150 µM picrotoxin and 1 µM TTX, ionomycin considerably increased the mPSC frequency in NG2 cells of all genotypes and age groups (Fig. 5.15A, compared to the baseline frequency in Fig. 5.14A). No mPSCs were recorded in the presence of 10 µM NBQX (Fig. 5.15B). Comparison of mean and cumulative probability distributions of mPSC amplitudes revealed no differences between the genotypes and age groups tested (Tab. 5.6, Fig. 5.15C).

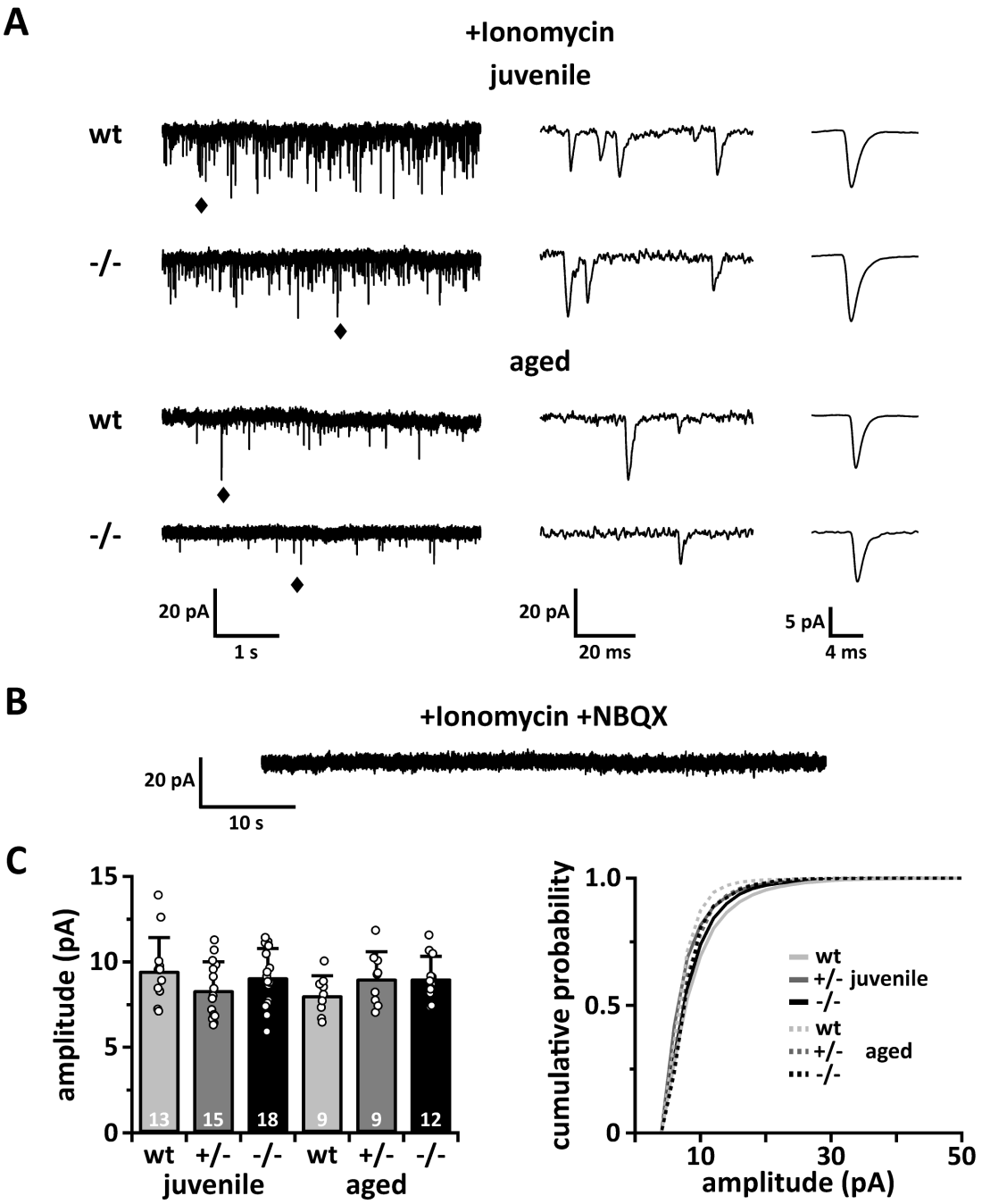

Fig. 5.15 Postsynaptic AMPA receptor density is unaffected by NG2 ablation. (A) Representative mPSC recordings from juvenile (p8, both) and aged (top: p447, bottom: p372) wt and -/- NG2 cells after application of 3 µM ionomycin to increase presynaptic vesicle fusion. Recordings were performed in the presence of 150 µM picrotoxin and 1 µM TTX using pipette solution 2 (section 3.4.3). Diamonds indicate regions shown at an expanded timescale (middle). Average of all mPSCs of the corresponding cell is given on the right. **(B)** In the presence of 10 µM NBQX no mPSCs were recorded upon ionomycin application in a juvenile (p10) -/- NG2 cell. **(C)** Summary bar graph of mean mPSC amplitudes (left) and cumulative probability distribution of mPSC amplitudes (right) in juvenile and aged wt, +/- and -/- NG2 cells. Differences between genotypes and age groups did not reach statistical significance. Cell numbers for each group indicated at the base of each bar (C, left also apply to C, right).

Tab. 5.6 Amplitudes of mPSCs in NG2 cells. Data are given as mean ± SD. Cell numbers are given in square brackets.

	juvenile			aged		
	wt	**+/-**	**-/-**	**wt**	**+/-**	**-/-**
amplitude (pA)	-9.49 ± 1.9 [13]	-8.36 ± 1.7 [15]	-9.11 ± 1.7 [18]	-8.06 ± 1.1 [9]	-9.04 ± 1.6 [9]	-9.04 ± 1.3 [12]

These data indicate that the postsynaptic AMPA receptor density is unaffected by NG2 ablation.

5.3.6 Influence of NG2 on kinetic properties of mPSCs in NG2 cells

As described in section 5.3.2, kinetic properties of postsynaptic AMPA receptor currents may vary depending on the presence of NG2. To investigate this, kinetic properties of averaged mPSCs measured after application of ionomycin were compared (detailed procedure in section 4.1.9.2). In agreement with the results gained for ePSCs (section 5.3.2), rise times and decay times of mPSCs were indistinguishable between the genotypes tested in both age groups (Tab. 5.7, Fig. 5.16). Furthermore, all genotypes displayed a developmental acceleration of rise times and decay times of mPSCs in NG2 cells as observed for ePSCs (section 5.3.2).

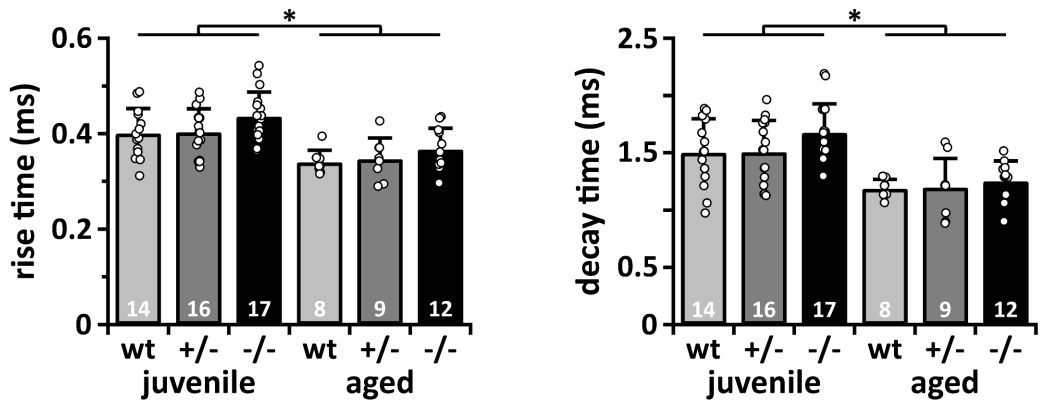

Fig. 5.16 Kinetic properties of mPSCs display no NG2-dependent alterations but experience developmental acceleration. Summary bar graphs of mPSC rise time (left) and decay time (right) of juvenile and aged wt, +/- and -/- NG2 cells. As for ePSCs, no differences were detected among the genotypes tested in both age groups. However, comparison of juvenile with aged mPSC kinetics revealed a developmental acceleration in all genotypes (test #1). Asterisks indicate statistically significant differences. Cell numbers for each group indicated at the base of each bar.

Tab. 5.7 Kinetic properties of mPSCs in NG2 cells. Data are given as mean ± SD. Cell numbers are given in square brackets.

	juvenile			aged		
	wt	**+/-**	**-/-**	**wt**	**+/-**	**-/-**
rise time (ms)	0.4 ± 0.05 [14]	0.4 ± 0.05 [16]	0.44 ± 0.05 [17]	0.34 ± 0.03 [8]	0.35 ± 0.04 [9]	0.37 ± 0.04 [12]
decay time (ms)	1.5 ± 0.3 [14]	1.51 ± 0.28 [16]	1.67 ± 0.25 [17]	1.19 ± 0.08 [8]	1.2 ± 0.25 [9]	1.25 ± 0.18 [12]

These findings confirm the results obtained from ePSCs suggesting that NG2 does not influence kinetic properties of postsynaptic AMPA receptor currents. However, an NG2-independent acceleration of these current kinetics during development was observed.

5.3.7 Expression analysis of neuroligins by NG2 cells

So far, little is known about the molecular structure of the postsynaptic compartment in NG2 cells (Bergles et al., 2010). Although the NG2 protein constituted a promising candidate to mediate synaptic cell adhesion to presynaptic neurons (Trotter et al., 2010), the preceding results did not support a critical role for NG2 in glutamatergic synaptic transmission at neuron-NG2 cell synapses.

In neurons, the binding of presynaptic neurexins to postsynaptic neuroligins was shown to be involved in the regulation of synapse number, synapse specificity and synaptic strength (section 1.6) (Missler et al., 2012). To test whether synaptic cell adhesion between neurons and NG2 cells might similarly be mediated by the neuroligin-neurexin complex, expression of postsynaptic neuroligins by NG2 cells was investigated by performing single-cell RT-PCR. Expression was compared between juvenile and aged +/- and -/- animals to test for potential developmental or NG2-dependent alterations in the expression pattern. Experiments concentrated on the identification of neuroligin-1-3, as neuroligin-4 is only found at glycinergic synapses (Hoon et al., 2011) that were so far not demonstrated for hippocampal NG2 cells.

Fluorescent NG2 cells of juvenile and aged +/- and -/- mice were analyzed in the whole-cell configuration. Subsequently, the cytoplasm was harvested and a two-round RT-PCR was performed with primers specific for neuroligin-1-3. PDGFα receptor expression served as a positive control and cell type-specific marker. Cells negative for PDGFα receptor mRNA were excluded (detailed procedure in section 4.2).

Neuroligin-1-3 were expressed by the majority of juvenile and aged NG2 cells in +/- and -/- mice (Tab. 5.8, Fig. 5.17). An NG2-dependent effect was only observed in aged NG2 cells where neuroligin-3 was less often detected in the absence of NG2 (Fig. 5.17). Expression frequencies were similar between age groups indicating no developmental changes.

Neuroligin-1 and -2 were coexpressed by 81% of all NG2 cells tested in juvenile and 59% in aged mice (+/- and -/- cells pooled; aged n = 21, juvenile n = 22). All cells positive for neuroligin-1 expressed the short splice variant lacking the insert at splice site A (Fig. 1.6) except for three juvenile NG2 cells expressing both, the long and short variant, and two aged NG2 cells expressing only the long variant.

Tab. 5.8 Expression of neuroligin-1-3 in juvenile and aged NG2 cells. Data are given as relative frequencies. Cell numbers are given in square brackets.

	juvenile		**aged**	
	+/-	**-/-**	**+/-**	**-/-**
neuroligin-1 (%)	90 [10]	73 [11]	91 [11]	73 [11]
neuroligin-2 (%)	100 [10]	91 [11]	82 [11]	73 [11]
neuroligin-3 (%)	90 [10]	82 [11]	82 [11]	36 [11]

These data demonstrate that juvenile and aged hippocampal NG2 cells frequently express the synaptic cell adhesion proteins neuroligin-1-3.

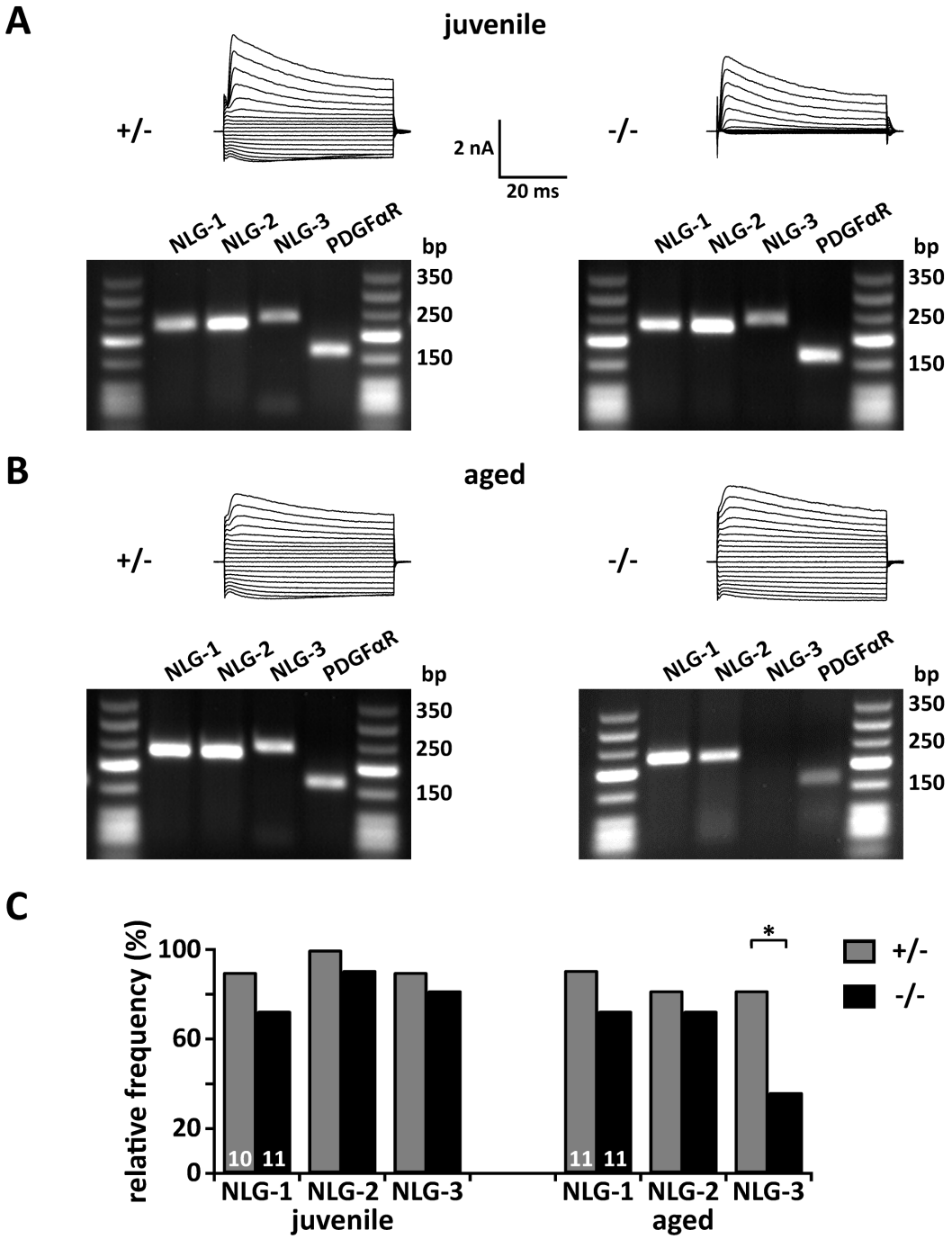

Fig. 5.17 Expression analysis of neuroligin-1-3 in NG2 cells by single-cell RT-PCR. (A, B) Agarose gels of neuroligin (NLG)-1-3 PCR-products of individual juvenile (A; left: p10, right: p9) and aged (B; left: p382, right: p372) +/- and -/- NG2 cells together with their respective current responses to de- and hyperpolarization between -160 and +20 mV (holding potential -80 mV). Expression of PDGFα receptor (PDGFαR) mRNA served as a positive control and cell type-specific marker. Low Molecular Weight DNA ladder served as size marker. **(C)** Summary bar graph of the relative frequency of NLG-1-3 expression of all NG2 cells tested. NLG-3 was less frequently detected in aged -/- NG2 cells (test #6). Asterisks indicate statistically significant differences. Cell numbers for each age group indicated at the base of bars.

6 Discussion

6.1 Functional properties of $GABA_A$ receptors in hippocampal NG2 cells

Research of the past decades has provided abundant evidence that glial cells are equally endowed with neurotransmitter receptors as neurons (Verkhratsky and Steinhäuser, 2000). However, their properties and physiological functions are still much less understood. Early studies identified $GABA_A$ receptors in oligodendrocytes and their precursors (putative NG2 cells) in culture (von Blankenfeld et al., 1991). These findings were later confirmed and complemented by *in situ* studies identifying $GABA_A$ receptor-mediated currents in complex cells (putative NG2 cells) in acute hippocampal slices (Steinhäuser et al., 1994a). Intriguingly, several groups even demonstrated that NG2 cells receive GABAergic synaptic input from interneurons in different brain regions (Lin and Bergles, 2004; Jabs et al., 2005; Velez-Fort et al., 2010). However, a detailed analysis of the physiological properties of NG2 cell $GABA_A$ receptors remained to be done (Bergles et al., 2010).

In the present study, molecular single-cell expression analysis combined with pharmacological modulation revealed that $GABA_A$ receptors of hippocampal NG2 cells resemble many properties of neuronal $GABA_A$ receptors in this brain region. Further experiments highlighted the selective presence of the γ2-subunit in synaptic $GABA_A$ receptors while being absent in extrasynaptic ones. For neocortical NG2 cells it could be demonstrated that the developmental switch from synaptic to extrasynaptic GABAergic transmission is accompanied by downregulation of γ2-subunit expression.

These results emphasize the significant role of the γ2-subunit in GABAergic synaptic signaling between neurons and NG2 cells and provide an important basis for future studies.

6.1.1 Hippocampal NG2 cells express a similar set of $GABA_A$ receptor subunits as neurons

The subunit composition of a given $GABA_A$ receptor determines its susceptibility to different modulators. Analysis of the modulatory action of a certain compound on $GABA_A$ receptor-mediated currents therefore allows the evaluation of the cell type-specific subunit

composition (overview in section 1.5.2) (Hevers and Luddens, 1998; Mehta and Ticku, 1999). By performing single-cell transcript analysis and pharmacological modulation *in situ*, the repertoire of $GABA_A$ receptor subunits expressed by hippocampal NG2 cells was investigated.

The barbiturate pentobarbital binds to the β-subunits that are present in all naturally occurring $GABA_A$ receptors described so far (Hadingham et al., 1993; Mehta and Ticku, 1999). As expected, pentobarbital enhanced the GABA-evoked response in all NG2 cells tested, demonstrating ubiquitous expression of functional β-subunit-containing $GABA_A$ receptors in hippocampal NG2 cells (Fig. 5.2). This is in line with a previous study showing potentiation of synaptic $GABA_A$ receptor currents by pentobarbital in hippocampal NG2 cells (Lin and Bergles, 2004).

Several modulators act via the benzodiazepine binding site located at the interface of α- and γ-subunits of $GABA_A$ receptors. As they usually prefer certain subunit combinations, they are frequently used to identify specific α- and γ-subunits. The benzodiazepine diazepam increased the GABA-evoked responses in all NG2 cells tested (Fig. 5.2) indicating frequent expression of the subunits α1-3 and 5 and γ1-3 and low expression of α-subunits 4 and 6 (Olsen and Sieghart, 2009). This is in line with the molecular expression analysis where the subunits α1 and 2 and γ1-3 were detected in the majority of cells while α4 was much less prevalent (Fig. 5.1). So far there are conflicting results in the literature concerning the modulation of NG2 cell $GABA_A$ receptors by benzodiazepines. While von Blankenfeld et al. (1991) demonstrated clear potentiation by diazepam, Williamson and colleagues (1998) claimed complete absence of benzodiazepine sensitivity of $GABA_A$ receptors in cultured oligodendrocyte precursor cells (putative NG2 cells). However, these studies were performed on different species (mice vs. rats) at different developmental time-points (embryo vs. neonatal) with different application techniques (fast vs. slow application), which might explain this discrepancy. Furthermore, both studies were performed in culture, while the present study investigated NG2 cells *in situ*. It is well known that different culture conditions may influence gene expression, which was demonstrated among others for neuronal $GABA_A$ receptors (Ives et al., 2002).

The non-benzodiazepine zolpidem particularly modulates $GABA_A$ receptors containing the subunits α1-3 and γ2 (Pritchett and Seeburg, 1990; Puia et al., 1991; McKernan et al., 1991;

Sanna et al., 2002) while being completely insensitive at γ1- and γ3-containing receptors (Wingrove et al., 1997). At high concentrations, zolpidem potentiated the GABA-evoked response in all NG2 cells tested. Application of a lower concentration which enhances the specificity for the α1-subunit (Pritchett and Seeburg, 1990; McKernan et al., 1991) still increased the response in the majority of NG2 cells (Fig. 5.2). These data indicate frequent expression of the subunits α1 and γ2 in hippocampal NG2 cells. However, the finding that high concentrations of zolpidem potentiated the currents in all cells, while lower concentrations also identified zolpidem-insensitive cells suggests that some NG2 cells express α2 and/or α3 at higher levels than α1 and γ1 and/or γ3 rather than γ2. This is supported by the RT-PCR data, showing high incidence of α1, α2 and γ1-3 expression (Fig. 5.1). These results are partly in conflict with data by Lin and Bergles (2004) who described diazepam-sensitive but zolpidem-insensitive $GABA_A$ receptors in hippocampal NG2 cells. This is characteristic for α5-containing receptors (Burgard et al., 1996). However, α5-transcripts were rarely detected in the present RT-PCR analysis (Fig. 5.1). It is worth noting, though, that Lin and Bergles (2004) worked with rats and used slightly older animals which might explain the diverging findings. Indeed, a developmental switch in zolpidem-sensitivity and subunit expression of $GABA_A$ receptors was recently shown for neocortical NG2 cells (Balia et al., 2013) and was partly investigated in the present study (section 5.2).

The inverse agonist DMCM usually decreases $GABA_A$ receptor currents, except at receptors containing the γ1- and/or β2/3-subunits where positive modulatory effects have been observed (Puia et al., 1991; Wafford et al., 1993; Stevenson et al., 1995). The effect of DMCM was variable in NG2 cells displaying potentiation, inhibition or no change in GABA-evoked currents (Fig. 5.2). This implies that receptors containing different β- and γ-subunits are coexpressed by individual NG2 cells at different levels. Positive and negative modulatory effects might then add up and possibly cancel each other out, as indicated by some cells showing no modulation by DMCM. This assumption is in line with the RT-PCR analysis where several cells coexpressed different β- and γ-subunits (section 5.1.1.1, Fig. 5.1). However, these results are in part contrary to earlier studies in cultured oligodendrocyte precursor cells (putative NG2 cells) that either showed inhibition in the majority of cells (von Blankenfeld et al., 1991) or no modulation at all in response to DMCM (Williamson et al., 1998). As mentioned before, these differences probably arise from the use of different

species (mice vs. rats), age groups (embryo/neonatal/postnatal) and experimental systems (culture vs. *in situ*). However, abundant presence of functional $GABA_A$ receptors containing the subunits β2/3 was further confirmed by the β2/3-subunit-specific modulator loreclezole (Wafford et al., 1994; Wingrove et al., 1994) which potentiated $GABA_A$ receptor currents in all NG2 cells tested (Fig. 5.2).

The endogenous $GABA_A$ receptor inhibitor Zn^{2+} is vesicularly released from a subset of glutamatergic terminals but was also found in GABA-containing vesicles of mossy fiber synapses (Ruiz et al., 2010; Toth, 2011). The inhibitory effect of Zn^{2+} on $GABA_A$ receptor currents is determined by the respective α- and γ-subunits of the receptor (Hevers and Luddens, 1998). Several studies using ectopic expression systems demonstrated that particularly γ2-subunit-containing $GABA_A$ receptors are much less inhibited by Zn^{2+} (Draguhn et al., 1990; Smart et al., 1991; Hosie et al., 2003). To test for a similar correlation of Zn^{2+} sensitivity with $GABA_A$ receptor subunit composition in NG2 cells, single-cell RT-PCR was performed subsequent to pharmacological modulation *in situ*. The results showed that hippocampal NG2 cells were heterogeneous with respect to their Zn^{2+} sensitivity. Despite some variability, NG2 cells lacking γ2 displayed a higher Zn^{2+} sensitivity compared to those expressing γ2 (Fig. 5.4) which is in line with the ectopic expression analysis mentioned before (Draguhn et al., 1990; Smart et al., 1991; Hosie et al., 2003). Similar results were obtained for cerebellar granule cells and cortical interneurons where pharmacological modulation combined with single-cell RT-PCR revealed that γ2-expressing cells were less Zn^{2+}-sensitive (Alsbo et al., 2001). The molecular analysis of the present study further showed that the subunits α1 and α5 were less abundant in the γ2-lacking Zn^{2+}-sensitive NG2 cells. This is consistent with a report demonstrating that α1/γ2-containing $GABA_A$ receptors are even less Zn^{2+}-sensitive compared to other α-subunits coexpressed with γ2 (White and Gurley, 1995).

However, the data collected here are in conflict with an earlier study investigating Zn^{2+} sensitivity in cultured oligodendrocyte precursor cells (putative NG2 cells) that reported a strong inhibition by Zn^{2+} in all cells tested. In the same study the authors performed RT-PCR of whole cultures showing expression of γ2 and a complete lack of α1 expression (Williamson et al., 1998). However, it has to be considered that they used purified cells from

neonatal rats (p1-2) and cultured these for 7 days prior to investigation. As discussed before, culture conditions (Ives et al., 2002) and species-dependent differences may account for the observed deviations. Moreover, it is particularly important to consider the difference in age since the present study demonstrated considerable developmental changes in $GABA_A$ receptor subunit expression of neocortical NG2 cells (section 5.2) that might as well occur in the hippocampus. A developmental decrease in Zn^{2+} sensitivity was also observed in hippocampal neurons (Martina et al., 1996) presumably due to increasing expression of the subunits α1/γ2 (Laurie et al., 1992; Brooks-Kayal et al., 1998).

In general, for the interpretation of these kind of experiments it has to be considered that the half-life of $GABA_A$ receptor subunit mRNA is substantially shorter than degradation of the corresponding protein (Lyons et al., 2000). This might have contributed to the variability in Zn^{2+} sensitivity among the γ2 mRNA-lacking cells. In this regard, one also has to keep in mind that a single transcript is theoretically sufficient to give a positive signal in RT-PCR, while the corresponding subunit might only be part of a minority of functional receptors.

Taken together, the results obtained from pharmacological modulation and molecular RT-PCR analysis suggest that juvenile hippocampal NG2 cells predominantly express the subunits α1, α2, β2, β3, γ1 and γ2. However, due to the observed heterogeneity of mRNA expression and pharmacological responses it is likely that transcellular differences in subunit expression among the juvenile NG2 cell population occur.

Among neurons, $GABA_A$ receptor subunit compositions vary substantially between different brain regions, types of neurons and individual cells. Even in the same cell $GABA_A$ receptors with different subunit compositions are coexpressed and are subject to differential subcellular localization (Farrant and Nusser, 2005). Immunohistochemical as well as electron microscopical studies support the notion that $GABA_A$ receptors of the subunit combination α1β2γ2 are the most abundant in the mammalian brain (Pirker et al., 2000; Olsen and Sieghart, 2009). In the hippocampus, developing neurons primarily express the subunits α1, α2, α5, β1 and γ2 (Brooks-Kayal et al., 1998). Similar results were obtained in the present study for juvenile hippocampal NG2 cells, as the subunits α1, α2, β1, β2 and γ2 were frequently expressed. The α5-subunit which is important for tonic currents in hippocampal neurons (Glykys et al., 2008) seems to be less relevant in NG2 cell, as it was rarely detected

by single-cell RT-PCR (Fig. 5.1). Nevertheless, $GABA_A$ receptors of hippocampal NG2 cells resemble many properties of neuronal receptors in this brain region. A detailed discussion on expression and pharmacological properties of synaptic vs. extrasynaptic receptors in NG2 cells vs. neurons is given in section 6.1.2.

$GABA_A$ receptors are also expressed by other glial cell types throughout the brain. Despite some similarities there are distinct differences in the $GABA_A$ receptor subunit expression pattern compared to NG2 cells.

Oligodendrocytes express functional $GABA_A$ receptors at lower levels as their precursors, NG2 cells (von Blankenfeld et al., 1991; Berger et al., 1992; Pastor et al., 1995). Experiments comparing pharmacological properties of oligodendrocytes and NG2 cells suggest that subunit composition is not markedly changed after maturation of NG2 cells to oligodendrocytes. $GABA_A$ receptor currents were still potentiated by pentobarbital and benzodiazepines and showed inhibition by DMCM in the majority of oligodendrocytes (von Blankenfeld et al., 1991). However, a more detailed analysis of the $GABA_A$ receptor subunit composition and function in oligodendrocytes is lacking so far.

In cultured astrocytes, $GABA_A$ receptor-mediated currents are potentiated by pentobarbital, diazepam and zolpidem (Bormann and Kettenmann, 1988; Bovolin et al., 1992; Rosewater and Sontheimer, 1994; Fraser et al., 1995). However, the modulatory action of DMCM was diverging in these studies. While Bormann and Kettenmann (1988) and Bovolin and colleagues (1992) demonstrated potentiation of $GABA_A$ receptor-mediated currents in cultured astrocytes, Rosewater and Sontheimer (1994) as well as Fraser and colleagues (1995) both showed differential effects with potentiation or inhibition by DMCM. These results indicate that astrocytes are heterogeneous with respect to the expression of different γ-subunits similar to NG2 cells as demonstrated in the present study. The subunits α1 and β1 were immunohistochemically detected on a subset of astrocytic processes (Fraser et al., 1995). Furthermore, primary astrocyte cultures were shown to express mRNA for the subunits α1, α2, β1, β3, and γ1 while lacking the subunit γ2 (Bovolin et al., 1992). This is in line with the idea that the γ2-subunit is mainly part of synaptic $GABA_A$ receptors (Lüscher and Keller, 2004) that are absent in astrocytes in contrast to NG2 cells (section 6.1.2) (Bergles et al., 2010). Bergmann glia, a type of astrocytes in the cerebellum, differ from

cerebral astrocytes and NG2 cells in $GABA_A$ receptor subunit composition as they were insensitive to diazepam indicating complete lack of γ-subunits in these cells (Müller et al., 1994). However, other groups demonstrated the presence of α2 and γ1 mRNA and protein on Bergmann glia cells (Wisden et al., 1989; Laurie et al., 1992; Riquelme et al., 2002). For glial Müller cells of the human and skate retina, $GABA_A$ receptor currents sensitive to pentobarbital and diazepam were shown (Qian et al., 1996; Biedermann et al., 2004). Intriguingly, in both studies these receptors were positively modulated by Zn^{2+} coapplication. A potentiation by Zn^{2+} is only known for AMPA receptors, while GABA, NMDA and kainate receptors are usually inhibited by Zn^{2+} (Toth, 2011). However, $GABA_A$ receptor expression in Müller cells seems to vary drastically between species as in other animals no $GABA_A$ receptor currents were detected in Müller cells at all (Biedermann et al., 2004).

The expression of $GABA_A$ receptors by microglia is still under debate. They do react to GABA or the specific $GABA_A$ receptor antagonist bicuculline by changes in their process motility. However, this is probably an indirect effect in response to GABA-evoked ATP-release from surrounding cells as microglia did not show direct membrane currents in response to GABA (Fontainhas et al., 2011). Only one report claimed the expression of $GABA_A$ receptor subunits α1, α3 and β1 in cultured human microglia (Lee et al., 2011). Nevertheless, no $GABA_A$ receptor-mediated currents were demonstrated in microglial cells so far doubting their functional expression.

It is worth mentioning that oligodendrocyte progenitors (putative NG2 cells) might also express functional $GABA_B$ receptors (Luyt et al., 2007). For neurons, it was demonstrated that $GABA_B$ receptors may activate K^+ conductances (Sodickson and Bean, 1996) and even modulate $GABA_A$ receptor-mediated currents in some brain regions (Tao et al., 2013). However, in hippocampal complex cells (putative NG2 cells) the $GABA_B$ receptor agonist baclofen did not elicit currents (Bekar et al., 1999). Furthermore, the present study revealed that coapplication of $GABA_B$ receptor antagonists did not affect GABA-evoked currents in hippocampal NG2 cells (Fig. 5.3). Thus, it is unlikely that $GABA_B$ receptors, if expressed by NG2 cells, modulate $GABA_A$ receptor-mediated currents in these cells.

In conclusion, $GABA_A$ receptors of hippocampal NG2 cells share many properties with neuronal $GABA_A$ receptors including prominent expression of the γ2-subunit. As opposed to this, other glial cell types largely lack this subunit. This is reasonable considering that the γ2-subunit is important for postsynaptic clustering of $GABA_A$ receptors and NG2 cells are the only glial cells that possess postsynaptic densities.

6.1.2 The γ2-subunit is selectively located in synaptic $GABA_A$ receptors

In neurons, $GABA_A$ receptors may localize to postsynaptic densities to mediate phasic synaptic currents or to extrasynaptic compartments to mediate tonic currents (Farrant and Nusser, 2005). While the presence of phasic $GABA_A$ receptor currents in NG2 cells was unambiguously demonstrated by several groups (Lin and Bergles, 2004; Jabs et al., 2005; Velez-Fort et al., 2010), the presence of tonic $GABA_A$ receptor currents is still under debate. Tong and colleagues (2009) showed prominent tonic $GABA_A$ receptor currents in juvenile hippocampal NG2 cells, which was not detected by Lin and Bergles (2004) and Mangin et al. (2008) in hippocampal or by Velez-Fort et al. (2010) in neocortical NG2 cells. In the present study, small but distinct tonic currents were observed in juvenile hippocampal NG2 cells. Intriguingly, application of the $GABA_A$ receptor antagonists bicuculline or gabazine differentially affected the tonic current amplitude. While bicuculline uncovered a tonic current in all cells tested, gabazine induced only a slight positive shift of the holding current in 4/6 NG2 cells (Fig. 5.5). Interestingly, the other study that detected tonic $GABA_A$ receptor currents in NG2 cells used bicuculline (Tong et al., 2009) while the studies that found no tonic $GABA_A$ receptor currents used gabazine (Lin and Bergles, 2004; Mangin et al., 2008; Velez-Fort et al., 2010). What could be the reason for the discrepancy between the two antagonists and the diverging results of the mentioned publications?

Several studies indicate that bicuculline and gabazine differentially influence $GABA_A$ receptor-mediated tonic currents. In summary, these reports demonstrate that in addition to ambient GABA, part of the tonic current is mediated by spontaneous channel opening. As bicuculline was able to block the GABA-induced as well as the spontaneous component of the tonic current, it was suggested that bicuculline acts as an inverse agonist being able to inhibit channel opening even in the absence of GABA. In contrast, gabazine only blocked the GABA-induced component indicating that it acts as a competitive antagonist with negligible

negative efficacy (Bai et al., 2001; Yeung et al., 2003; McCartney et al., 2007; Wlodarczyk et al., 2013). On the basis of these findings, the results of the present study suggest that the small positive shift induced by gabazine might represent the tonic current evoked by extracellular GABA, while the bicuculline-sensitive tonic current might additionally include the component mediated by spontaneous channel opening. However, to clarify this issue additional experiments are required. Interestingly, although denying the presence of a tonic current in neocortical NG2 cells, Velez-Fort and colleagues (2010) did detect a small positive shift by gabazine which is similar to the value of the present study (here: 2.5 ± 1.1 pA in 4/6 cells tested, section 5.1.3, Fig. 5.7; Velez-Fort et al. (2010): 2.5 ± 9.8 pA, n = 4). Furthermore, their value displays a considerable standard error of the mean indicating that some cells did exhibit a distinct tonic current. Furthermore, Balia and colleagues (2013) demonstrated a gabazine-sensitive tonic current in juvenile neocortical NG2 cells in the presence of 5 µM GABA in the extracellular solution. This further supports the idea that NG2 cells are able to sense ambient GABA via extracellular $GABA_A$ receptors. Nevertheless, the presence and role of tonic $GABA_A$ receptor currents in the CNS is still controversially debated and the results between studies vary drastically depending on cell type, brain region and experimental approach (Farrant and Nusser, 2005). The present study, however, revealed that NG2 cells are able to sense elevations of the ambient GABA concentration which might influence their physiology under normal and pathological conditions.

It is known that the synaptic vs. extrasynaptic targeting of $GABA_A$ receptors is influenced by the specific subunit composition of the receptor. Synaptic $GABA_A$ receptors of neurons in different brain regions are primarily composed of α1-3, β2/3 and γ2, while α4-6 and δ are considered to be mainly extrasynaptic (Lüscher and Keller, 2004; Farrant and Nusser, 2005). Especially the γ2-subunit was subject to intensive investigation since it was identified as an important interacting partner of the synaptic scaffolding protein gephyrin. γ2-knockout animals displayed severe deficits in GABAergic synaptic signaling due to a dysfunction of postsynaptic $GABA_A$ receptor clustering (Essrich et al., 1998; Schweizer et al., 2003). However, the γ2-subunit is not necessary for the assembly of functional $GABA_A$ receptors and extrasynaptic expression of other $GABA_A$ receptor subunits was largely unaltered in γ2-knockout cells (Günther et al., 1995).

In the present study, pharmacological modulation of synaptic vs. extrasynaptic $GABA_A$ receptor currents with diazepam and zolpidem was performed to investigate whether a preferential occurrence of the γ2-subunit in synaptic $GABA_A$ receptors similarly applies to NG2 cells. As discussed in the preceding section, modulation by diazepam is rather independent of the particular α- and γ-subunits while zolpidem at low concentrations is highly specific for α1/γ2-containing receptors and thus suited to detect presence of the γ2-subunit (Olsen and Sieghart, 2009). To facilitate a better resolution of potential modulatory effects on the tonic current, the extracellular GABA concentration was enhanced by blocking GABA transporters. This resulted in a considerable increase in the bicuculline-sensitive current (section 5.1.3) further corroborating the finding that NG2 cells are able to sense ambient GABA via extrasynaptic $GABA_A$ receptors.

The pharmacological modulation revealed that the majority of NG2 cells possess synaptic $GABA_A$ receptors that are sensitive to diazepam and zolpidem. In contrast, extrasynaptic $GABA_A$ receptor currents were potentiated by diazepam but not by zolpidem (section 5.1.3, Fig. 5.6 and Fig. 5.7). Hence, these data suggest that NG2 cells exhibit synaptic $GABA_A$ receptors that mostly contain the subunits α1 and γ2 while extrasynaptic receptors lack these subunits. The finding that diazepam modulated tonic $GABA_A$ receptor currents shows that extrasynaptic receptors contain γ-subunits as they are necessary for occurrence of the benzodiazepine binding site. Therefore, it is likely that γ1- and γ3-containing receptors are present in extracellular $GABA_A$ receptors in hippocampal NG2 cells. Similar results were obtained from studies comparing the differential subcellular localization of $GABA_A$ receptor subunits in neurons, although cell type-specific differences occurred. Potentiation of synaptic $GABA_A$ receptor currents by diazepam (or midazolam, a similar benzodiazepine) and zolpidem was shown by several studies on neurons of different brain regions (Bai et al., 2001; Hamann et al., 2002; Nusser and Mody, 2002; Caraiscos et al., 2004). This is expected since the γ2-subunit is essential for clustering of synaptic $GABA_A$ receptors (Essrich et al., 1998; Schweizer et al., 2003). The effect of these drugs on tonic currents, however, is variable. Some studies found no effect of diazepam and zolpidem on tonic currents (Hamann et al., 2002; Nusser and Mody, 2002; Caraiscos et al., 2004; Mangan et al., 2005). This might be explained by the fact that in some cell types and brain regions the γ-subunit is replaced by the δ-subunit in extrasynaptic receptors removing the benzodiazepine-binding site

(Farrant and Nusser, 2005). In the juvenile hippocampus the δ-subunit is rarely expressed suggesting that extrasynaptic $GABA_A$ receptors contain γ-subunits at this developmental time point (Laurie et al., 1992). Accordingly, Bai and colleagues (2001) as well as Yeung and colleagues (2003) demonstrated midazolam-sensitive tonic currents in hippocampal neurons indicating the presence of extrasynaptic $GABA_A$ receptors containing γ-subunits. Another study even demonstrated the presence of zolpidem-sensitive tonic currents in hippocampal interneurons, while CA1 pyramidal neurons lacked this sensitivity. Additionally, in both cell types zolpidem modulated synaptic $GABA_A$ receptor currents. These results indicate presence of the γ2-subunit in synaptic and extrasynaptic $GABA_A$ receptors of hippocampal interneurons while CA1 pyramidal neurons contain this subunit solely in synaptic receptors (Semyanov et al., 2003).

Only a few studies investigated the subunit composition of synaptic $GABA_A$ receptors in NG2 cells and no information about extrasynaptic receptors is available so far. In agreement with the present study, Balia and colleagues (2013) demonstrated diazepam- and zolpidem-sensitive synaptic $GABA_A$ receptors in juvenile neocortical NG2 cells, indicating presence of the subunits α1 and γ2. However, another study showed diazepam-sensitive but zolpidem-insensitive synaptic $GABA_A$ receptors in hippocampal NG2 cells. Based on this pharmacology, the authors suggested presence of the α5-subunit in these receptors (Lin and Bergles, 2004). α5-subunit-containing synaptic $GABA_A$ receptors were also demonstrated for the majority of neocortical NG2 cells (Balia et al., 2013). The molecular analysis of the present study did not support prominent expression of the α5-subunit in juvenile hippocampal NG2 cells (Fig. 5.1). However, the experiments combining Zn^{2+} sensitivity and single-cell RT-PCR revealed frequent coexpression of γ2 with α1 and α5 in a subset of NG2 cells (Fig. 5.4). Considering that α5 expression was variable in neocortical NG2 cells as well (Balia et al., 2013), these data might indicate that the α5-subunit is expressed by a subpopulation of juvenile NG2 cells in different brain regions. In this respect, it is interesting that a study by Ali and Thomson (2008) revealed that α1- and α5-containing $GABA_A$ receptors are located in different synapses in neocortical pyramidal cells. Intriguingly, they further demonstrated that these postsynaptic sites are targeted by different types of interneurons. A similar mechanism might also be valid for NG2 cells as they potentially possess α1- as well as α5-subunit-

containing synaptic $GABA_A$ receptors in the hippocampus and neocortex (Lin and Bergles, 2004; Passlick et al., 2013; Balia et al., 2013).

The data from the present study demonstrate that synaptic $GABA_A$ receptors in juvenile hippocampal NG2 cells contain the subunits α1 and γ2. Furthermore, NG2 cells are able to sense ambient GABA with extrasynaptic receptors lacking these subunits.

6.1.3 GABA causes depolarization in NG2 cells

GABA may act as an inhibitory/hyperpolarizing or excitatory/depolarizing neurotransmitter, depending on $[Cl^-]_i$. In neurons, a developmental switch from a depolarizing to a hyperpolarizing action of GABA was demonstrated during the first two postnatal weeks (Cherubini et al., 1991). The underlying change in $[Cl^-]_i$ is due to the upregulation of KCC2 that transports Cl^- out of the cell and the downregulation of NKCC1 that transports Cl^- into the cell (Rivera et al., 1999; Stein et al., 2004; Yamada et al., 2004; Khirug et al., 2008). The expression of KCC2 was also demonstrated for oligodendrocytes of the optic nerve (Malek et al., 2003), while any evidence for expression by NG2 cells is lacking so far. In contrast, it was shown that juvenile and adult NG2 cells express NKCC1 indicative of a high $[Cl^-]_i$ (Price et al., 2006; Tong et al., 2009). A few studies tried to estimate the $[Cl^-]_i$ of NG2 cells by performing voltage-clamp-based reversal potential analysis of $GABA_A$ receptors. Although all studies demonstrated that $GABA_A$ receptor activation causes depolarization in NG2 cells, the measured reversal potentials and thus the calculated $[Cl^-]_i$ were inconsistent (Lin and Bergles, 2004; Tong et al., 2009; Tanaka et al., 2009). Furthermore, an increasing negative shift of the reversal potential during GABA application indicative of a change in $[Cl^-]_i$ was observed in the voltage-clamp configuration (Passlick et al., 2013). In neurons it was shown that $GABA_A$ receptor activation is sufficient to induce persistent changes in $[Cl^-]_i$ (DeFazio and Hablitz, 2001; Karlsson et al., 2011). Thus, depletion of intracellular Cl^-, due to the permanent $GABA_A$ receptor activation while clamping the cell to the holding potential, might account for this shift (Passlick et al., 2013). In the present study, brief pulses of the specific $GABA_A$ receptor agonist muscimol were applied while clamping the cell to different potentials. Between each voltage step, the cell was kept at -70 mV for 15 s to reequilibrate the $[Cl^-]_i$. However, the value gained by this approach was even more negative compared to

the shifted one (-56 and -45 mV, respectively) (Passlick et al., 2013). This indicates that depletion of Cl^- could probably not be circumvented by the modified experimental conditions. By using a similar approach Lin and Bergles (2004) measured a reversal potential of -44 mV, corresponding to a $[Cl^-]_i$ of ~21 mM for juvenile hippocampal NG2 cells which is close to the shifted value of -45 mV (Passlick et al., 2013). The risk of depletion of intracellular Cl^- may be reduced by employing a current-clamp-based approach to estimate the reversal potential. A similar experiment resulted in a reversal potential of -27 mV corresponding to a $[Cl^-]_i$ of ~50 mM in juvenile hippocampal NG2 cells, which considerably deviates from the voltage-clamp-based experiments (Passlick et al., 2013).

Another study analyzing the $[Cl^-]_i$ of juvenile hippocampal NG2 cells reported a reversal potential of -34 mV with a corresponding $[Cl^-]_i$ of ~35 mM (Tong et al., 2009). The authors also demonstrated that blocking NKCC1 shifted the reversal potential to more negative values suggesting that NKCC1 is responsible for the high $[Cl^-]_i$, similar to immature neurons (Yamada et al., 2004). Tanaka and colleagues (2009) investigated adult cortical NG2 cells and demonstrated a reversal potential of -31 mV corresponding to a $[Cl^-]_i$ of ~45 mM, which is close to the value measured in the current-clamp mode for juvenile hippocampal NG2 cells (Passlick et al., 2013), although they employed a voltage-clamp-based experiment.

The variability of the $[Cl^-]_i$ values in the different studies illustrates the difficulty in determining the correct reversal potential/$[Cl^-]_i$. Besides the already mentioned phenomenon of depletion or accumulation of Cl^-, other issues have to be considered. Naturally, these experiments have to be performed in the perforated patch configuration leaving the $[Cl^-]_i$ intact. To ensure this, the experiment should be repeated with the same pipette and bath solution in the whole-cell configuration. In this case, internal and external Cl^- concentrations are known and the exact reversal potential can be calculated. The measured reversal potential should then be close to the calculated one but different to the one measured in perforated patch configuration, thereby suggesting that $[Cl^-]_i$ was not disturbed. Furthermore, this control experiment provides information whether the membrane potential was accurately corrected for liquid junction potential. Incorrect adjustment of the membrane potential leads to completely different and false reversal potentials and thereby $[Cl^-]_i$. Consequently, with this control experiment two big pitfalls associated with the correct determination of the reversal potential/$[Cl^-]_i$ can be avoided and

should be performed in every study. In the present study, this control experiment yielded the calculated reversal potential suggesting that perforated patch recordings and correction of liquid junction potential were adequately performed (Fig. 5.8).

The comparison of voltage-clamp- with current-clamp-based reversal potential analysis revealed that the $[Cl^-]_i$ of juvenile hippocampal NG2 cells is presumably higher than previously thought (Passlick et al., 2013). Furthermore, perforated patch experiments by Tanaka et al. (2009) as well as Tong et al. (2009) demonstrated that GABA activity remains excitatory during development in different brain regions. This is in contrast to neurons, which show a switch from an excitatory to an inhibitory action of GABA during postnatal development (Cherubini et al., 1991) and might have important consequences for NG2 cell physiology (discussed in section 6.1.5).

6.1.4 $GABA_A$ receptor subunit expression is developmentally regulated in neocortical NG2 cells

Vélez-Fort and colleagues (2010) recently demonstrated that NG2 cells in the somatosensory neocortex experience a switch from a synaptic to an extrasynaptic mode of GABAergic transmission during postnatal development. The authors showed that GABAergic synaptic activity decreases during the second postnatal week and completely disappears at later developmental stages. This decline in synaptic connectivity is accompanied by the emergence of a type of extrasynaptic communication that is mediated by GABA spillover. Whether this functional switch is accompanied by molecular changes was unknown so far and investigated in the present study.

Synaptic and extrasynaptic $GABA_A$ receptors are assembled from different subunits, thus varying in their pharmacological and biophysical properties. Although no exclusively synaptic or extrasynaptic subunits were identified so far, most reports suggest that the subunits α1-3, β2/3 and γ2 are mainly synaptic, while α4-6 and δ are primarily located at extrasynaptic sites (Lüscher and Keller, 2004; Farrant and Nusser, 2005). The γ2-subunit is particularly important for synaptic $GABA_A$ receptors as it interacts with the synaptic scaffolding protein gephyrin and is essential for clustering of synaptic $GABA_A$ receptors (Essrich et al., 1998; Schweizer et al., 2003). In line with this, the present study revealed a distinct subcellular

localization pattern of γ2-containing $GABA_A$ receptors in juvenile hippocampal NG2 cells. While synaptic $GABA_A$ receptors largely contain the γ2-subunit, it is virtually absent in extrasynaptic ones (section 5.1.3). Assuming that γ2-containing $GABA_A$ receptors of neocortical NG2 cells exhibit a similar localization pattern, then γ2-expression should be dispensable and thus downregulated during development when synaptic GABAergic activity decreases (Velez-Fort et al., 2010).

So far no data on the subunit composition of neocortical NG2 cells during development are available. Therefore, in this study single-cell RT-PCR was employed to compare the $GABA_A$ receptor subunit expression of neocortical NG2 cells in the second and fourth postnatal week. The transcript analysis revealed a certain degree of heterogeneity among the NG2 cell population in both age groups. However, distinct developmental changes in subunit expression were identified. In juvenile NG2 cells the subunits α1, α2, α5, β2, β3, γ1, γ2 and γ3 were most frequently detected, which is similar to juvenile hippocampal NG2 cells (section 5.1.1). In the fourth postnatal week α2 and α5 transcripts were less often detected while the expression of α3 and α4 increased compared to the second postnatal week. Importantly, the analysis revealed a pronounced decrease of γ2-subunit expression (Fig. 5.9). These changes on the transcript level were also reflected by the properties of functional $GABA_A$ receptors during postnatal development as shown by Balia and colleagues (2013). Indeed, the authors demonstrated that the subunits α5 and γ2 were frequently present in synaptic $GABA_A$ receptors of neocortical NG2 cells during the second postnatal week as ePSCs were sensitive to the α5-specific modulator α5IA and the α1/γ2-specific modulator zolpidem. During the fourth postnatal week the same modulators were ineffective, indicating that $GABA_A$ receptors lacked α5 and γ2 at this later stage. The upregulation of the α4-subunit could not be verified by pharmacology as the specific modulator furosemide had no effect on evoked GABAergic currents in the second and fourth postnatal week. However, the effect of furosemide on α4-containing $GABA_A$ receptors was investigated on α4γ2- and α4δ-containing receptors (Wafford et al., 1996). The present RT-PCR analysis suggested frequent expression of γ1 and γ3 while γ2 and δ were virtually absent in older NG2 cells. Therefore, the pharmacology determined by Wafford and colleagues (1996) might not be valid for α4γ1- or α4γ3-containing receptors as they probably occur in adult neocortical NG2 cells.

In general, the developmental changes in expression profile of $GABA_A$ receptor subunits by NG2 cells are similar to the overall changes in the neocortex during this period (Laurie et al., 1992; Golshani et al., 1997). Over the first two postnatal weeks transcripts for α1, α2, α5, β1, β2, β3, and γ1 are most abundant in the neocortex. Afterwards, α2, α5, and β1 expression decreases, while α1, β2, β3, and γ1 levels remain constant in adult animals. Expression of the δ-subunit, present in extrasynaptic $GABA_A$ receptors in some neurons, is low in the juvenile neocortex but increases at later developmental stages (Laurie et al., 1992). This expression pattern is similar to what was found in the present study for neocortical NG2 cells, however, the subunits α3, α4, γ2 and γ3 exhibit a cell type-specific developmental regulation differing from the overall neocortex. While α3 and α4 were rarely detected during the second postnatal week in NG2 cells, their expression increased during the fourth postnatal week. In the neocortex α3 and α4 expression peaks during the first two postnatal weeks and declines thereafter. γ3 expression was constant in NG2 cells, while it is low in the adult neocortex (Laurie et al., 1992). As the γ2-subunit is important for clustering of postsynaptic $GABA_A$ receptors (Essrich et al., 1998; Schweizer et al., 2003) its expression stays high throughout development in the neocortex (Laurie et al., 1992) which is in contrast to NG2 cells exhibiting a strong decrease in γ2 expression.

As discussed earlier (section 6.1.1), data on expression of the γ2-subunit by NG2 cells is controversial in the literature so far. Experiments by von Blankenfeld and colleagues (1991) suggested the presences of the γ2-subunit in $GABA_A$ receptors of NG2 cells, whereas results by Williamson et al. (1998) claimed its absence. The present study, however, revealed heterogeneity of γ2 expression in juvenile hippocampal and neocortical NG2 cells. Furthermore, a developmental downregulation of the γ2-subunit in neocortical NG2 cells was shown. This heterogeneity and age-dependence probably contributed to the inconsistency in the literature.

In summary, these data demonstrate that the switch from synaptic to extrasynaptic GABAergic transmission in neocortical NG2 cells is accompanied by the downregulation of the γ2-subunit, a major component of synaptic $GABA_A$ receptors. Furthermore, neocortical NG2 cells exhibit a specific subunit expression profile differing from other cells in this brain region. These findings widen and complement other data of the present study on the

specific localization of the γ2-subunit in synaptic $GABA_A$ receptors of hippocampal NG2 cells. Consequently, expression of the γ2-subunit may be considered as a molecular marker of direct synaptic GABAergic transmission onto NG2 cells and provides an interesting target for future studies investigating the role of GABAergic signaling in NG2 cells. The developmental changes also indicate that GABAergic transmission has a distinct function in juvenile vs. adult neocortical NG2 cells.

6.1.5 Possible roles of $GABA_A$ receptor signaling in NG2 cells

While the role of GABAergic signaling is quite established in neuronal networks, its function in NG2 cell physiology remains largely unknown. Similar to immature neurons, $GABA_A$ receptor activation causes depolarization in NG2 cells (section 6.1.3) (Lin and Bergles, 2004; Tanaka et al., 2009; Tong et al., 2009; Passlick et al., 2013). This depolarization was shown to elicit Ca^{2+} transients in NG2 cells, although the cellular mechanism of this Ca^{2+} elevation is still under debate. Several groups demonstrated that depolarization by focal application of GABA activates voltage-gated Ca^{2+} channels in NG2 cells (Kirchhoff and Kettenmann, 1992; Tanaka et al., 2009; Haberlandt et al., 2011). In contrast, Tong and colleagues (2009) recently suggested that GABA evokes a persistent Na^+ current which induces Ca^{2+} influx through the reversed activity of the Na^+/Ca^{2+} exchanger (NCX). This discrepancy is even intensified by the fact that Haberlandt et al. (2011) demonstrated that blocking NCX has no effect on depolarization-induced Ca^{2+} currents while Tong et al. (2009) and Ge et al. (2006b) claimed that NG2 cells lack voltage-gated Ca^{2+} channels. Although it is difficult to estimate whether these mechanisms functionally exist *in vivo*, the primary finding that GABA-evoked depolarizations induce Ca^{2+} elevations in NG2 cell is generally accepted as also shown by further studies (Bernstein et al., 1996; Velez-Fort et al., 2010).

For immature neurons where GABA is depolarizing, it was shown that GABA-induced Ca^{2+} signaling facilitates diverse crucial functions. In premature and adult neuronal progenitors excitation by GABA was reported to regulate neurogenesis and neural differentiation (Nguyen et al., 2003; Tozuka et al., 2005; Ge et al., 2006a; Young et al., 2012). Another study even demonstrated that depolarization of neuronal progenitors by GABA directly modified DNA synthesis indicating an influence on the cell cycle of developing neurons (LoTurco et al., 1995). Similarly, GABA might regulate proliferation and differentiation of NG2 cells. This idea

is supported by the observation that GABAergic innervation of neocortical NG2 cells is higher during the second postnatal week when oligodendrogenesis peaks as compared to later developmental stages (Velez-Fort et al., 2010). However, GABAergic signaling in NG2 cells mainly occurs in gray matter areas where oligodendrogenesis is less prominent suggesting that GABA rather inhibits differentiation. This is consistent with the observation that NG2 cells maintain GABAergic innervation during cytokinesis and that synapses are equally allocated to the daughter cells (Kukley et al., 2008; Ge et al., 2009). In contrast, studies by Gallo and colleagues (1996) and Yuan and colleagues (1998) argue against a role of $GABA_A$ receptor activation in proliferation and differentiation as incubation of cultured NG2 cells or cerebellar slices with GABA or $GABA_A$ receptor antagonists did not influence these processes in NG2 cells.

Besides proliferation and differentiation, other studies demonstrated a role for $GABA_A$ receptor-mediated excitation in migration of neuronal progenitors (Fueshko et al., 1998; Bolteus and Bordey, 2004; Wang and Kriegstein, 2009). During development and in response to injury NG2 cells migrate over considerable distances (Sugimoto et al., 2001; Aguirre and Gallo, 2004; Aguirre et al., 2007). The guiding cues, however, are largely unknown. Tong and colleagues (2009) suggested a role for $GABA_A$ receptor activation in NG2 cell migration, although the cellular mechanism that was proposed is controversial, as discussed before (Haberlandt et al., 2011). However, it is well known that GABA is one of the most abundant neurotransmitters in the developing CNS (Nguyen et al., 2001). Apart from the concrete intracellular signaling cascade, it might well be that GABA gradients influence the migration and physiology of NG2 cells.

In immature neurons, GABA exerts many of its effects via depolarization-induced activation of voltage-gated Ca^{2+} channels (Ben-Ari et al., 2007). It is, however, uncertain how NG2 cells *in vivo* may sufficiently be depolarized to activate voltage-gated Ca^{2+} channels. Due to the very negative resting potential and the small GABAergic synaptic depolarizations received at low frequency, it seems unlikely that synaptic innervation under normal conditions is sufficient to reach the threshold for the activation of voltage-gated Ca^{2+} channels. This notion is supported by the finding that physiological stimulation is insufficient to elicit Ca^{2+} signals at the soma of NG2 cells (Velez-Fort et al., 2010; Haberlandt et al., 2011). However, in the thin processes of these cells the depolarization might be high enough to activate local

Ca^{2+} microdomains. This process might even be amplified by Ca^{2+}-induced Ca^{2+}-release from local endoplasmatic reticulum stores (Haberlandt et al., 2011). Such local Ca^{2+} elevations could for example regulate cytoskeletal dynamics of the highly motile NG2 cell processes (Haberlandt et al., 2011; Hughes et al., 2013). Similar mechanisms are known from immature neurons where $GABA_A$ receptor activation leads to local Ca^{2+} elevations from endoplasmatic reticulum stores that regulate the motility of dendritic filopodia (Lohmann et al., 2005). This is further supported by another study which demonstrated that preventing the depolarizing effect of tonic and phasic $GABA_A$ receptor activation on immature neurons leads to impaired morphological maturation of these cells (Cancedda et al., 2007). Therefore, it is quite reasonable that $GABA_A$ receptor activation elicits local Ca^{2+} transients that mediate the activity-dependent movement of NG2 cell processes.

Activation of voltage-gated Ca^{2+} channels due to GABA-evoked depolarization was also shown to increase the expression of the transcription factor NeuroD, a positive modulator of neuronal differentiation in immature neurons (Tozuka et al., 2005). Similarly, another study observed for NG2 cells that GABA-evoked Ca^{2+} transients induced the production of the brain-derived neurotrophic factor (BDNF), a molecule known to promote axonal growth and guidance (Tanaka et al., 2009). Furthermore, NG2 cells express several proteins of the vesicular release machinery (Haberlandt et al., 2011). Local Ca^{2+} elevations induced by GABA depolarization might therefore not only induce the production but also the release of neuroactive substances. This would provide a bidirectional communication pathway between neurons and NG2 cells where GABAergic synaptic innervation induces the production and/or release of neuroactive substances to the innervating neuron.

Studies investigating immature neurons also suggested that GABAergic synaptic innervation which precedes glutamatergic synaptic innervation during development is important for normal formation of excitatory synapses (Ben-Ari, 2002; Wang and Kriegstein, 2008). Similar mechanisms are also conceivable for NG2 cells although a systematic analysis of the time course of GABAergic vs. glutamatergic innervation has not been performed so far.

Beyond these physiological functions, GABAergic signaling might also enable NG2 cells to sense pathophysiological activity patterns in the CNS. As discussed so far, it is unclear which conditions could sufficiently depolarize NG2 cells to reach the threshold for voltage-gated Ca^{2+} channel activation *in vivo*. One possibility is that the depolarization by $GABA_A$ receptor

activation might be enhanced by the extracellular accumulation of K^+ after strong neuronal activity. This is supported by the observation that high frequency stimulation of neuronal fibers was sufficient to increase the intracellular Ca^{2+} concentration in NG2 cell somata while physiological stimulation failed to do so (Haberlandt et al., 2011). In this respect it is interesting that during normal gamma oscillations NG2 cells experienced an increase in synaptic activity without considerable summation of these currents. In contrast, during epileptic-like discharges NG2 cells received transient bursts of synaptic events with significant summation (Mangin et al., 2008). These might be sufficient to induce larger Ca^{2+} increases compared to normal activity, enabling NG2 cells to perceive pathological activity in the CNS. Indeed, Wennström and colleagues (2003) reported increased proliferation of NG2 cells after electroconvulsive seizures indicating a functional response of NG2 cells to abnormal neuronal activity.

Other studies showed that activation of NG2 cell $GABA_A$ receptors induced a long-lasting inhibition of resting K^+ conductances concomitant to the $GABA_A$ receptor-mediated Cl^- current (Pastor et al., 1995; Bekar et al., 1999). A decrease in K^+ conductance apparently increases the membrane resistance. As a result, subsequent activation of $GABA_A$ receptors might produce larger depolarizations possibly reaching the threshold to activate voltage-gated Ca^{2+} channels.

Furthermore, Lin and Bergles (2004) reported that activation of $GABA_A$ receptors led to a decrease in membrane conductance and an enduring change in $[Cl^-]_i$. This concomitantly decreased the size of AMPA receptor-mediated currents, similar to the known phenomenon of shunting inhibition in neurons. $GABA_A$ receptor signaling might thereby contribute to the signal integration of different synaptic inputs in NG2 cells.

Despite all these reasonable hints about the function of $GABA_A$ receptors in NG2 cells, we do not yet understand what physiological role they serve. The data from the present study, however, provide a promising framework for future studies aiming at elucidating the function of GABAergic signaling in NG2 cells.

6.2 Role of the NG2 protein in glutamatergic neuron-NG2 cell synaptic signaling

Glutamatergic synaptic innervation is unique to glial NG2 cells and displays one of their key features throughout the brain (De Biase et al., 2010). In neurons it has been shown that transsynaptic cell adhesion proteins are crucial for proper synapse formation, maturation and plasticity (Dalva et al., 2007; Missler et al., 2012). However, for neuron-NG2 cell synapses the cellular basis of these fundamental processes is completely unknown (Bergles et al., 2010). The structural similarities of the NG2 protein to the synaptic cell adhesion protein neurexin and the finding that it binds via GRIP to AMPA receptors suggested a critical role for the NG2 protein in neuron-NG2 cell synaptic physiology (Stegmüller et al., 2003; Trotter et al., 2010). Surprisingly, the present results demonstrate that functional glutamatergic neuron-NG2 cell synapses form in the absence of NG2 and display properties indistinguishable from control cells in juvenile and aged mice. However, the shown expression of neuroligins suggests that synaptic cell adhesion at neuron-NG2 cell synapses is similarly regulated as known from synapses between neurons.

6.2.1 NG2 is not necessary for glutamatergic neuron-NG2 cell synaptic signaling

Functional glutamatergic neuron-NG2 cell synapses form in the absence of NG2

Sequence-analysis of the NG2 protein revealed structural similarities to the synaptic cell adhesion protein neurexin, including extracellular LNS domains and an intracellular PDZ-binding motif (Nishiyama et al., 1991; Missler and Südhof, 1998). The LNS domains of neurexins are important for transsynaptic binding to neuroligins (Missler et al., 2012). This neuroligin-neurexin complex was found to be an important regulator of synapse number, synapse specificity and synaptic strength in neurons (Missler et al., 2012). Intriguingly, *in vitro* it was shown that expression of neuroligins in non-neuronal cells is sufficient to induce presynaptic differentiation in co-cultured neurons (Scheiffele et al., 2000; Graf et al., 2004). Vice versa, the expression of neurexins led to the assembly of postsynaptic specializations in neurons (Nam and Chen, 2005). Moreover, triple-knockout of either the α-neurexin or the neuroligin-1, -2 and -3 genes both led to early postnatal lethality due to respiratory failure caused by impaired synaptic transmission. However, for both proteins single or double

knockout animals displayed milder phenotypes revealing at least in part functional redundancy among the different isoforms (Missler et al., 2003; Varoqueaux et al., 2006). With respect to the NG2 protein, several lines of NG2 knockout mice have been generated and in all studies, NG2-lacking animals were viable and showed no obvious phenotype (Grako et al., 1999; Karram et al., 2008; Hill et al., 2011). This indicates that NG2 is either not essential for survival or other proteins are able to replace its function.

Although neurexins are located presynaptically while NG2 is a postsynaptic protein, NG2 might still bind to a yet unknown presynaptic binding partner via its LNS domains and regulate synaptogenesis (Trotter et al., 2010). Intriguingly, it was recently shown that even one membrane-tethered LNS domain is sufficient to trigger synapse formation (Gokce and Südhof, 2013), emphasizing its intrinsic synaptogenic capability. Other studies investigating differentiation of NG2 cells to oligodendrocytes showed that neuron-NG2 cell synapses are disassembled as soon as differentiation is initiated. Strikingly, this disassembly coincides with the downregulation of NG2 expression (De Biase et al., 2010; Kukley et al., 2010).

However, the results of the present study show that neuron-NG2 cell synapses readily form in the absence of NG2 as AMPA receptor-mediated ePSCs and mPSCs were measured from hippocampal NG2 cells of juvenile and aged -/- mice (Fig. 5.12 and Fig. 5.15). These findings demonstrate that the NG2 protein is not necessary for the formation of functional glutamatergic synapses between neurons and NG2 cells.

Kinetic properties of synaptic AMPA receptor-mediated currents are unaffected by NG2

It has been shown that synaptic cell adhesion molecules are among others involved in synapse maturation and synapse morphology (Dalva et al., 2007; Wittenmayer et al., 2009). This might change the time course of glutamate concentration in the synaptic cleft which in turn influences the kinetic properties of AMPA receptor-mediated currents as shown for neuron-neuron synapses (Jonas, 2000; Cathala et al., 2005; Takahashi, 2005). Furthermore, AMPA receptor current kinetics also depend on subunit composition of the receptor and their association with auxiliary subunits (Jonas, 2000; Lu et al., 2009; Jackson and Nicoll, 2011). As it was shown that NG2 is in a complex with GluA2/3 containing AMPA receptors (Stegmüller et al., 2003), it might also influence subunit composition of synaptic AMPA receptors. To test for possible alterations of one of these parameters by NG2, the kinetic

properties of synaptic AMPA receptor-mediated currents were compared. However, no differences were observed for rise times and decay times of ePSCs and mPSCs between -/- and control animals in both age groups (Fig. 5.12 and Fig. 5.16). These results suggest that ablation of NG2 does not lead to severe changes in synapse morphology, AMPA receptor subunit composition or association with auxiliary AMPA receptor subunits.

However, when comparing kinetic properties between the two age groups investigated, a developmental acceleration of AMPA receptor-mediated ePSC and mPSC rise times and decay times was detected (Fig. 5.12 and Fig. 5.16). This observation is in line with a previous study investigating NG2 cells of the dentate gyrus, showing an acceleration of AMPA receptor current kinetics during the first three postnatal weeks (Mangin et al., 2008). In this regard, other studies suggested that this acceleration might be caused by a switch in subunit composition as reported for NG2 cells during postnatal development in different brain regions (Seifert et al., 1997; Seifert et al., 2003; Ge et al., 2006b; Ziskin et al., 2007). Furthermore, a maturation effect of synaptic structure that influences the time course of glutamate concentration in the synaptic cleft as shown for developing neuron-neuron synapses might be valid for neuron-NG2 cell synapses, too (Takahashi, 2005; Cathala et al., 2005). Results from our laboratory (Passlick, S. and Seifert, G., unpublished observations) as well as a study by Zonouzi et al. (2011) showed that NG2 cells express AMPA receptor auxiliary subunits that influence glial AMPA receptor current kinetics. Developmental changes of their expression pattern might also be responsible for the acceleration. However, besides AMPA receptor kinetics, several other properties have been shown to change in NG2 cells during development, such as expression of $GABA_A$ receptor subunits (section 5.2) (Balia et al., 2013), passive membrane properties and K_{ir} channel expression (section 5.3.1) (Kressin et al., 1995; Bordey and Sontheimer, 1997; Maldonado et al., 2013). These profound changes in NG2 cell physiology might indicate that juvenile and adult NG2 cells serve distinct functions during development.

Short-term synaptic plasticity is preserved in NG2 lacking cells

As discussed before, it was shown that the triple-knockout of all α-neurexins led to early postnatal lethality due to respiratory failure. Surprisingly, the authors found that not the initial formation of synapses was disturbed but that the Ca^{2+}-triggered neurotransmitter

release was impaired due to reduced synaptic Ca^{2+} channel function. These data suggest that α-neurexins organize presynaptic terminals by functionally coupling Ca^{2+} channels to the presynaptic machinery (Missler et al., 2003). Another study found that even postsynaptic neuroligins are able to regulate presynaptic release probability by retrograde signaling to the presynaptic side (Futai et al., 2007). Thereby, the neuroligin-neurexin complex is able to modulate short-term synaptic plasticity.

The results of the present study show that hippocampal neuron-NG2 cell synapses display paired-pulse facilitation similar to excitatory neuronal CA3-CA1 synapses. These data confirm earlier studies investigating neuron-NG2 cell synapses in the hippocampus (Bergles et al., 2000; Ge et al., 2006b). More importantly, short-term synaptic plasticity was not influenced by NG2 as animals of the different genotypes showed similar PPR values (Fig. 5.13). Therefore, it can be concluded that NG2 is probably not involved in retrograde modulation of presynaptic release probability and short-term synaptic plasticity.

NG2 does not influence synaptic connectivity

Several studies investigated the effect of NG2 on neurite outgrowth. The results, however, are conflicting, describing NG2 either as being inhibitory or permissive for neurite outgrowth (Jones et al., 2003; Ughrin et al., 2003; Tan et al., 2005; de Castro et al., 2005; Tan et al., 2006). Furthermore, it was shown that NG2 cells are actively contacted by neurons while oligodendrocytes are not (Yang et al., 2006). This indicates that NG2 cells exhibit properties making them more attractive for neuronal contacts than oligodendrocytes.

As discussed before, the NG2 protein possesses structural similarities to neurexins. For neurexins and their binding partner neuroligins it was shown that they are able to influence the number of functional synapses *in vitro* (Prange et al., 2004; Levinson et al., 2005). Overexpression of neuroligins in cultured neurons led to more functional synapses as measured by an increase in mPSC frequency (Prange et al., 2004). Strikingly, this effect was inhibited when soluble neurexin was applied to the culture demonstrating that the neuroligin-neurexin binding is responsible for the increase in synapse number (Levinson et al., 2005). *In vivo* knockout studies of all neurexins or neuroligins initially supported the *in vitro* findings as mPSC frequency was reduced in both cases, indicating a reduction in synaptic innervation. However, detailed analysis showed that not the number of synapses

was altered, but that a failure in synaptic maturation led to impaired Ca^{2+}-triggered neurotransmitter release (Missler et al., 2003; Varoqueaux et al., 2006). As discussed in the preceding section, this is clearly not the case for NG2, as presynaptic release properties were unaffected by NG2 ablation (Fig. 5.13). Therefore, in this study the frequency of mPSCs could be regarded as an indirect measure of synaptic connectivity.

Considering the effect of NG2 on neurite outgrowth and its similarities to neurexins, NG2 might influence synapse number of NG2 cells. However, comparison of mPSC frequency between the genotypes and age groups tested revealed no NG2-dependent alterations, indicating that synaptic connectivity is preserved and unchanged in the absence of NG2 (Fig. 5.14).

A recent study by Kwon and colleagues (2012) established an interesting new model for the regulation of synapse number by neuroligin-1 *in vivo*. They demonstrated that not the absolute neuroligin-1 level is relevant but that synapse number depends on transcellular differences in the relative amount of neuroligin-1. In other words, those cells expressing higher levels of neuroligin-1, catch more synapses compared to a neighboring cell expressing lower levels of neuroligin-1. This also explains the finding that the number of functional synapses is unaltered in neuroligin-1 knockout mice as obviously no transcellular differences occur. Certainly, a similar mechanism cannot be excluded for the NG2 protein, however, recently it was shown that NG2 cells achieve their even distribution by self-avoidance with non-overlapping territories (Hughes et al., 2013), making it rather unlikely that they compete for synapses.

Even though synaptic connectivity was not influenced by NG2, a drastic decrease in mPSC frequency during development was observed (Fig. 5.14). These findings are in line with previous results, showing a relatively low mPSC frequency in juvenile mice (Jabs et al., 2005; Kukley et al., 2010) that further decreased in young adult mice (De Biase et al., 2011). However, the present study extends this work by demonstrating that even aged NG2 cells (> 9 months) still receive synaptic input, albeit to a much lower extent than juvenile ones. This supports the notion that synaptic innervation is a hallmark of NG2 cells throughout life and must serve some important yet unknown function (De Biase et al., 2010).

Synaptic AMPA receptor density is unaffected by NG2

The NG2 protein was found to be in a complex with GRIP and GluA2/3 containing AMPA receptors (Stegmüller et al., 2003). In neurons it was shown that the interaction of GRIP with GluA2-containing AMPA receptors is important for synaptic accumulation of AMPA receptors (Osten et al., 2000). Furthermore, synaptic neuroligin-neurexin contacts specifically recruit GluA2-containing AMPA receptors (Heine et al., 2008) and are able to capture surface diffusing AMPA receptors into synapses (Mondin et al., 2011). A recent study by Aoto and colleagues (2013) discovered that different splice variants of presynaptic neurexins transsynaptically control postsynaptic AMPA receptor density.

To test for a potential effect of NG2 ablation on synaptic AMPA receptor density, mPSC amplitudes were compared between juvenile and aged -/- and control mice. The analysis revealed no NG2-dependent alterations of mPSC amplitudes (Fig. 5.15), indicating that AMPA receptors are trafficked into postsynaptic densities of NG2 cells with the same efficacy when NG2 is lacking.

mPSC amplitudes did not change from juvenile to adult NG2 cells suggesting that postsynaptic AMPA receptor density stays constant throughout development (Fig. 5.15). This is in line with and complements other reports investigating mPSC amplitudes in NG2 cells at different developmental time points (Bergles et al., 2000; De Biase et al., 2010; De Biase et al., 2011).

6.2.2 NG2 cells express neuroligins

Given that synaptic transmission was unaltered in the absence of the NG2 protein other factors must mediate and regulate the physiology of neuron-NG2 cell synapses. As discussed before, the neuroligin-neurexin complex was identified as a crucial mediator of several key features of synaptic transmission in neurons including the regulation of synapse number, synapse specificity and synaptic strength (Missler et al., 2012). Single-cell transcript analysis of the present study revealed that NG2 cells similarly express the postsynaptic cell adhesion proteins neuroligin-1, -2 and -3 (Fig. 5.17). These data suggest that the neuroligin-neurexin complex operates for neuron-NG2 cell synapses analog to neuron-neuron synapses.

NG2 cells are innervated by glutamatergic and GABAergic neurons (Bergles et al., 2010). For the different neuroligins, it was shown that neuroligin-1 is preferentially found at

glutamatergic synapses (Song et al., 1999), while neuroligin-2 is mainly present at GABAergic synapses (Varoqueaux et al., 2004). Neuroglin-3 by contrast shows no preference (Budreck and Scheiffele, 2007). Consequently, it is likely that neuroligin-1 and -2 are similarly located at glutamatergic and GABAergic postsynaptic sites in NG2 cells, respectively. However, this selective localization also depends on alternative splicing (Dalva et al., 2007). The present study revealed that the majority of NG2 cells express the short form of neuroligin-1 lacking the insertion at splice site A. This is in contrast to the overall expression in the mouse brain showing similar levels of neuroligin-1 lacking or exhibiting the insertion at splice site A. Presence of the A-insert was shown to shift the preference from glutamatergic to GABAergic terminals. However, critical for the final localization seems to be splice site B, as neuroligin-1 exhibiting both inserts displays a strong preference for glutamatergic terminals (Chih et al., 2006). Since primers used in the present study did not flank the B-insert, no information about its presence in NG2 cells is available so far.

Especially the function and properties of neuroligin-1 were intensively investigated. Although it was suggested that neuroligins are not essential for the initial formation of synapses but rather control synaptic function (Südhof, 2008), a recent study demonstrated that neuroligin-1 is a pivotal determinant of the number of functional synapses (Kwon et al., 2012). The authors showed that transcellular differences in the expression level of neuroligin-1 determine the number of functional excitatory synapses revealing a novel competitive mechanism of synapse formation between neighboring neurons. Considering now the findings from the present study demonstrating abundant expression of neuroligins by NG2 cells, these data might indicate that neurons not only compete with each other for synapses but possibly also with NG2 cells evenly distributed throughout the CNS. In this respect it is interesting that NG2 cells actively survey their microenvironment with highly motile processes (Haberlandt et al., 2011; Hughes et al., 2013). By binding and releasing neurexins on presynaptic boutons, neuroligins on NG2 cells might influence the number of boutons available for neurons and thereby affect synapse number. Furthermore, expression of neuroligins in cultured non-neuronal cells was sufficient to induce the assembly of presynaptic specializations in co-cultured neurons (Scheiffele et al., 2000), suggesting that neuroligins expressed on the surface of NG2 cells might be able to do so as well. However, the synaptic connectivity of NG2 cells is several magnitudes lower as compared to neurons.

A potential competitive effect between neurons and NG2 cells is therefore likely to be rather small.

Interestingly, the present study revealed an NG2-dependent decrease in the expression frequency of neuroligin-3 in aged NG2 cells. Neuroligin-3 knockout mice were recently shown to exhibit impaired mGluR-dependent synaptic plasticity (Baudouin et al., 2012) and dysfunctional tonic endocannabinoid signaling (Földy et al., 2013). Furthermore, specific mutations in the neuroligin-3 gene were implicated in the pathogenesis of autism (Tabuchi et al., 2007). Whether NG2 cells or the NG2 protein itself contribute to this phenotype needs to be clarified.

By demonstrating that NG2 cells express neuroligins similar to neurons, the present study provides promising targets for interfering with neuron-NG2 cell synaptic transmission to unravel the function of this unique type of neuron-glia communication.

6.2.3 Passive membrane properties are influenced by NG2 – a hint to other roles?

Results from several studies supported the hypothesis that the NG2 protein might play a role in synaptic transmission between neurons and NG2 cells (Trotter et al., 2010). However, data from the present study revealed that NG2 is not necessary in this context. But what is the function of the NG2 protein? Why is it expressed by NG2 cells but downregulated in oligodendrocytes?

The comparison of passive membrane properties of -/- NG2 cells with control cells in juvenile and aged mice revealed besides the known age-dependent changes (Kressin et al., 1995; Maldonado et al., 2013) also NG2-dependent changes. The membrane resistance was increased when NG2 levels were reduced in aged animals, indicating changes in expression or function of K^+ channels (Fig. 5.11). Furthermore, an increase in membrane capacitance of juvenile -/- cells was detected, suggesting an influence of NG2 on cell size (Fig. 5.11).

In this context, it is interesting that NG2 cells migrate over considerable distances during development and in response to injury in adult animals (Sugimoto et al., 2001; Aguirre and Gallo, 2004; Kessaris et al., 2006; Aguirre et al., 2007). Furthermore, NG2 cells were shown to maintain their density by self-avoidance and that loss of NG2 cells leads to rapid proliferation and migration of adjacent NG2 cells to restore their density (Hughes et al.,

2013). Besides migration, recent studies also revealed that even in a resting state NG2 cells displayed highly motile processes with which they surveyed their local environment (Haberlandt et al., 2011; Hughes et al., 2013). Migration as well as process motility rely on reorganization of the cytoskeleton and lead to changes in cell morphology and thereby cell size (Bacon et al., 2007; Biname et al., 2010). Interestingly, several publications demonstrated that the NG2 protein is involved in cytoskeletal reorganization and directional migration of NG2 cells (Niehaus et al., 1999; Fang et al., 1999; Makagiansar et al., 2004; Makagiansar et al., 2007; Biname et al., 2013). Furthermore, it was shown that the ectodomain of NG2 binds to components of the extracellular matrix. Especially the binding to collagen VI leads to changes in the actin cytoskeleton and cell spreading (Burg et al., 1996; Tillet et al., 2002). The findings that NG2 affects cytoskeletal dynamics and that polarization of NG2 cells is disturbed in NG2 knockout mice (Biname et al., 2013), might explain the NG2-dependent changes in cell size found in the present study (Fig. 5.11). Furthermore, AMPA receptor activation was implicated in NG2 cell migration mediated by a complex consisting of AMPA receptors, myelin proteolipid proteins and a_v integrins (Gudz et al., 2006). Since it was shown that NG2 cell AMPA receptors are associated with the NG2 protein (Stegmüller et al., 2003), NG2 might well be implicated in this mechanism. In that case, ablation of NG2 could complicate the formation of this functional complex and thereby migration. This idea would also provide a reason for NG2 being in a complex with AMPA receptors.

Other studies implicated the NG2 protein in proliferation and differentiation. The PDGFα receptor, a growth factor receptor known to regulate cell proliferation and differentiation, is exclusively expressed by NG2 cells in the CNS (Noble et al., 1988; Nishiyama et al., 2009). It was shown that NG2 interacts with the PDGFα receptor and that this interaction is necessary for the correct response of the receptor to its ligand PDGF-AA (Nishiyama et al., 1996; Goretzki et al., 1999). Independent groups demonstrated that PDGFα receptor or PDGF-AA knockout animals display reduced numbers of NG2 cells and oligodendrocytes (Fruttiger et al., 1999; Murtie et al., 2005). Intriguingly, Kucharova and Stallcup (2010) showed a very similar phenotype in NG2 knockout mice, as the density of NG2 cells as well as oligodendrocytes was reduced during development. Moreover, when NG2 cells differentiate to oligodendrocytes, expression of NG2 and PDGFα receptor is downregulated at the same time (Kukley et al., 2010). These data strongly suggest that NG2 and PDGFα receptor form a

functional complex important for proliferation and differentiation of NG2 cells. Importantly, this is not limited to the CNS as NG2, being expressed by multiple progenitor cells in different tissues, has been shown to mediate PDGFα receptor function also in smooth muscle cells (Grako et al., 1999). This indicates a general role for NG2 in proliferation and differentiation of progenitor cells in all types of tissue it is expressed.

Another study even revealed a dual role for NG2 in proliferation and motility/migration (Makagiansar et al., 2007). They showed that differential phosphorylation of the NG2 protein affects its cell surface localization thus balancing proliferation and migration of NG2 cells (Makagiansar et al., 2007). Moreover, Sugiarto and coworkers (2011) demonstrated that polarized localization of the NG2 protein during mitosis is required for proper asymmetric cell division of NG2 cells and that decreased NG2 asymmetry might have tumor-initiating potential. Thus, NG2 might be an important regulator to adjust proliferation and motility/migration of progenitor cells by various interactions with intra- and extracellular communication partners.

It should be noted though that this study investigated NG2 cells of the gray matter where differentiation to oligodendrocytes is much less relevant compared to the highly myelinated white matter regions (Dimou et al., 2008). Regarding this, it was shown that NG2 cells of white matter but not gray matter proliferate in response to PDGF (Hill et al., 2013). Furthermore, it was demonstrated that although NG2 cells from both regions share a set of core properties (De Biase et al., 2010), they also exhibit region-dependent variations, e.g. in K_{ir} channel density (Chittajallu et al., 2004). In this respect it is interesting that the level of K_{ir} conductances might be implicated in proliferation and differentiation of NG2 cells to oligodendrocytes (Sontheimer et al., 1989; Neusch et al., 2001; Djukic et al., 2007). Thus, it might be worth investigating whether the NG2-dependent changes in passive membrane properties of NG2 cells found in the present study likewise occur in white matter regions and, if so, whether these changes contribute to alterations in proliferation and differentiation of NG2 cells.

In the light of the results from the present study it is rather unlikely that NG2 plays a vital role in glutamatergic synaptic transmission between neurons and NG2 cells. Instead, the identification of neuroligins in NG2 cells suggests that synaptic cell adhesion of neuron-NG2 cell synapses is similarly regulated as in neurons. Furthermore, published data on the function of the NG2 protein rather point towards a role in motility, migration and proliferation. However, the fact that ablation of NG2 only leads to a mild phenotype might also indicate compensatory mechanisms by redundancy of function of other proteins.

7 Summary

NG2 cells are nowadays considered as a fourth class of glial cells in the CNS. They express various types of voltage-gated ion channels and neurotransmitter receptors and exhibit the capability to proliferate and differentiate. Intriguingly, NG2 cells receive direct synaptic input from glutamatergic and GABAergic neurons throughout the CNS. However, the role of this synaptic innervation remains largely unknown.

Within the framework of the present study three main aspects of the synaptic transmission between neurons and NG2 cells were investigated.

(i) *Functional characterization of $GABA_A$ receptors in hippocampal NG2 cells.*

The fact that NG2 cells express functional $GABA_A$ receptors has been known for a long time. However, the subunit composition and functional properties were less evident. The present study revealed that NG2 cells are endowed with a similar set of $GABA_A$ receptor subunits as hippocampal neurons. Detailed analysis showed that in addition to synaptic $GABA_A$ receptor currents, NG2 cells are able to sense ambient GABA via extrasynaptic receptors. In this context, it could be demonstrated that the γ2-subunit is selectively located in synaptic $GABA_A$ receptors while being absent in extrasynaptic ones. Since the γ2-subunit is crucial for clustering of synaptic $GABA_A$ receptors in neurons, it probably serves a similar role in NG2 cells.

(ii) *Analysis of the $GABA_A$ receptor subunit expression pattern of neocortical NG2 cells during postnatal development.*

Neocortical NG2 cells experience a switch from synaptic to extrasynaptic GABAergic transmission during postnatal development. The present study revealed that this switch is accompanied by the downregulation of the γ2-subunit of $GABA_A$ receptors. Together with the data summarized in (i), these findings suggest that the γ2-subunit is a pivotal component of synaptic $GABA_A$ receptors in NG2 cells of different brain regions.

(iii) *Role of the NG2 protein in neuron-NG2 cell synaptic signaling.*

The NG2 protein exhibits structural similarities to the synaptic cell adhesion proteins neurexins. Furthermore, it is complexed with AMPA receptors via the synaptic protein GRIP. These findings suggested that the NG2 protein might serve as a synaptic cell adhesion protein at neuron-NG2 cells synapses and possibly influence AMPA receptor clustering in the glial postsynaptic density. To test this, synaptic transmission between hippocampal neurons and NG2 cells was investigated in juvenile and aged NG2 knockout animals. The findings revealed that AMPA receptor current kinetics and amplitudes as well as synaptic connectivity and short-term synaptic plasticity were unaltered in the absence of the NG2 protein. Based on these results it can be concluded that the NG2 protein is not necessary for synaptic communication between neurons and NG2 cells. However, further single-cell expression analysis demonstrated frequent expression of the postsynaptic cell adhesion proteins neuroligins in juvenile and aged NG2 cells. This finding indicates similar mechanisms of synaptic cell adhesion at neuron-NG2 cell synapses as known from neuron-neuron synapses.

The results of the present study provide important new insights into the physiology of neuron-NG2 cell synapses. They may thus serve as a valuable basis for future studies aiming at elucidating the function of this unique type of neuron-glia communication.

8 Perspective

Although various studies investigated the physiology and function of NG2 cells, their role in the adult CNS is still enigmatic. The most remarkable but likewise obscure feature of NG2 cells is their synaptic innervation by neurons. As it is a common characteristic of these cells in different brain regions, it might represent an elementary and possibly essential feature of NG2 cells to accomplish their function. Unraveling the role of synaptic transmission between neurons and NG2 cells should therefore constitute a central objective of future research. For this purpose, the present study provides important information about the physiology of neuron-NG2 cell synapses.

In the first part of this study it could be demonstrated that the γ2-subunit is selectively located in synaptic $GABA_A$ receptors in NG2 cells. Furthermore, the second part revealed that the γ2-subunit is downregulated when direct synaptic innervation diminishes. Due to its essential function in clustering of synaptic $GABA_A$ receptors in neurons, the γ2-subunit might play a similar role in NG2 cells. To tackle this question, NG2 cell-specific γ2-knockout mice should be investigated. Assuming that the γ2-subunit is indeed crucial for synaptic $GABA_A$ receptor clustering in NG2 cells, this approach would provide further information about the general role of GABAergic synaptic innervation in NG2 cells. Since $GABA_A$ receptors assemble from a variety of subunits, the generation of NG2 cell-specific total $GABA_A$ receptor knockout mice would be a daunting task. However, targeting the putatively essential γ2-subunit constitutes an easier to realize strategy to investigate the role of the GABAergic synaptic innervation of NG2 cells.

The third part of the study revealed that despite promising assumptions, the NG2 protein is not necessary for synaptic transmission between neurons and NG2 cells. Instead, it could be shown that NG2 cells express the synaptic cell adhesion proteins neuroligins. For these proteins an intriguing new mechanism for the regulation of synapse number was recently proposed. Up- and downregulation of neuroligin-1 expression in single neurons *in vivo* demonstrated that transcellular differences in the relative expression of neuroligin-1 determine synapse number (Kwon et al., 2012). The new data from the present study

indicate that neurons probably also compete with neighboring NG2 cells for synapses depending on the relative expression of neuroligins. Similar experiments including overexpression or downregulation of neuroligins in single NG2 cells would therefore provide important information about synapse formation in these cells. Furthermore, this might uncover mechanisms how neurons and NG2 cells interact or compete with each other when it comes to synapse formation.

A more global but straight forward approach to investigate the function of neuron-NG2 cell synapses would be the knockout of AMPA receptors in NG2 cells. However, since they presumably express all four GluA subunits, the generation of such knockout mice would expend a large effort. Nevertheless, synaptic signaling in NG2 cells might be the key to uncover their function and thus justify such an elaborate experiment.

9 Zusammenfassung

NG2 Zellen werden heutzutage als vierter Glia-Zelltyp im ZNS angesehen. Sie exprimieren verschiedene spannungsgesteuerte Ionenkanäle und Neurotransmitter-Rezeptoren und besitzen die Fähigkeit zu proliferieren und differenzieren. Interessanterweise erhalten NG2 Zellen direkten synaptischen Input von glutamatergen und GABAergen Neuronen im gesamten ZNS. Die Rolle dieser synaptischen Innervation ist jedoch weitestgehend unbekannt. Im Rahmen der vorliegenden Studie wurden drei Hauptaspekte der synaptischen Transmission zwischen Neuronen und NG2 Zellen untersucht.

(i) *Funktionelle Charakterisierung von $GABA_A$-Rezeptoren in hippokampalen NG2 Zellen.*
Die Tatsache, dass NG2 Zellen funktionelle $GABA_A$-Rezeptoren exprimieren, ist seit langer Zeit bekannt. Die genaue Untereinheiten-Zusammensetzung und die funktionellen Eigenschaften wurden jedoch noch nicht bestimmt. Die vorliegende Arbeit konnte zeigen, dass NG2 Zellen eine ähnliche Ausstattung an $GABA_A$-Rezeptor Untereinheiten aufweisen wie hippokampale Neurone. Im Rahmen weitergehender Analysen stellte sich heraus, dass NG2 Zellen neben synaptischen $GABA_A$-Rezeptorströmen auch umgebendes GABA über extrasynaptische $GABA_A$-Rezeptoren wahrnehmen können. In diesem Zusammenhang konnte nachgewiesen werden, dass die γ2-Untereinheit bevorzugt in synaptischen $GABA_A$-Rezeptoren vorhanden ist, während sie in extrasynaptischen fehlt. In Neuronen ist die γ2-Untereinheit eine Voraussetzung für den dichten Zusammenhalt synaptischer $GABA_A$-Rezeptoren. Es ist daher anzunehmen, dass die γ2-Untereinheit diese Funktion auch in NG2 Zellen wahrnimmt.

(ii) *Analyse der $GABA_A$-Rezeptor Untereinheiten Expression von neokortikalen NG2 Zellen während der postnatalen Entwicklung.*
In neokortikalen NG2 Zellen findet während der postnatalen Entwicklung eine Umstellung von synaptischer auf extrasynaptische Transmission statt. Die vorliegende Studie konnte zeigen, dass diese Umstellung mit der Runterregulierung der γ2-Untereinheit einhergeht. Im Zusammenhang mit den unter Punkt (i) zusammengefassten Daten, deutet dies darauf hin, dass die γ2-Untereinheit eine entscheidende

Komponente von synaptischen $GABA_A$-Rezeptoren in NG2 Zellen in verschiedenen Hirnregionen ist.

(iii) *Rolle des NG2 Proteins bei der synaptischen Signalübertragung zwischen Neuronen und NG2 Zellen.*

Das NG2 Protein besitzt strukturelle Ähnlichkeit zu den synaptischen Zelladhäsionsproteinen Neurexinen. Außerdem ist es durch Interaktion mit dem synaptischen Protein GRIP in einem Komplex mit AMPA-Rezeptoren zu finden. Diese Ergebnisse deuteten darauf hin, dass das NG2 Protein als synaptisches Zelladhäsionsprotein an Neuron-NG2 Zell Synapsen fungiert und möglicherweise die enge Zusammenlagerung von AMPA-Rezeptoren in der glialen postsynaptischen Membranregion beeinflusst. Um dies zu überprüfen, wurde die synaptische Transmission zwischen hippokampalen Neuronen und NG2 Zellen in jungen und gealterten NG2-Knockout Mäusen untersucht. Die Ergebnisse zeigten, dass AMPA-Rezeptor Kinetiken und Amplituden sowie synaptische Konnektivität und Kurzzeit-Plastizität in der Abwesenheit von NG2 unverändert sind. Diese Daten deuten darauf hin, dass das NG2 Protein für die synaptische Kommunikation zwischen Neuronen und NG2 Zellen nicht notwendig ist. Weiterführende Einzelzell-Expressionsanalysen zeigten hingegen die häufige Anwesenheit der postsynaptischen Zelladhäsionsproteine Neuroligine in jungen und gealterten NG2 Zellen. Dies könnte bedeuten, dass die synaptische Zelladhäsion an Neuron-NG2 Zell Synapsen ähnlich wie bei Neuron-Neuron Synapsen reguliert ist.

Die Daten der vorliegenden Studie liefern neue Erkenntnisse über die Physiologie von Neuron-NG2 Zell-Synapsen. Sie stellen daher eine wertvolle Basis für zukünftige Studien dar, die die Funktion dieser besonderen Neuron-Glia Kommunikation aufdecken wollen.

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11 Appendix

11.1 Erklärung

Hiermit versichere ich, dass die vorliegende Dissertation von mir selbst und ohne unerlaubte Hilfe angefertigt worden ist. Es wurden keine anderen als die angegebenen Hilfsmittel verwendet. Ferner erkläre ich, dass die vorliegende Arbeit an keiner anderen Hochschule als Dissertation eingereicht worden ist.

Bonn, den 03.04.2014

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(Stefan Paßlick)