Role of polysialic acid and NCAM in interneuron development

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Erklärung zur Dissertation

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel „Role of polysialic acid and NCAM in interneuron development“ selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistung herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

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Zusammenfassung

Das neurale Zelladhäsionsmolekül NCAM und seine außergewöhnliche Glykosylierung mit Polysialinsäure (PolySia) sind eng mit der Entwicklung und Plastizität des Nervensystems verbunden. PolySia verringert die Zelladhäsion auf unspezifische Art und Weise und schafft dadurch permissive Bedingungen, z. B. für die Migration neuraler Vorläuferzellen. PolySia reguliert jedoch auch spezifische, NCAM-vermittelte Interaktionen. Im Unterschied zur wohlbekannten Migrationsstörung der Interneuron-Vorläuferzellen des olfaktorischen Bulbus in PolySia-defizienten Mäusen, ist noch nicht geklärt, ob PolySia möglicherweise deren Differenzierung sowie die Entwicklung kortikaler Interneurone beeinflusst.

Die hier vorliegende Doktorarbeit beinhaltet zwei Studien zur Rolle von NCAM und PolySia in der Interneuronentwicklung *in vitro* und *in vivo*. In der ersten Studie konnte mittels Primärkulturen von Neuroblasten der subventrikulären Zone der Einfluss von PolySia auf die Differenzierung von Vorläuferzellen untersucht und von der fördernden Wirkung auf die Migration getrennt werden. Das Entfernen von PolySia förderte die Entstehung von Neuriten und die Reifung zu Calbindin-positiven Interneuronen. Diese Reaktion konnte durch den Kontakt mit NCAM imitiert und durch ein NCAM-bindendes Peptid unterbunden werden. Dies deutet auf eine Beteiligung NCAM-spezifischer Interaktionen hin. Darüber hinaus führte die Inkubation von Vorläuferzellen aus NCAM-*knockout* Mäusen mit einem löslichen NCAM-Fc Fusionsprotein zu einer erhöhten Differenzierung, ein Befund, der darauf hinweist, dass diese Reaktion durch heterophile NCAM Interaktionen hervorgerufen wurde.

Abnorme Expression von NCAM und polySia wurde mit der Pathophysiologie von Schizophrenie und anderen neuropsychiatrischen Erkrankungen in Zusammenhang gebracht. Markante Veränderungen bei Schizophrenen betreffen die Dichte der Interneuronen im präfrontalen Kortex und im Hippocampus, sowie die Größe des olfaktorischen Bulbus. Im zweiten Teil meiner Arbeit wurde untersucht, wie sich der Verlust von polySia auf bestimmte Interneuronpopulationen auswirkt. Um unterscheiden zu können, ob Reduktion von NCAM, Abnahme von polySia oder unzureichende Polysialylierung von NCAM oder eventuell weiteren polysialylierten Molekülen zu Veränderungen führt, wurden Mäuse mit unterschiedlich kombinierten *Ncam1*- und Polysialyltransferase-Mutationen analysiert. Eine Auswertung maßgeblicher Interneuron-Marker ergab, dass die Dichte Parvalbumin-positiver Zellen im präfrontalen Kortex und Calbindin-positiver Zellen im olfaktorischen Bulbus in allen polySia- oder NCAM-defizienten Mausstämmen verringert war. Dagegen nahm die Dichte Parvalbumin-positiver Zellen in den CA Feldern des Hippocampus zu.

Zusammengefasst belegen diese Ergebnisse, dass Polysialinsäure auf NCAM für die Entwicklung spezifischer GABAerger Interneuronsubtypen im Vorderhirn der Maus unerlässlich ist und deuten darauf hin, dass PolySia die Migration und Differenzierung von Interneuron-Vorläuferzellen über zwei unterschiedliche Mechanismen beeinflusst.

Summary

The neural cell adhesion molecule NCAM and its unique glycosylation with polysialic acid (polySia) are tightly associated with neural development and plasticity. PolySia attenuates cell adhesion in a non-specific manner and creates permissive conditions for e.g. neural precursor migration. Alternatively, polySia acts as a specific regulator of NCAM-mediated interactions. In contrast to the well-known migration deficits of olfactory bulb interneuron precursors in polySia-deficient mice, the potential impact of polySia on their differentiation as well as on the development of cortical interneurons is unresolved.

The thesis presented here comprises two studies analyzing the role of NCAM and polySia on interneuron development *in vitro* and *in vivo*. Using primary cultures of subventricular zone-derived neuroblasts, the first study dissects the influence of polySia on precursor differentiation from its function as a promoter of neuroblast migration. Removal of polySia enhanced neuritogenesis and maturation into calbindin-positive interneurons. This response was mimicked by exposure to NCAM and could be blocked by a NCAM-binding peptide, pointing towards an involvement of NCAM specific interactions. Moreover, the incubation of precursors derived from NCAM-knockout mice with a soluble NCAM-Fc fusion protein resulted in a higher degree of differentiation, indicating that heterophilic NCAM binding partners mediate the differentiation response.

Aberrant NCAM and polySia expression have been linked to the pathophysiology of schizophrenia and other neuropsychiatric disorders. Prominent findings in schizophrenia are altered interneuron densities in the prefrontal cortex and in the hippocampus as well as smaller olfactory bulbs. In the second part of my thesis, I investigated the effect of polySia deficiency on selected interneuron populations. To dissect, whether effects were caused by loss of NCAM, loss of polySia, or reduced polysialylation of either NCAM or additional polySia carriers, mice with differently combined *Ncam1* and polysialyltransferase deletions were comparatively analyzed. Evaluation of major interneuron markers revealed a reduced density of parvalbumin-positive cells in the prefrontal cortex and of calbindin-positive cells in the olfactory bulb of all polySia- or NCAM-deficient strains, whereas densities of parvalbumin-positive cells in the CA-fields of the hippocampus were increased.

These results prove that NCAM-bound polySia is essential for the development of specific GABAergic interneuron subtypes and indicate that polySia affects migration and differentiation of interneuron precursors by two distinct mechanisms.

Chapter 1 – General Introduction

1.1 NCAM isoforms

In the brain of higher vertebrates billions of neurons form complex networks. For the development of these circuitries a precise temporal and spatial control of cellular interactions is essential. Cell adhesion molecules (CAMs) are important players in that field. The first CAM identified in vertebrates was the neural cell adhesion molecule NCAM (Thiery et al. 1977), which was originally described as a synaptic membrane glycoprotein termed D2 (Jorgensen and Bock 1974). NCAM is a cell surface glycoprotein belonging to the immunoglobulin (Ig) superfamily. The Ig superfamily is a heterogenic group of proteins that share a common fold, a sandwich of two β-sheets, called the Ig fold (Halaby and Mornon 1998). NCAM is encoded by a single gene located on chromosome 11q23.1 in humans (official gene name *NCAM1*; Nguyen et al. 1986; Walsh et al. 1986) and on chromosome 9 in mice (official gene name *Ncam1*; D'Eustachio et al. 1985). By alternative splicing three major isoforms are generated, which differ in their C-terminal regions and, according to their apparent molecular weight, are named NCAM-180 (180kDa), NCAM-140 (140kDa) and NCAM-120 (120kDa) (Fig. 1A; Murray et al. 1986; Cunningham et al. 1987; Walsh and Dickson 1989). NCAM-180 and NCAM-140 are type II transmembrane molecules with intracellular domains of different lengths, whereas NCAM-120 lacks an intracellular domain and is attached to the membrane via a glycosylphosphatidylinositol (GPI) anchor. The N-terminal (extracellular) region of all NCAM isoforms consists of five immunoglobulin (Ig)-like domains and two fibronectin type III (FnIII)-like repeats. Structural variations in the extracellular part result from alternative splicing of the small exons VASE ("variable alternatively spliced exon", in the fourth Ig domain), MSD1a, b, c (muscle specific domain 1), and AAG (all four between the two FnIII-like repeats, Fig. 1A; Ronn et al. 1998; Walmod et al. 2004). In addition to the membrane bound isoforms, NCAM also exists in a secreted form produced by the expression of the small so-called SEC-exon. This exon contains a stop-codon therefore resulting in a truncated form of the extracellular part of the NCAM molecule (Bock et al. 1987; Gower et al. 1988). Furthermore, soluble NCAM can be produced via ectodomain shedding from the membrane-bound isoforms. Proteolytic cleavage mediated by a disintegrin and metalloprotease (ADAM) family metalloprotease results in the release of the entire NCAM extracellular region (NCAM-EC) as a soluble fragment (Vawter et al. 2001; Hübschmann et al. 2005; Hinkle et al. 2006; Kalus et al. 2006; Brennaman and Maness 2008a).

1.2 Developmental regulation and posttranslational modification of NCAM

NCAM-180 and NCAM-140 are expressed by neurons, whereas NCAM-120 is primarily found in glia (Noble et al. 1985; Dityatev et al. 2000; Maness and Schachner 2007). As shown for rodents, the expression of all NCAM isoforms is developmentally regulated. In the mouse NCAM-180 and NCAM-140 first appear at embryonic day 8 (E8; Probstmeier et al. 1994). Both are highly expressed during fetal and early postnatal development, and persist at lower levels into adulthood (Chuong and Edelman 1984; Gennarini et al. 1986; Oltmann-Norden et al. 2008). In contrast, NCAM-120 is hardly detectable until postnatal day 5 (P5). But parallel to the progression of myelination a massive up-regulation of this characteristic isoform of oligodendrocytes and myelin sheaths has been observed during the second and third postnatal week (Bhat and Silberberg 1986; Bhat and Silberberg 1988; Oltmann-Norden et al. 2008). A recent study revealed a similar developmental regulation pattern of the major NCAM isoforms in the human prefrontal cortex (PFC; Cox et al. 2009).

NCAM can be posttranslationally modified by phosphorylation and palmitoylation of the intracellular domain (Sorkin et al. 1984; Little et al. 1998; Ponimaskin et al. 2008) and by glycosylation of its extracellular part (Geyer et al. 2001; Liedtke et al. 2001; von der Ohe et al. 2002). Six potential N-glycosylation sites have been detected in the Ig-like domains of NCAM (Fig. 1; Albach et al. 2004). To all of these sites variable glycans can be attached giving rise to a high structural diversity (Liedtke et al. 2001). The most prominent modification of NCAM is its glycosylation with polysialic acid (polySia). The term “polysialic acid” denotes polymers of sialic acids, which comprise derivates of the nine carbon sugers neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galacto-2-nonulosonic acid, Neu) or KDN (3-deoxy-D-glycero-D-galacto-2-nonulosonic acid). With over 50 naturally occurring derivatives identified so far sialic acids are highly diverse (Angata and Varki 2002). PolySia on NCAM consists of a linear homopolymer of α2,8-glycosidically linked *N*-acetylneuraminic acid (Neu5Ac) with typically up to 50-60 residues (Galuska et al. 2008). One or more polySia chains can be attached to the 5th and 6th *N*-glycosylation site in the 5th Ig-like domain of all three major NCAM isoforms (Fig. 1; Nelson et al. 1995; Franceschini et al. 2001; Liedtke et al. 2001; Hildebrandt et al. 2008). However, the predominant carriers of polySia in the brain are NCAM-140 and NCAM-180, whereas the majority of NCAM-120 remains in a polySia-free state (Oltmann-Norden et al. 2008).

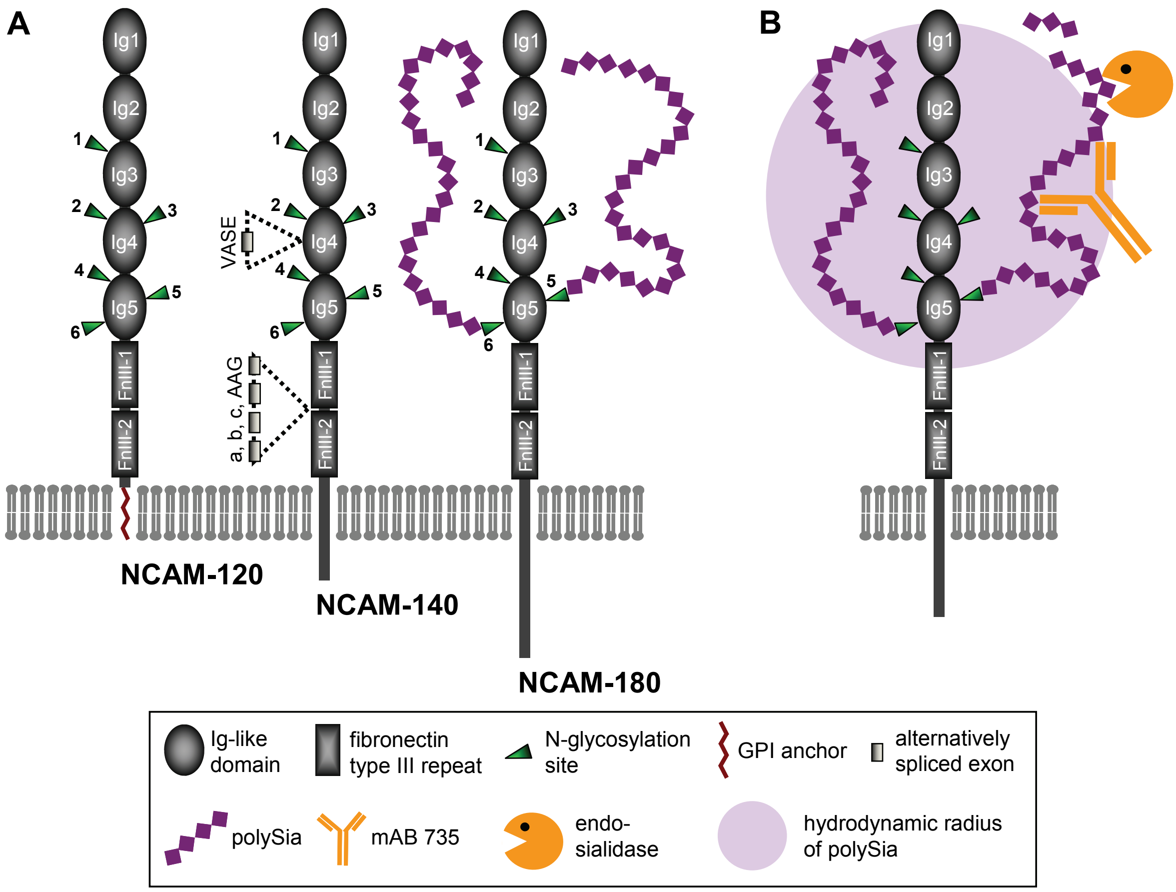

Figure 1: Structure of the neural cell adhesion molecule NCAM and polysialic acid. **A:** Schematic illustration of the three major NCAM isoforms. In the extracellular part all NCAM isoforms consist of five immunoglobulin (Ig)-like domains and two fibronectin type III repeats. NCAM-140 and NCAM-180 are transmembrane proteins, which differ in the length of their intracellular part, whereas NCAM-120 is attached to the plasmamembrane via a glycosylphosphatidylinositol (GPI) anchor. Structural variations can result from alternative splicing of small exons, depicted exemplarily at NCAM-140. NCAM contains six N-glycosylation sites and *in vivo* polysialylation is confined to the 5th and 6th N-glycosylation site. **B:** Polysialic acid (polySia)-specific tools. Two important tools for the analysis of polySia functions are the monoclonal antibody 735 and the phage-derived endosialidases, which specifically cleave polySia. Due to its negative charge polySia is highly hydrated and therefore considerably expands the hydrodynamic radius of NCAM, as indicated by the purple sphere.

1.3 PolySia biosynthesis

In mammals, the biosynthesis of polySia is catalyzed by two polysialyltransferases (polySTs), termed ST8SiaII (STX) and ST8SiaIV (PST; Eckhardt et al. 1995; Kojima et al. 1995; Nakayama et al. 1995) which are both independently capable of synthesizing polySia on NCAM (Kojima et al. 1996; Mühlenhoff et al. 1996b; Angata and Fukuda 2003). The two closely related Golgi-resident enzymes show 59% identity on the amino acid sequence level and were classified as typical members of the mammalian sialyltransferase family (Harduin-Lepers et al. 2001). They are type II transmembrane glycoproteins with a short N-terminal cytoplasmic tail, a transmembrane domain, a stem region, and a large C-terminal catalytic domain turned towards the lumen of the Golgi-apparatus. The catalytic domain includes three consensus sequences (the sialymotives L, S, and VS) which are conserved in all animal sialyltransferases and known to be involved in binding of donor and acceptor substrate, and in the transfer of sialic acid (Datta and Paulson 1995; Harduin-Lepers et al. 2005). The polySTs contain a structurally unique polybasic motif of 32 amino acids, called the polysialyltransferase domain (PSTD), which is absent in other members of the sialyltranferasse family and is essential for their polysialylation capability (Nakata et al. 2006). Both enzymes catalyze the transfer of multiple α2,8-linked sialic acid residues to terminally α2,3- or α2,6-sialylated galactose residues that are bound in α1,4-linkage to N-acetyl glucosamine (Mühlenhoff et al. 1996b; Angata et al. 1998). Whereas most glycosyltransferases modify glycan structures irrespective of the carrier protein the polySTs are highly selective for NCAM, which is by far the major polySia acceptor. So far, only a limited number of other polysialylated proteins have been found in the mammalian system. These proteins include the polySTs themselves, which autopolysialylate their own *N*-glycosylation sites (Mühlenhoff et al. 1996a; Close and Colley 1998), the α-subunit of the voltage-gated sodium channel in rat brain (Zuber et al. 1992), the scavenger receptor CD36 in human and mouse milk (Yabe et al. 2003), and neuropilin-2 on human dendritic cells (Curreli et al. 2007). Most recently, SynCAM 1 was identified as a novel polysialylated protein in brains from NCAM-deficient and wildtype mice (Galuska et al. 2009).

1.4 Developmental regulation of polysialylation

Polysialylation of NCAM is highly regulated during brain development. PolySia is detectable in mouse starting at E9, shortly after the first appearance of NCAM (Probstmeier et al. 1994; Ong et al. 1998). Then, polySia expression increases reaching a maximum in the perinatal phase, when almost all NCAM is polysialylated (Probstmeier et al. 1994; Oltmann-Norden et al. 2008; Schiff et al. 2009). Postnatally, polySia declines rapidly by about 70% between P9 and P17 (Oltmann-Norden et al. 2008). The down-regulation of polySia and the resulting increase of polySia-free NCAM coincide with the completion of major morphogenetic events during postnatal brain development. However, polySia expression persists into adulthood at sites of ongoing neurogenesis or plasticity, like the subventricular zone (SVZ) of the lateral ventricles or the subgranular zone (SGZ) of the hippocampal formation (Doetsch 2003; Bonfanti 2006).

The total amount of polySia, the chain length distribution, the ratio of polysialylated to non-polysialylated NCAM, and the amount of polySia per NCAM molecule can be affected by alterations in the expression of the two polySTs (Galuska et al. 2006; Hildebrandt et al. 2008). In this way, the degree of NCAM polysialylation may be adjusted by variation of the ST8SiaII and ST8SiaIV levels. Although there is a considerable overlap, differences in tissue- and time-specific mRNA expression patterns suggest an independent regulation of ST8SiaII and ST8SiaIV at the transcriptional level. In the perinatal mouse brain transcript levels of ST8SiaII exceed those of ST8SiaIV (Galuska et al. 2006; Oltmann-Norden et al. 2008; Schiff et al. 2009). From P1 to P21, ST8SiaII transcript levels drop rapidly, whereas ST8SiaIV declines gradually (Oltmann-Norden et al. 2008). At P9 both polySTs reach identical transcript levels, and thereafter, ST8SiaIV becomes the predominant enzyme (Oltmann-Norden et al. 2008). Thus, ST8SiaII is prevailing during embryonic and early postnatal development, while ST8SiaIV is the major polysialyltransferase of the adult brain.

1.5 NCAM interactions and NCAM-mediated neurite outgrowth

One NCAM molecule is able to interact with another NCAM molecule (homophilic interaction) on the same cell (in *cis*) or on opposing cells (*in trans*; for review see: Soroka et al. 2008). As known for long, NCAM is involved in homophilic *trans*-

interactions (Rutishauser et al. 1982). However, the exact nature of homophilic binding is still under discussion and several models exist including binding via the Ig3 domains, between Ig1 and Ig2 or the involvement of all five Ig-like domains (Rao et al. 1992; Rao et al. 1993; Rao et al. 1994; Ranheim et al. 1996; Kiselyov et al. 1997; Atkins et al. 1999; Jensen et al. 1999; Kasper et al. 2000; Johnson et al. 2004; Johnson et al. 2005a; Johnson et al. 2005b). A recent model for NCAM homophilic adhesion, based on the crystal structure of a fragment consisting of NCAM Ig1-Ig2-Ig3, postulates two different zipper-like arrays of NCAM molecules (Soroka et al. 2003; Walmod et al. 2004; Soroka et al. 2008).

NCAM can also be involved in heterophilic interactions with other proteins and extracellular matrix molecules, thereby modulating diverse biological processes including cell adhesion, migration, proliferation, differentiation, survival and synaptic plasticity (Amoureux et al. 2000; Ronn et al. 2000a; Prag et al. 2002; Ditlevsen et al. 2003; for review see: Hinsby et al. 2004a; Walmod et al. 2004). Among the heterophilic binding partners of NCAM are other members of the Ig-superfamily. A functional cooporation between NCAM and closely related cell adhesion molecule L1 in *cis* has been demonstrated. By inducing phosphorylation of tyrosine and serin residues in L1 this interaction seems to be involved in basal neurite outgrowth (Kadmon et al. 1990a; Kadmon et al. 1990b; Horstkorte et al. 1993; Heiland et al. 1998). NCAM has also been found to be a high-affinity ligand of the transiently expressed axonal surface glycoprotein-1 (TAG-1; Milev et al. 1996). In addition, NCAM interacts with several components of the extracellular matrix (ECM) like the glycosaminoglycan heparin (Cole et al. 1986; Cole and Akeson 1989), heparan sulfate proteoglycans (HSPGs) including agrin (Grumet et al. 1993; Burg et al. 1995), and chondroitin sulfate proteoglycans (CSPG) including phosphacan and neurocan. NCAM binding to phosphacan and neurocan interferes with NCAM homophilic interactions and inhibits neuronal adhesion and neurite outgrowth (Grumet et al. 1993; Friedlander et al. 1994; Milev et al. 1994; Retzler et al. 1996). NCAM was also observed to bind to collagens. However, this binding is probably indirect and mediated by NCAM interactions with the ECM via the heparin-binding site (Probstmeier et al. 1989; Probstmeier et al. 1992; Kiselyov et al. 1997).

The most prominent and widely studied function of NCAM is the promotion of neurite outgrowth (e.g. Doherty et al. 1990; for review see: Walsh and Doherty

1997). This activity is presumably mediated through an interaction and activation of the fibroblast growth factor receptor (FGFR) in response to homophilic NCAM interactions (Williams et al. 1994; Saffell et al. 1997; Hinsby et al. 2004b). Data from pancreatic tumor cells indicate that NCAM initiates the assembly of a signaling complex consisting of NCAM, N-cadherin and FGFR-4 at the cell surface and a number of intracellular adaptor and signaling proteins. The formation of this complex seems to activate FGFR-4 and downstream signaling cascades (Cavallaro et al. 2001). Independent from FGF receptor activation, NCAM may also stimulate neurite outgrowth by acting as an alternative signaling receptor for members of the GDNF (glial cell line-derived neurotrophic factor) ligand family (Paratcha et al. 2003; Paratcha and Ledda 2008; Nielsen et al. 2009). Both GDNF and the GPI-anchored GDNF family receptor α1 (GFRα1) have been demonstrated to bind directly to NCAM. Association of NCAM with GFRα1 downregulates NCAM-mediated cell adhesion and promotes high-affinity binding of GDNF to NCAM-140. The resulting activation of the cytoplasmic Src-like kinase Fyn and the focal adhesion kinase FAK seems to promote neurite outgrowth and Schwann cell migration (Paratcha et al. 2003; Sariola and Saarma 2003). Finally, NCAM itself, at least the transmembrane isoforms NCAM-140 and NCAM-180 can also take part in a number of direct or indirect interactions with various intracellular molecules (reviewed in: Walmod et al. 2004; Buttner and Horstkorte 2008). Amongst others, associations with the cytoskeletal linker-protein spectrin (Pollerberg et al. 1986; Pollerberg et al. 1987; Leshchyns'ka et al. 2003) or the src-family tyrosine kinase fyn and the focal adhesion kinase FAK (Beggs et al. 1997) have been demonstrated and were implicated in NCAM-induced neurite outgrowth.

1.6 Tools for the analysis of polySia and NCAM functions

PolySia-specific antibodies and polySia-degrading enzymes turned out to be essential tools for the analysis of polySia functions (Fig. 1B). The monoclonal antibody 735 binds specifically to α-2,8-linked Neu5Ac with a chain length of at least eight residues (Frosch et al. 1985; Husmann et al. 1990; Hayrinen et al. 2002). PolySia can be specifically degraded by the phage-derived enzymes endosialidase (endo-*N*-acetylneuraminidase, endoN) E or F (Gerardy-Schahn et al. 1995; Stummeyer et al. 2005). For binding to polySia-NCAM, endoNE seems to require a minimum of eight α-2,8-linked Neu5Ac residues with a minimum of three residues on the nonreducing end (distal side) and a minimum of five residues on the reducing end (proximal side). Thus, after enzymatic cleavage, at least five sialic acid residues remain on NCAM (Finne and Makela 1985). By use of these reagents *in vitro* and *in vivo* important functions of polySia and NCAM in migration and differentiation of neuronal precursor cells, axon growth and pathfinding, neuronal plasticity and repair have been mapped (for review see: Kleene and Schachner 2004; Bonfanti 2006; Gascon et al. 2007b; Hildebrandt et al. 2007; Maness and Schachner 2007; Rutishauser 2008).

To unravel NCAM functions and for use as pharmacological tools, a number of synthetic NCAM mimetic peptides have been developed (for review see: Berezin and Bock 2004). One example is the C3 peptide, which binds to the Ig1-domain of NCAM (Ronn et al. 1999). In the absence of NCAM interactions, a dendrimeric tetramer of this peptide (C3d) has been shown to mimic NCAM activity by inducing neurite outgrowth *in vitro* (Ronn et al. 1999; Ronn et al. 2000b). In low-density cultures of hippocampal neurons, this neuritogenic response has been demonstrated to be dose- and incubation time-dependent (Kiryushko et al. 2003). On the other hand, the C3d peptide inhibits NCAM-induced neurite growth (Ronn et al. 1999; Ronn et al. 2000b). Important in the context of this thesis, C3d has been shown to abolish the response of neuroblastoma cells to endoN treatment (Seidenfaden et al. 2003; Seidenfaden et al. 2006) indicating that this compound can block NCAM interactions induced by polySia removal.

1.7 Mode of polySia function

Models of NCAM interactions described so far mostly refer to NCAM, irrespective of its polysialylation status. PolySia, however, drastically changes NCAM properties. The classical model of polySia function is the “steric repulsion”. Due to its negative charge and high water binding capacity polySia forms a large and repulsive structure and therefore increases the distance between apposing cell membranes (Fig. 1B; Yang et al. 1992; Yang et al. 1994; Johnson et al. 2005b). Thereby, polySia is supposed to interfere with NCAM homo- and heterophilic interactions in *cis* and *trans* and to attenuate binding of other cell contact-dependent receptors, such as cadherins, leading to reduced cell adhesion and cell contact-dependent signaling (Rutishauser 1998; Fujimoto et al. 2001; Rutishauser 2008). Recently, the analysis of polySia deficient mice (see below for details) revealed that a major function of polySia is to mask NCAM and to guarantee that NCAM mediated contacts take place in a highly organized, time- and site-specific manner (reviewed in: Hildebrandt et al. 2007; Mühlenhoff et al. 2009). The assumption that polySia acts as a control element of specific NCAM interactions and that down-regulation of polySia initiates NCAM signaling is supported by *in vitro* studies. In neuroblastoma cells, loss of polySia has been shown to initiate NCAM trans-interactions at cell-cell contact sites, leading to reduced proliferation but enhanced neuronal differentiation and survival by activation of the ERK/MAP-kinase (extracellular signal-related/mitogen activated protein-kinase) pathway (Seidenfaden et al. 2003; Seidenfaden et al. 2006).

In addition to the repulsive activity of polySia and its role as a regulator of NCAM interactions, polySia-specific functions have been suggested. In the presence of polySia, hippocampal and hypothalamic neurons were more sensitive to brain-derived neurotrophic factor (BDNF) or ciliary neurotrophic factor (CNTF), resulting in enhanced neuronal survival (Muller et al. 2000; Vutskits et al. 2001; Vutskits et al. 2003). Recently, it has been shown that polySia can bind directly to a BDNF-dimer to form a large complex (Kanato et al. 2008). Furthermore, it has been demonstrated that oligodendrocyte precursors require the presence of polySia for directed migration in a gradient of the platelet-derived growth factor (PDGF; Zhang et al. 2004). Other types of direct polySia action were indicated by binding of polySia to heparan sulfate proteoglycans of the cell surface or the extracellular matrix, which may be involved in synaptogenesis and remodeling of synapses (Storms and Rutishauser 1998; Dityatev et al. 2004) or by the effects of NCAM-bound or free polySia on ionotropic glutamate receptors and synaptic plasticity (Vaithianathan et al. 2004; Hammond et al. 2006; Senkov et al. 2006).

An important, but yet poorly defined aspect of polySia functions is its role in the regulation of neural progenitor differentiation. Removal of polySia with endoN reduces migration of oligodendrocyte preprogenitors and induces their differentiation *in vitro* and *in vivo* (Decker et al. 2000; Decker et al. 2002). Similarly, removal of polySia from the subventricular zone (SVZ) blocks cell migration and leads to a premature onset of neuronal differentiation of precursors *in vivo* and in explant cultures (Petridis et al. 2004). In these studies, however, it remains open, whether reduced migration is causally linked to the observed differentiation and it is completely unclear, which of the above discussed modes of polySia action are accuntable for these responses.

1.8 PolySia in postnatal neurogenesis

PolySia is commonly used as a marker for postnatal neurogenesis (Doetsch 2003; Kempermann et al. 2004; Bonfanti 2006). The two main regions with persistent neurogenesis in adulthood are the subventricular zone (SVZ) and the subgranular zone in the hippocampal dentate gyrus (SGZ; Gage 2000). A role of polySia in migration and neuronal differentiation of progenitor cells in the SGZ has been shown only recently (Burgess et al. 2008). In contrast, several studies, including the analyses of the polysialylation- or NCAM-deficient mouse models (discussed below), deal with the role of polySia in the SVZ neurogenic system. The SVZ lines the lateral walls of the lateral ventricles and is the largest germinal zone of the adult rodent brain (Fig. 2; Conover and Allen 2002). It can be divided into an anterior (SVZa) and a posterior part. Most cells derived from the posterior part develop into astrocytes and oligodendrocytes (Privat 1975; Luskin and McDermott 1994), whereas virtually all cells derived from the SVZa differentiate into neurons (Luskin 1993; Luskin et al. 1997). Initially, ependymal cells were considered as neural stem cells (NSCs) in the SVZ (Morshead et al. 1994; Johansson et al. 1999), later glial fibrillary acidic protein expressing (GFAP) SVZ astrocytes were identified as NSCs (Doetsch et al. 1999; Alvarez-Buylla and Garcia-Verdugo 2002), but the identity of the NSCs is still under debate (Chojnacki et al. 2009).

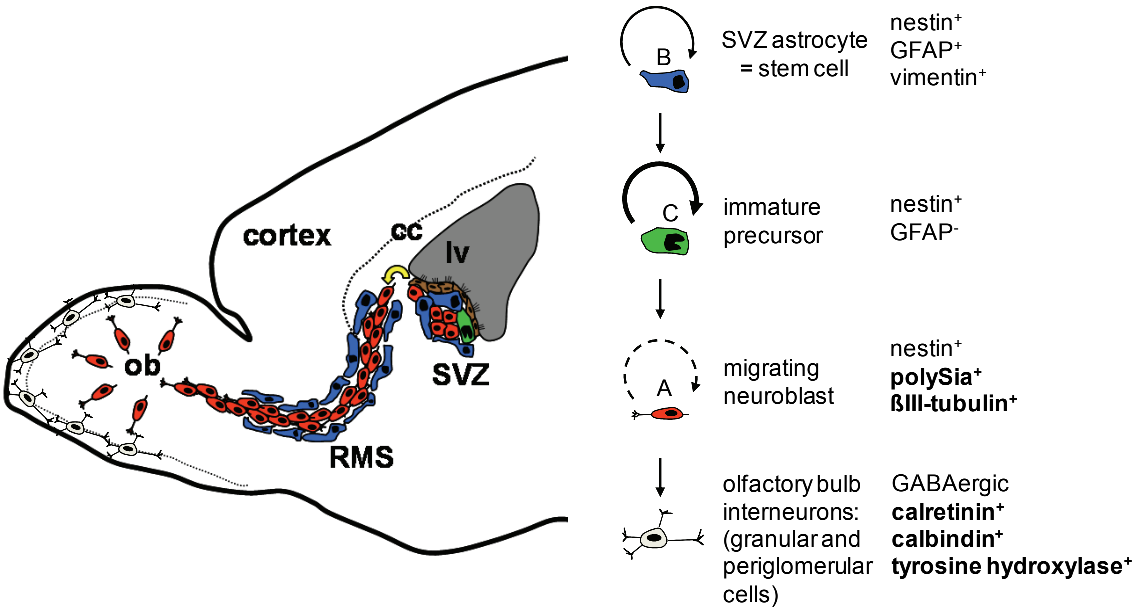

Figure 2: Adult neurogenesis in the subventricular zone (SVZ). The SVZ lines the lateral wall of the lateral ventricles (lv). In the SVZ stem cells (type B cells) give raise to transient amplifying precursors (type C cells) that produce neuroblasts (type A cells). The polySia-positive neuroblasts migrate along the rostral migratory stream (RMS) into the olfactory bulb (OB) where they become interneurons. To the right, the major markers of the different cell types are shown. Abbreviations: cc: corpus callosum, GABA: γ-aminobutyric acid, GFAP: glial fibrillary acidic protein.
Based on: (Doetsch et al. 1997; Doetsch et al. 1999; Doetsch 2003)

According to the current model, slowly dividing stem cells within the SVZ (SVZ astrocytes, type B cells) give rise to transient amplifying precursors (type C cells), which then produce migratory neuroblasts (type A cells) characterized by expression of class III β-tubulin and polySia (Doetsch and Alvarez-Buylla 1996; Doetsch et al. 1997; Doetsch et al. 1999; Morshead et al. 2003). These neuroblasts migrate tangentially in chain-like structures independent from radial glia along a well defined pathway, the rostral migratory stream (RMS) towards the olfactory bulb (OB), where they detach from the chains and differentiate into granule and periglomerular interneurons (Fig. 2; Luskin 1993; Rousselot et al. 1995; Lois et al. 1996; Wichterle et al. 1997). Chains of migrating neuroblasts are ensheated by tube-like structures formed by astrocytes (Jankovski and Sotelo 1996; Lois et al. 1996; Peretto et al. 1997). The function of the glial tunnels is not known and they are not essential for chain migration (Wichterle et al. 1997), but factors secreted by astrocytes appear to enhance the migration of SVZ neuroblasts (Mason et al. 2001). As outlined below, polySia plays a crucial role in neuroblast migration, as shown by its genetic or enzymatic deletion (Tomasiewicz et al. 1993; Cremer et al. 1994; Ono et al. 1994; Hu et al. 1996; Chazal et al. 2000; Weinhold et al. 2005). In addition, polySia was found to be important in controlling cell-contact dependent differentiation (Petridis et al. 2004) and survival of SVZ derived neuroblasts (Gascon et al. 2007a; Gascon et al. 2008).

The SVZ/RMS has become an important model to study the molecular and cellular mechanisms involved in adult neurogenesis in rodents (Alvarez-Buylla and Garcia-Verdugo 2002; Hagg 2005; Lledo et al. 2006). In humans however, the RMS has been elusive until Curtis et al. (2007) demonstrated its existence. The SVZ is regarded as a potential source of adult neural stem cells and neuronal precursors that could be applied in brain repair in neurodegenerative disease like Parkinson or after stroke. To utilize these cells, it is essential to understand the mechanisms and molecular determinants that regulate their differentiation into specific neurons. Despite the evidence of polySia being involved in precursor migration and differentiation it is not clear, if differentiation after removal of polySia is a consequence of impaired migration or due to altered NCAM properties or other cell surface interactions that may be affected by polySia.

1.9 Transgenic approaches to NCAM and polySia functions

Major insights in polySia and NCAM functions were obtained from transgenic mouse models. In the last years diverse mouse strains lacking NCAM, either one or both polySTs or NCAM and the two polySTs were bred (Fig. 3). All mice differ in their phenotype and the comparison of shared and individual phenotypic traits allows for a dissection of NCAM and polySia functions during brain development.

1.9.1 NCAM-deficient mice

In 1994, Cremer et al. described a mouse model, which is deficient for all NCAM isoforms ($N^{-/-}$; Fig. 3D), and in addition is almost completely devoid of polySia due to the absence of its major protein carrier. Unexpectedly, these mice displayed a rather mild phenotype. They appeared to be healthy and fertile and showed only a small reduction in brain weight, whereas the overall cytoarchitecture, with only a few exceptions, was normal. The most prominent finding was the reduced size of the olfactory bulbs. This is consistent with the phenotype of mice with a specific deletion of the NCAM-180 isoform and has been explained by disturbed tangential migration of SVZ-derived neuronal precursors along the RMS towards the olfactory bulb (OB; Tomasiewicz et al. 1993; Cremer et al. 1994; Hu et al. 1996). This impaired neuroblast migration is phenocopied by enzymatic removal of polySia and therefore due to polySia deficiency (Ono et al. 1994). Another prominent morphological defect of $N^{-/-}$ mice is a delamination of the mossy fiber tract in the hippocampus. Moreover, $N^{-/-}$ mice showed deficits in spatial learning when tested in the Morris water maze and long-term potentiation (LTP) was severely impaired at mossy fiber-CA3 synapses and Schaffer collateral-CA1 synapses of hippocampal organotypic slice cultures (Cremer et al. 1994; Muller et al. 1996; Cremer et al. 1998).

1.9.2 Polysialyltransferase single knockout mice

To unravel the individual role of the two polySTs in NCAM polysialylation, single knockout mice for each of the two polySTs have been generated (Eckhardt et al. 2000; Angata et al. 2004). Both strains are viable and fertile but differ in their phenotype.

In *St8siaIV*-knockout mice ($IV^{-/-}$*;* Fig. 3C), the mossy fiber tract arising from the dentate gyrus was found to be devoid of polySia but, unlike in NCAM-deficient animals, displays a normal morphology and LTP at mossy fiber-CA3 synapses is unaffected. In contrast, LTP and long-term depression (LTD) are impaired at Schaffer collateral-CA1 synapses of adult $IV^{-/-}$ mice (Eckhardt et al. 2000). Thus, alterations of activity-induced synaptic plasticity in the CA1 region are similar in $IV^{-/-}$ and $N^{-/-}$ animals. Consistent with the lack of apparent neurodevelopmental defects, polySia levels in the brains of newborn $IV^{-/-}$ mice are analogous to the wildtype situation. This can be explained by compensation due to the high expression of ST8SiaII in early postnatal stages. In contrast, polySia expression is strongly reduced in adult ST8SiaIV-deficient animals corresponding to the predominance of ST8SiaIV in adult wildtype mice (Eckhardt et al. 2000; Oltmann-Norden et al. 2008).

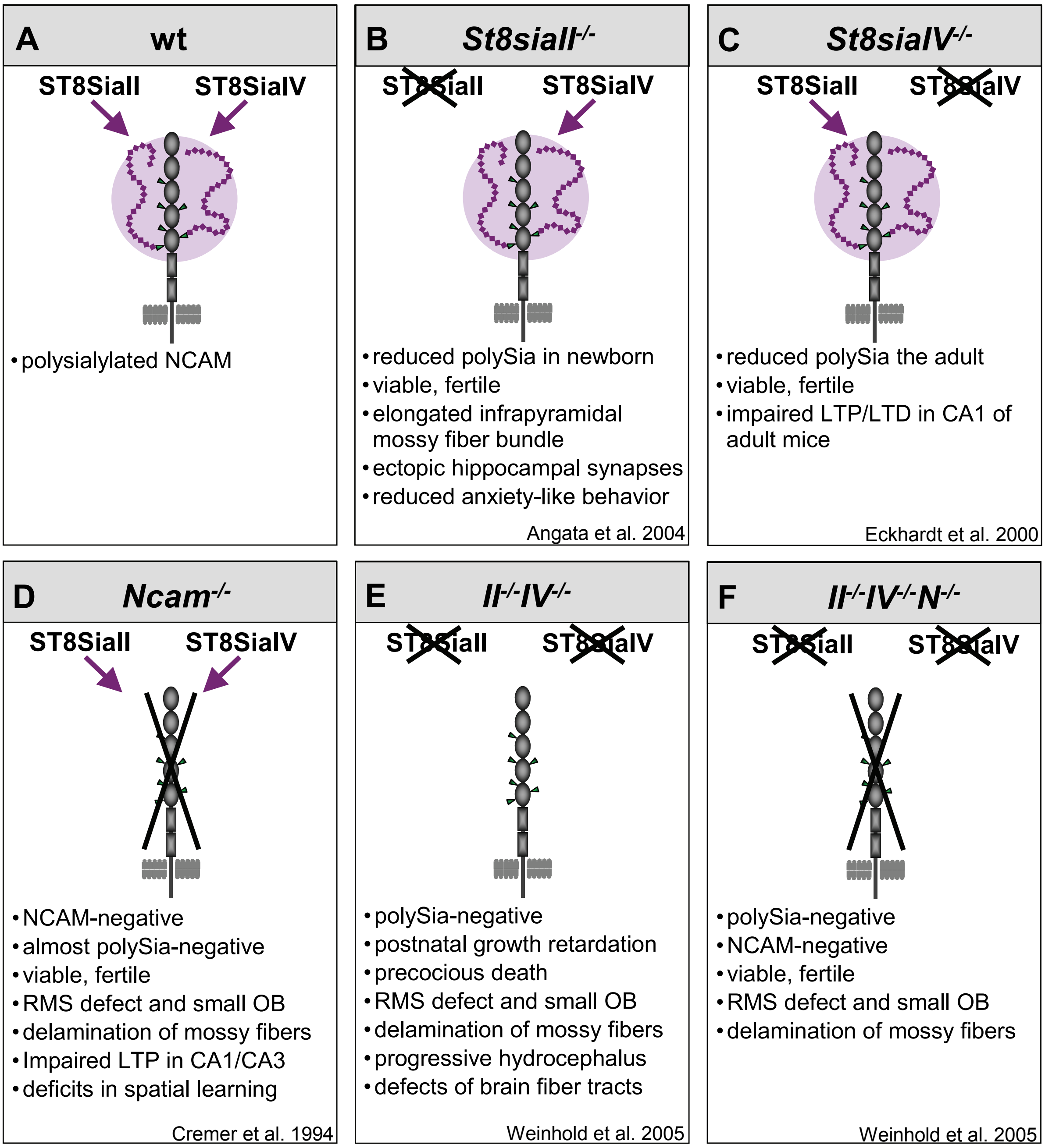

Figure 3: Overview of the different mouse models and synopsis of their phenotypes. **A**: wildtype mouse. **B**: *St8siaII* knockout mouse. **C**: *St8siaIV* knockout mouse. **D**: *Ncam* knockout mouse: **E**: *St8siaII* and *St8siaIV* double knockout mouse. **F**: *St8siaII*, *St8siaIV* and *Ncam* triple knockout mouse. Abbreviations: CA: cornu ammonis subfield of the hippocampus (Ammon's horn), LTD: long-term depression, LTP: long-term potentiation, OB: olfactory bulb, RMS: rostral migratory stream.

In *St8siaII*-knockout mice (*II*$^{-/-}$; Fig. 3B) polySia levels are reduced in newborn animals while they are hardly affected in adults (Angata et al. 2004; Galuska et al. 2006; Oltmann-Norden et al. 2008). Similar to the phenotype of NCAM-deficient mice, a morphological defect of the mossy fiber tract has been described in *II*$^{-/-}$

mice. Its infrapyramidal bundle is elongated and mistargeting of mossy fibers is indicated by the presence of ectopic synapses. However, in contrast to *IV*$^{-/-}$ mice, the mossy fiber tract of adult *II*$^{-/-}$ animals is polySia-positive, pointing towards a developmental origin of this defect. These mossy fiber alterations have been linked to the higher exploratory drive and reduced behavioral responses to Pavlovian fear conditioning observed in *II*$^{-/-}$ mice (Angata et al. 2004).

As judged by non-quantitative immunostaining, no obvious decrease of polySia expression in the SVZ and RMS of *II*$^{-/-}$ or *IV*$^{-/-}$ mice has been detected. From the regular pattern of migratory cells in the RMS of *IV*$^{-/-}$ and the unaltered gross morphology of RMS and OB in *II*$^{-/-}$ mice, it was concluded that precursor migration along this pathway is not impaired (Eckhardt et al. 2000; Angata et al. 2004). However, no detailed analysis has been performed so far and therefore minor, yet undetected defects due to slightly altered polysialylation patterns can not be excluded.

1.9.3 Polysialyltransferase double knockout mice

The simultaneous deletion of both polysialyltransferase genes (*II*$^{-/-}$*IV*$^{-/-}$; Fig. 3E) resulted in mice completely devoid of polySia but retaining normal levels of NCAM protein (Weinhold et al. 2005). *II*$^{-/-}$*IV*$^{-/-}$ mice recapitulate the major morphological phenotype of *N*$^{-/-}$ mice, i.e. smaller olfactory bulbs, a migration deficit in the RMS and delamination of mossy fibers (Weinhold et al. 2005; Angata et al. 2007). This indicates that these defects are caused by the lack of polySia, independent of the presence or absence of NCAM. In contrast to *NCAM*, *ST8SiaII* or *ST8SiaIV* single knockout mice the *II*$^{-/-}$*IV*$^{-/-}$ animals display additional defects resulting in a severe phenotype. Although *II*$^{-/-}$*IV*$^{-/-}$ mice are indistinguishable from double-heterozygous littermates at P1, their postnatal growth is drastically retarded and less than 20% survive for more than four weeks. *II*$^{-/-}$*IV*$^{-/-}$ mice have a high incidence of a progressive hydrocephalus with massive dilatation of the lateral and third ventricles in conjunction with thinning of cortex and corpus callosum as well as deformation of the hippocampal formation and fimbria. Independent of hydrocephalus formation, *II*$^{-/-}$*IV*$^{-/-}$ mice show defects of several fiber tracts. The most striking findings, so far, were the complete absence of the anterior commissure as well as hypoplasia of the internal capsule, the corticospinal and mammillothalamic tracts, and a reduced rostrocaudal extent of the corpus callosum (Weinhold et al. 2005; Hildebrandt et al. 2009). Since these *II*$^{-/-}$*IV*$^{-/-}$ specific defects are fully reversed by the additional deletion of the *NCAM* gene in triple knockout mice (*N*$^{-/-}$*II*$^{-/-}$*IV*$^{-/-}$; Fig. 3F), it has been concluded that they are caused by a gain of NCAM functions (Weinhold et al. 2005). Therefore, one function of polySia is to mask NCAM and prevent premature interactions. This is supported by a recent study, which revealed that the extent of fiber tract deficiencies observed in mice with selected combinations of mutant NCAM and polysialyltransferase alleles correlates strictly with the level of polySia-free NCAM during brain development (Hildebrandt et al. 2009). More important, this correlation indicates that also minor imbalances of NCAM polysialylation can cause deficits in brain connectivity.

Angata et al. (2007) provide evidence that the migration of undefined precursors is impaired during cortical development of *II*$^{-/-}$*IV*$^{-/-}$ mice. This study also reports reduced numbers of calbindin (CB)-positive interneurons in the cerebral cortex of adult *II*$^{-/-}$ *IV*$^{-/-}$ animals. Since no other interneuron populations were evaluated, the specificity of this effect remains elusive. Moreover, the observed defect may be not related to impaired migration during development but could be secondary to hydrocephalus formation, which results in cortical thinning as observed in the animals that were evaluated in this study.

1.10 PolySia and NCAM in neuropsychiatric disorders

Numerous studies link dysregulation of NCAM to the pathophysiology of schizophrenia and other neuropsychiatric disorders (reviewed in Vawter 2000; Sullivan et al. 2007; Brennaman and Maness 2008b). Elevated levels of a soluble NCAM fragment have been detected in the cerebrospinal fluid and in postmortem brains of schizophrenic patients, and fragment concentrations were found to correlate with severity and duration of the disease (Poltorak et al. 1995; van Kammen et al. 1998; Vawter et al. 2001; Sullivan et al. 2007). Furthermore, reduced polySia expression was observed in the hilus region of the hippocampus in schizophrenic brains (Barbeau et al. 1995). The neurodevelopmental hypothesis of schizophrenia implicates altered neuronal development in disrupted brain connectivity and cognitive dysfunction (Lewis and Levitt 2002; Rapoport et al. 2005; Fatemi and Folsom 2009). Since NCAM and polySia play a crucial role in cell migration and differentiation they are candidate factors for schizophrenia. NCAM1 as well as both polysialyltransferase genes map to chromosomal regions considered to be involved in genetic predisposition to schizophrenia (11q23.1, 15q26, and 5q21 for NCAM1, ST8SIA2 and ST8SIA4, respectively; Lewis et al. 2003; Lindholm et al. 2004; Maziade et al. 2005). Single nucleotide polymorphisms (SNPs) in the promoter region of ST8SIA2 showed a significant association with schizophrenia (Arai et al. 2006) and two recent studies suggested also an association between SNPs in the NCAM1 gene and schizophrenia (Atz et al. 2007; Sullivan et al. 2007).

The striking analogies between the phenotype of NCAM- or polysialylation-deficient mice and the pathophysiological findings in schizophrenia further support the possibility that NCAM polysialylation may be relevant to etiological aspects of schizophrenia. Ventricular enlargement as shown for NCAM-180 or polySia deficient ($II^{-/-}IV^{-/-}$) mice (Wood et al. 1998; Weinhold et al. 2005) is one of the most characteristic abnormalities in schizophrenia (Hyde and Weinberger 1990). Also similar to $N^{-/-}$ or $II^{-/-}IV^{-/-}$ mice, patients with schizophrenia have a reduced size of the olfactory bulb (Turetsky et al. 2000). $N^{-/-}$ mice show deficits in spatial learning and LTP (Cremer et al. 1994; Cremer et al. 1998), which correlates with cognitive impairment, another hallmark finding in schizophrenia (Heinrichs and Zakzanis 1998). Furthermore, in schizophrenic patients a reduction of corpus callosum size and length as well as a decreased size of the internal capsule has been reported (Innocenti et al. 2003; Hulshoff Pol et al. 2004; Douaud et al. 2007; Mitelman et al. 2007; Begre and Koenig 2008). This is in correlation with the fiber tract deficits observed in polysialylation compromised mice (Hildebrandt et al. 2009). Major abnormalities of schizophrenic brains concern alterations of specific GABAergic interneurons, most notably of the parvalbumin-positive subtype, in the prefrontal cortex (PFC) and hippocampus (Reynolds et al. 2001; Eyles et al. 2002; Heckers and Konradi 2002; Zhang and Reynolds 2002; Sakai et al. 2008). As the combined evidence indicates the possibility that altered NCAM polysialylation contributes to a neurodevelopmental predisposition to schizophrenia, it appears mandatory to analyze, if NCAM- or polysialylation-deficient mice display aberrant compositions of interneurons, similar to those observed in schizophrenia.

1.11 Objectives

In vitro data indicate that loss of polySia enhances differentiation of progenitor cells. In addition, enzymatic removal of polySia *in vivo* has been shown to induce premature differentiation of neuronal precursors in the subventricular zone and subgranular zone. However, since polySia also plays a crucial role in neuroblast migration in the rostral migratory stream, the cause for the differentiation response remained elusive. Differentiation could also result from impaired chain migration leading to altered interactions with the cellular environment along the migratory path. Therefore, the objective of the first study of this thesis was to investigate the role of polySia and NCAM in neuroblast differentiation *in vitro*, independent from a possibly confounding influence of migration.

As outlined above, polySia and NCAM seem to be involved in interneuron migration and differentiation on the one hand as well as in the pathophysiology of schizophrenia on the other. Since alterations of GABAergic interneurons are frequently observed in schizophrenia, one important open question is, if any of the major interneuron populations is affected by altered NCAM polysialylation. The second study of this thesis addresses this question by evaluating selected interneuron populations of the olfactory bulb, prefrontal cortex and hippocampus in mouse models with impaired polysialylation capacity or NCAM deficiency.

Chapter 2 - Polysialic acid controls NCAM-induced differentiation of neuronal precursors into calretinin-positive olfactory bulb interneurons

- Manuscript originally published in Developmental Neurobiology -

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Preface – About this manuscript

In the absence of polysialic acid (polySia) neuroblast migration in the rostral migratory stream (RMS) is impaired. Together with migration deficits, enhanced neuronal differentiation has been observed in the RMS after enzymatic removal of polySia, but the cause for the differentiation response remained elusive and could result from halted chain migration leading to altered interactions with the cellular environment along the migratory path. Therefore, the first study of my thesis aimed at analyzing the impact of polySia and of the neural cell adhesion molecule NCAM on differentiation under controlled conditions in vitro and independent from its firmly established role in chain migration.

For this purpose, primary cultures of subventricular zone (SVZ) derived precursors from early postnatal wildtype, NCAM knockout and polysialylation-deficient mice were generated. The wildtype cultures were treated with NCAM- or polySia-specific reagents and the differentiation response was analyzed by evaluation of neuritogenesis and the expression of biochemical differentiation markers. To confirm the absence of chain migration in the primary cultures, time-lapse recording was performed.

My contributions to this manuscript comprised the preparation and culture of neuroblasts, the immunofluorescent staining, microscopy, and all evaluations. Prof. H. Hildebrandt and I designed the experiments and wrote the paper.

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Polysialic Acid Controls NCAM-Induced Differentiation of Neuronal Precursors into Calretinin-Positive Olfactory Bulb Interneurons

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ABSTRACT: **Understanding the mechanisms that regulate neurogenesis is a prerequisite for brain repair approaches based on neuronal precursor cells. One important regulator of postnatal neurogenesis is polysialic acid (polySia), a post-translational modification of the neural cell adhesion molecule NCAM. In the present study, we investigated the role of polySia in differentiaion of neuronal precursors isolated from the subventricular zone of early postnatal mice. Removal of polySia promoted neurite induction and selectively enhanced maturation into a calretinin-positive phenotype. Expression of calbindin and Pax6, indicative for other lineages of olfactory bulb interneurons, were not affected. A decrease in the number of TUNEL-positive cells indicated that cell survival was slightly improved by removing polySia. Time lapse imaging revealed the absence of chain migration and low cell motility, in the presence and absence of polySia. The changes in survival and differentiation, therefore, could be dissected from the well-known function of polySia as a promoter of precursor migration. The differentiation response was mimicked by exposure of cells to soluble or substrate-bound NCAM and prevented by the C3d-peptide, a synthetic ligand blocking NCAM interactions. Moreover, a higher degree of differentiation was observed in cultures from polysialyltransferase-depleted mice and after NCAM exposure of precursors from NCAM-knockout mice demonstrating that the NCAM function is mediated via heterophilic binding partners. In conclusion, these data reveal that polySia controls instructive NCAM signals, which direct the differentiation of subventricular zone-derived precursors towards the calretinin-positive phenotype of olfactory bulb interneurons**. © 2008 Wiley Periodicals, Inc. Develop Neurobiol 68: 1170–1184, 2008

Keywords: **neurogenesis; subventricular zone; olfactory bulb; neural cell adhesion molecule; cell surface glycosylation**

INTRODUCTION

Neurogenic systems of the postnatal brain provide a reservoir of neuronal precursor cells with potential use in cell-based brain repair approaches (Lindvall et al., 2004; Falk and Frisen, 2005). In particular, ongoing neurogenesis from stem cells in the subventricular zone (SVZ) of the postnatal rodent brain has become an important model to study the molecular and cellular mechanisms that contribute to the generation of new neurons (Alvarez-Buylla and Garcia-Verdugo, 2002; Hagg, 2005; Lledo et al., 2006). *In vivo*, slowly dividing stem cells give rise to transient

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amplifying precursors, which then produce migratory neuroblasts characterized by expression of class III β-tubulin and polysialic acid (polySia) (Doetsch and Alvarez-Buylla, 1996; Doetsch et al., 1997, 1999; Morshead et al., 2003). These neuroblasts migrate in chain-like structures towards the olfactory bulb (OB), where they detach from the chains and differentiate into granule and periglomerular interneurons. Although the majority of granule cells are homogeneously GABAergic, sub-populations of interneurons in the glomerular layer can be distinguished by differential expression of markers for GABAergic and dopaminergic neurons, or the calcium-binding proteins calretinin and calbindin (Kosaka et al., 1995; Brinon et al., 1999; Kohwi et al., 2007; Parrish-Aungst et al., 2007). So far, little is known about the molecular cues regulating neuronal differentiation and maturation of SVZ-derived neuroblasts. Their neuronal commitment has been assumed, but recent evidence indicates conversion and differentiation into glial cell types upon ectopic transplantation (Seidenfaden et al., 2006a). Thus, one challenge for using the reservoir of SVZ-derived precursors in cell-based or endogenous brain repair approaches is to explore new molecular cues to direct their neuronal differentiation and maturation.

In rodents and humans, polySia is intimately linked to postnatal neurogenesis from the SVZ (Alvarez-Buylla and Garcia-Verdugo, 2002; Curtis et al., 2007). This carbohydrate polymer of α2,8-linked sialic acids is found almost exclusively as a post-translational modification of the neural cell adhesion molecule, NCAM (Mühlenhoff et al., 1998; Angata and Fukuda, 2003). Mice deficient in NCAM also lack polySia (Tomasiewicz et al., 1993; Cremer et al., 1994) and are characterized by impaired migration of SVZ-derived neuroblasts along the rostral migratory stream (RMS; Ono et al., 1994; Hu et al., 1996; Chazal et al., 2000). The migration phenotype must be explained by the loss of polySia, because it also developed in mice with genetic ablation of poly-Sia synthesis (Weinhold et al., 2005; Angata et al., 2007) and could be copied by enzymatic removal of polySia using endo-N-acetyl-neuraminidase (endoN) *in vivo* and in SVZ explant cultures (Ono et al., 1994; Hu et al., 1996). Together with migration deficits, enhanced neuronal differentiation has been observed in the RMS after endoN treatment (Petridis et al., 2004), but the cause for the differentiation response remained elusive and could result from halted chain migration leading to altered interactions with the cellular environment along the migratory path. This possibility is highlighted by recent work showing neuronal differentiation as a consequence of a cell-intrinsic block of neuroblast migration in the RMS of doublecortin-deficient mice with uncompromised polySia expression (Koizumi et al., 2006). The present study was designed to analyze the impact of polySia and NCAM on differentiation independent from its firmly established role in chain migration. Using primary cultures of neuroblasts derived from single cell suspensions of the SVZ, chain migration was not observed and the overall low cell motility was not affected by enzymatic removal of polySia. Morphometric analyses and evaluation of biochemical markers revealed that loss of polySia initiates NCAM trans-interactions, which promote the differentiation of SVZ-derived precursors into a calretinin-positive phenotype.

METHODS

Mice

C57BL/6J and transgenic mice were bred at the central animal facility at Hannover Medical School. All protocols for animal use were in compliance with German law and approved by the responsible animal welfare officer. *St8sia-II* and *St8sia-IV* single knockout strains, which have been backcrossed with C57BL/6J mice for at least six generations, were intercrossed to obtain double knockout *St8sia-II*$^{-/-}$ *St8sia-IV*$^{-/-}$ animals (Weinhold et al., 2005). *Ncam*$^{-/-}$ mice were obtained from H. Cremer (Developmental Biology Institute of Marseille, Marseille, France; Cremer et al., 1994) and backcrossed to C57BL/6J mice for at least six generations. Genotyping was performed by PCR as previously described (Weinhold et al., 2005).

NCAM- and PolySia-Specific Reagents

Recombinant endo-N-acetylneuraminidase (endoN) specifically degrading polySia was isolated as described (Gerardy-Schahn et al., 1995; Stummeyer et al., 2005) and used in the cell culture medium at a concentration of 60 ng/mL to remove polySia from the cell

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surface. C3d, a synthetic dendrimeric undeca peptide which binds to the first Ig-like module of NCAM and its inactive variant C3d2ala (Ronn et al., 1999) were kindly provided by E. Bock (Panum Institute, Copenhagen, Denmark) and used at 1 μ*M*.

NCAM-Fc used in this study contains the extracellular domain of human NCAM (amino acids 1–705) fused to the constant (Fc) part of human IgG_1. For construction of the expression plasmid the human NCAM-120 cDNA (in the absence of the small alternatively spliced exons) was used as a template and a fragment comprising nucleotides 1–2,115 was amplified by PCR with the primers NCAMFwd (5'-CCCAAGCTTACAATGCTGCAAACTAAG GATC-3') and NCAMRev (5'-ACGGATCC ACTTACCTGTATTGCCTCCCAAG-3') containing *Hind*III and *Bam*HI restriction sites, respectively. Endonuclease restriction sites are underlined. After digestion, the *Hind*III-*Bam*HI fragment was ligated into the corresponding sites of pcDNA3.1-Ig upstream of the DNA sequence encoding the human Fc part of IgG_1. The vector pcDNA3.1-Ig was kindly provided by H. Volkmer (NMI Reutlingen, Germany). The resulting plasmid pcDNA3.1-N-Fc was stably expressed in polysialylation-deficient CHO-2A10 cells (Eckhardt et al., 1995). Secreted, polySia-free NCAM-Fc was affinity purified from cell culture supernatants by protein A-Sepharose chromatography. The human IgG_1-Fc fragments used for control experiments were isolated on protein A-Sepharose from the supernatants of CHO-2A10 cells stably transfected with pSecTagC (Invitrogen, Germany) containing the *Hind*III-*Not*I fragment from pcDNA3.1-Ig. Recombinant NCAM-Fc and Fc protein were used at 1 μg/mL.

Culture of Primary Neuroblasts

Brains dissected from postnatal day 2 mice (C57BL/6J or knockout strains as indicated in the result part) were immediately sliced into 400 μm coronal vibratome sections, transferred to 1x HBSS (Hanks' Balanced Salt Solution, GIBCO, Germany), and the anterior part of the SVZ was isolated from the striatal wall of the lateral ventricle. SVZ tissue was minced and incubated with 10 mg/mL Trypsin type IX (Sigma, Germany), 0.5 mg/mL DNAse I (Roche, Germany) at 37°C for 10 min. During the second half of the incubation period, 0.5 mg/mL DNAse I and 12 m*M* $MgCl_2$ were added. After gentle trituration cells were collected by centrifugation with 280g for 10 min at 4°C and the pellet was resuspended in Dulbecco's modified Eagle Medium (high glucose), containing 2 m*M* Glutamax, 1% (v/v) N2 supplement, 2% (v/v) B27 (all from GIBCO, Germany), 10 μg/mL Insulin (Sigma, Germany), 10% (v/v) horse serum (Biochrom, Germany) and 5 μg/mL gentamycin (GIBCO, Germany). If not stated otherwise, single cell suspensions were seeded at densities of 100,000 cells/cm^2 in 12 or 24 well plates containing glass coverslips coated with poly-D-lysine (100 μg/mL). Reagents were added when cells were firmly attached (~2 h after start of culture). Fixation and immunostaining of cells followed 48 h after addition of test reagents.

Culture of Neuroblasts on Cellular Substrate Layers

Co-culture experiments were used to test the influence of NCAM presented at the cell surface of substrate cell layers on the neuroblast differentiation. To generate NCAM-positive cell layers, the clone LBN was used, representing a subclone of the murine fibroblast cell line L-929 stably expressing nonpolysialylated human NCAM-140 (lacking the alternatively spliced exons VASE, a, b, c and AAG; Kasper et al., 1996). NCAM-negative layers were established with the mock transfected L-929 clone LVN. Mocktransfected LVN and NCAM-140 expressing LBN cells were kindly provided by E. Bock (Panum Institute, Copenhagen, Denmark). The LBN cells were subcloned, screened by immunocytochemistry with NCAM-specific mAb 123C3, and a clone with homogeneous NCAM immunoreactivity was used. Western blot analysis confirmed that only non-polysialylated NCAM was expressed by LBN cells. To form the substrate layers, LBN or LVN cells were grown in 12-well plates on 20 mm diameter glass coverslips precoated with poly-D-lysine (100 μg/mL). In the experimental situation, primary neuroblasts were seeded on confluent monolayers.

Time-Lapse Microscopy and Measurements of Cell Motility

For time-lapse live-cell imaging cells were seeded in poly-D-lysine coated Lab-Tek two-chamber slides (Nunc, Germany) and placed in a humidified, CO_2- and temperaturecontrolled incubation chamber mounted on a Zeiss Axiovert 200 M inverted microscope equipped with a motorized stage, AxioCam MRm digital camera and AxioVison software (Carl Zeiss, Germany). Five frames per chamber were recorded in both chambers simultaneously and images were acquired over a 48 h period at a rate of 10 images/h. To assess cell motility the displacement of the center of the observed cell soma was tracked using the interactive measurement module of the AxioVison software. Cell movements in µm/h were calculated from the length of the recorded track, given that the individual cell remained viable and could be traced over the entire observation time (48 h).

Immunocytochemistry

Primary cultured neuroblasts and mouse fibroblasts were fixed with 4% paraformaldehyde for 30 min, blocked with 2% BSA for 1 h at RT and incubated with primary antibodies for 2 h at RT or overnight at 4°C. The following monoclonal (mAb) or polyclonal antibodies (pAb) were used: polySia-specific mouse mAb 735 (IgG_{2a}, 10 µg/mL), rat mAb H28 recognizing all isoforms of mouse NCAM (IgG_{2a}, 7.5 µg/mL), and mouse mAb 123C3, reactive with all isoforms of human NCAM (IgG_1, 5 µg/mL). The following mono- and polyclonal antibodies (mAb, pAb) were applied according to the manufacturers' instructions: Beta-III-tubulin-specific mouse mAb (IgG_{2b}), glutamate decarboxylase (GAD65/67)-specific rabbit pAb, glial fibrillary acidic protein-specific rabbit pAb (all Sigma, Germany), calretinin- and calbindin-specific rabbit pAb (Swant, Switzerland), tyrosine hydroxylase-specific rabbit pAb, A2B5-specific mouse mAb (both Chemicon, CA), and Pax6-specific rabbit pAb (Covance, CA). For staining of intracellular markers, cells were permeabilized with 0.1% Triton X-100. In some of the experiments, the rate of proliferation was addressed by incorporation of 5-bromo-deoxyuridine (BrdU, Roth, Germany). Cells were incubated for 2 h with 10 µ*M* BrdU prior to fixation. After incubating with 2*N* HCl for 15 min at 378C followed by 0.1*M* borate, pH 8.5 for 10 min, BrdU was detected with rat anti-BrdU antibody (clone BU1/75, Accurate Chemical and Scientific Corp., NY) diluted 1:100 for immunocytochemistry. Rabbit, Rat, and mouse IgG-specific and subtype-specific Cy3- (Chemicon, CA), Alexa488-, Alexa568- (Molecular Probes, The Netherlands), and FITC-conjugated secondary antibodies (Rockland, CA) were used as suggested by the suppliers. In double stained immune-fluorescence samples, specificity was controlled by omitting one of the two primary antibodies. Cross-reactivity was not observed for any of the secondary antibodies. Cells were coverslipped in Vectashield mounting medium with DAPI (Vector Laboratories, CA). Microscopy was performed using a Zeiss Axiovert 200 M equipped with AxioCam MRm digital camera and AxioVison software (Carl Zeiss, Germany).

Terminal Deoxynucleotidyl Transfer-ase-Mediated dUTP Nick end Labeling

DNA strand breaks were detected by terminal deoxynucleotidyl transferase-mediated Dig-oxigenin-dUTP nick end labeling (TUNEL) as described by Herzog et al. (2007). After preincubation in 1x terminal deoxytransferase (TdT) buffer containing 0.2 *M* cacodylate, 25 m*M* Tris-HCl, 1 m*M* $CoCl_2$ and 0.01% Triton X-100 (Fermentas, Germany), cells were labeled using 1x TdT buffer, 4 units TdT, 1 µ*M* DigdUTP and 0.1 m*M* dTTP for 1 h at 37°C. The reaction was stopped by washing with 2x SSC (sodium citrate buffer). After that cells were rinsed with PBS and Digoxygenin was visualized using an anti-Dig-Rhodamin antibody (Roche, Germany).

Evaluation of Neuritogenesis and of Immunocytochemical Markers

From each well with cultured neuroblasts between 3 and 20 randomly selected frames (0.14 or 0.04 mm^2) were scanned and evaluated in a blinded procedure using AxioVison software. Per frame, the mean length of all *β*-III-tubulin-positive processes exceeding 10 µm was determined and the number of processes was evaluated relative to the total number of neuroblasts. In addition, the number of neurite branch points and the number of processes per cell were counted in some of the experiments. Although neuritis were frequently touching each other, the use of *β*-III-tubulin staining enables the assignment of each neurite to a particular cell. For evaluation of neurochemical markers, the number of calretinin-, calbindin-, or Pax6-

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positive cells was counted relative to the total number of neuroblasts identified by *β*-III-tubulin or polySia staining. TUNEL positive cells were evaluated against total numbers of DAPI stained nuclei. Data were plotted as means (± s.e.m.) of values from at least three independently treated cultures per experimental group. Statistical analyses were performed using Graphpad Prism software. Differences between two groups were evaluated with Student's t test (two-tailed). For more than two groups to compare, one way ANOVA with Newman-Keuls multiple comparison post hoc test (two-tailed) was applied.

RESULTS

Characterization of SVZ-Derived Neuroblast Cultures

Dissociated cells isolated from the SVZ of 2-day old wildtype mice were plated as single cell suspension on poly-D-lysine coated glass coverslips [Fig. 1(A,B)]. During attachment many of the cells aggregated in small clusters [Fig. 1(C)] and more than 95% of the adherently growing cells were immunopositive for *β*-III-tubulin and polySia [Fig. 1(D,E)], two markers indicative for the neuroblast stage of neuronal precursor cells (Doetsch and Alvarez-Buylla, 1996; Lim and Alvarez-Buylla, 1999). Together with its protein carrier NCAM, polySia was found to be enriched at cell-cell contacts [Fig. 1(G,H)], and some of the cells started to develop polySia-positive neuritis [Fig. 1(I)]. Incubation of cultures with the polySia-specific phage-derived enzyme endoN (Stummeyer et al., 2005) efficiently removed polySia from the surface of adherently growing primary cultured neuroblasts and no re-expression of polySia could be detected during a 2-day culture period [Fig. 1(F)]. Consistent with the observation, that migrating precursors within the RMS express the GABAergic marker glutamic acid decarboxylase (GAD; Wang et al., 2003), all polySia- and *β*-III-tubulin-positive cells in the adherent cultures also stained positive for GAD-65/67 [Fig. 1(J)]. In a parallel control experiment, cells from the same preparations which gave rise to the homogenously *β*-III-tubulin- and polySiapositive neuroblasts were cultured under nonadherent conditions. Under these conditions proliferating, BrdU incorporating neurospheres were formed (Supplementary Fig. S1). In line with published data (Gritti et al., 1996; Doetsch et al., 1999; Dizon et al., 2006), these neurospheres consisted of cells expressing markers of astrocytes and oligodendrocyte precursors (GFAP, A2B5) together with *β*-III-tubulin and polySia-positive neuroblasts (Supplementary Fig. S1). In contrast, no A2B5-positive cells were detected after 2 days under adherent culture conditions and in the presence or absence of endoN the number of GFAP-positive cells was invariably below 2%.

Unlike for the neurospheres, a 2 h pulse of BrdU yielded no labeled cells in the adherently growing cultures. The lack of cell proliferation was confirmed by time-lapse live-cell imaging (for examples, see Supplementary Material, Video 1 and 2). Over an observation period of 48 h, less than 1% of the cells divided. In addition, the time-lapse recordings revealed that no migrating chains were formed in the adherent neuroblast cultures. Importantly, removal of polySia with endoN had no effect on the overall low cell motility and sporadic saltatory movements were observed in both, control and endoN-treated cultures (Fig. 2 and Supplementary Material, Video 1 and 2). In summary, these data demonstrate that under adherent culture conditions the influence of polySia removal on neuroblast differentiation could be tested without the risk of being superimposed by potential effects on proliferation or chain migration.

Removal of PolySia Enhances Neuroblast Survival

Further assessment of adherently growing neuroblasts revealed that cell numbers were slightly increased after 2 days of culture in the presence of endoN [Fig.3(A)]. Because no proliferation was observed, this effect of polySia removal must be due to enhanced survival, which was confirmed by an evaluation of apoptotic cells using TUNEL staining [Fig. 3(B–D)]. The slight but statistically significant decrease of TUNEL-positive cells after endoN treatment was inversely proportional to the increase of cell numbers.

Neuritogenic Effects of PolySia Removal and Trans-Interacting NCAM

All processes in the SVZ-derived cultures stained positive for *β*-III-tubulin identifying them as neuritis [Fig. 4(A–D)]. At day two of

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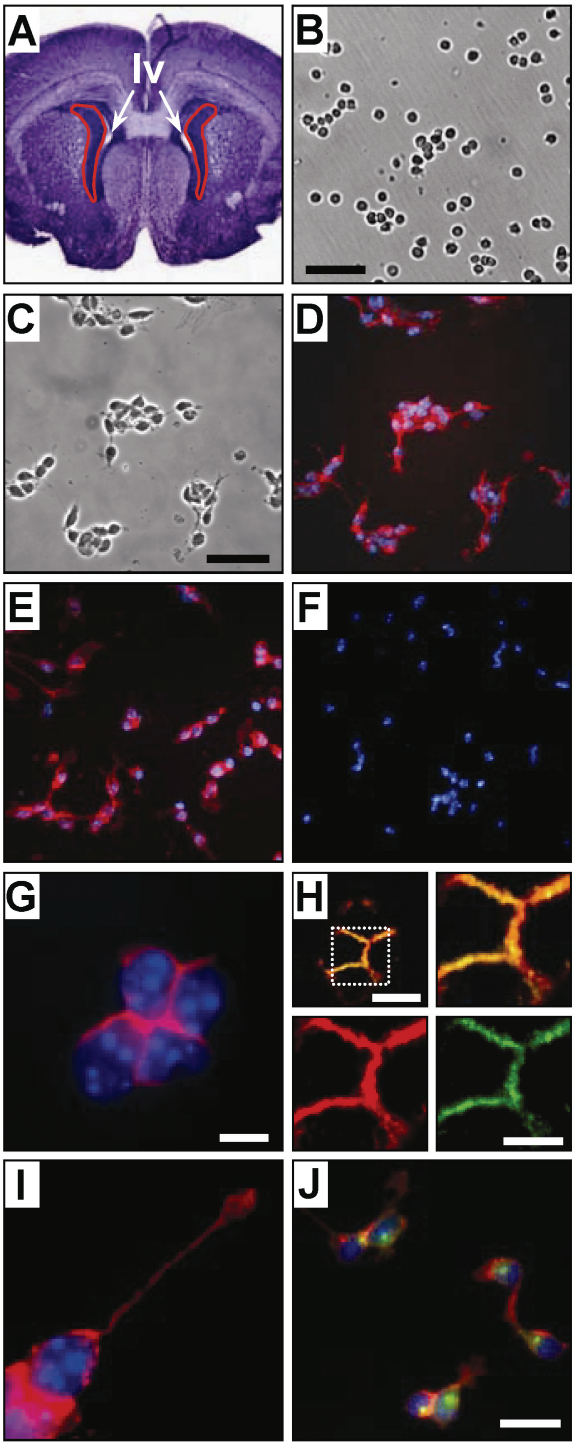
endoN treatment the number of neurites was significantly higher than in control cultures, while the mean length of the neuritis remained constant [Fig. 4(A–F)]. Further analyses established that the neuritogenic response was borne of an increase in neurite-bearing cells rather than in the number of neurites per cell or in neurite branching [Fig. 4(G–I)]. Identical to the situation observed in cultures grown on poly-D-lysine, polySia-NCAM- and β-III-tubulin-positive cells were abundant, when SVZ cells were seeded on monolayers of NCAM-negative fibroblasts [LVN, Fig. 4(J,L)]. However, if NCAM-positive, polySia-negative fibroblasts [LBN, Fig. 4(K,M)] were used in the substrate layer, substantially higher amounts of neurites were obtained [Fig. 4(N)]. As with endoN treatment, no changes in neurite lengths, but enhanced amounts of cells with one or more neurites, and a slight, though not statistically significant, increase in the number of neuritis per cell and of neurite branches was observed [Fig. 4(O–R)]. Combining growth on polySia-free NCAM with endoN treatment elicited no additive effects [Fig. 4(S)]. These data show that both, unmasking of NCAM by removal of polySia and exposure of SVZ cultures to polySia-free NCAM, result in a comparable neuritogenic response.



Nonpolysialylated NCAM Promotes Maturation into a Calretinin-Positive Phenotype

We next asked, whether polySia removal affects the maturation of SVZ-derived neuroblasts into a specific interneuron subtype.

Figure 1 Characterization of SVZ-derived neuroblast cultures. A: Coronal section of a P2 mouse brain stained with Cresyl violet/ thionine (Nissl stain). The subventricular zone (SVZ) of the striatal wall of the lateral ventricle (lv) is outlined in red to illustrate the area dissected to obtain single cell suspensions as described under “experimental methods”. B: Phase contrast image of cells 1 h after plating. C–J: SVZ-derived cells grown for 2 days under adherent conditions on poly-D-lysine coated glass coverslips. Phase contrast image (C) and corresponding immunofluorescence staining for β-III-tubulin (D, red) indicative for the neuroblast stage. Close to 100% of the adherent cells express polySia (E, red). After 2 days of endoN treatment (60 ng/mL), the same staining revealed complete removal of polySia (F). Close-up views showing small cell clusters with polySia-immuno-reactivity (red) enriched at cell-cell contacts sites (G) and co-localized with NCAM (H; NCAM green; merged color, yellow), as well as polySia on an outgrowing neurite (I). Double-immunofluorescence (J), showing co-expression of β-III-tubulin (red) and the GABAergic marker GAD-65/67 (green). In D, E-G, I, and J, DAPI stain was used to visualize nuclei (blue). Scale bars: 50 µm in B and C (for C–F), 20 µm in J, 10 µm in H (upper left), and 5 µm in G (for G, I) and H (lower right). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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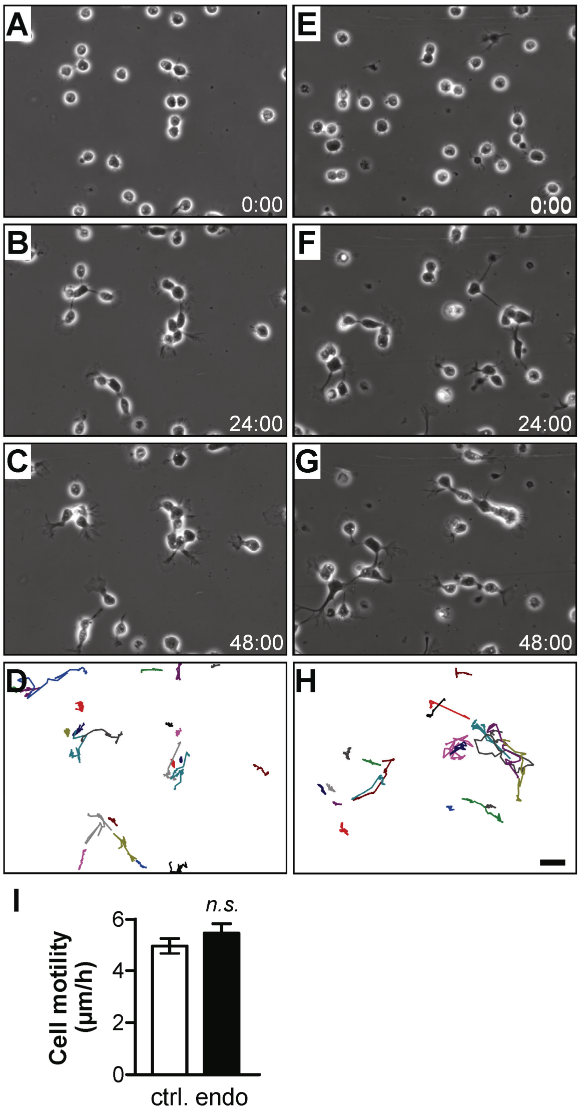

Figure 2 No effect of polySia removal on cell motility. A–H: Time-lapse recordings of two representative frames from control (left column) and endoN-treated cultures (right column). Images at 0, 24, and 48 h recording time and tracks of cell movements over 48 h are shown. Scale bar: 20 µm. Movies are available online as supplementary material. I: Evaluation of cell motility in control versus endoN treated cultures. Means ± s.e.m. from n = 100 cells, each. n.s., difference statistically not significant (t test, $p > 0.1$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

In the OB, three nonoverlapping interneuron populations can be distinguished by their differential expression of dopaminergic markers or the calcium-binding proteins calbindin and calretinin (Kosaka et al., 1995; Brinon et al., 1999; Kohwi et al., 2007; Parrish-Aungst et al., 2007). Despite evaluation of eight independent cultures and >3000 cells, immunoreactivity for the dopaminergic marker tyrosine hydroxylase was never detected in the SVZ-derived neuroblasts. To address commitment to the dopaminergic lineage, protein expression of the paired homeobox transcription factor Pax6 was analyzed, which has been shown to be specifically involved in the generation of dopaminergic OB interneurons (Dellovade et al., 1998; Hack et al., 2005; Kohwi et al.,

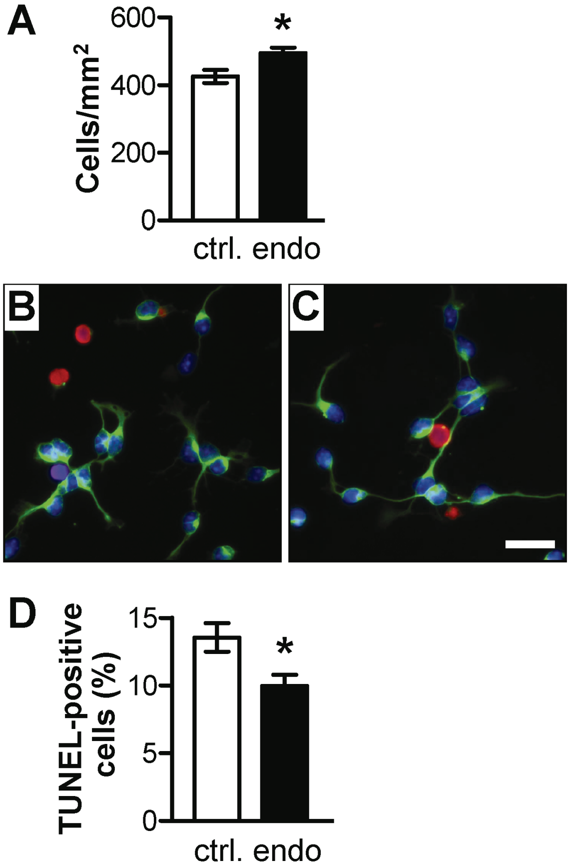

Figure 3 Effect of polySia removal on cell survival. A: Cell counts per area ±s.e.m. after 2 days in vitro (d.i.v.) under control conditions (ctrl.) or in the presence of 60 ng/mL endoN (endo). Means ± s.e.m. for five independent experiments with a minimum of 6 frames or 0.84 mm^2 evaluated per experiment and treatment; $p < 0.05$, t test. B, C: TUNEL-labeling (red) combined with nuclear DAPI stain (blue) and immunofluorescence with antibodies against β-III-tubulin (green) of cultures under control conditions (B) and after 2-days incubation with 60 ng/mL endoN (C). Scale bar: 20 µm. D: Percentage of TUNEL-positive cells in control (ctrl.) and endoN-treated cultures (endo). Means ± s.e.m. from 10 independently treated cultures with a minimum of five frames evaluated per culture. *, significant difference (t test, $p < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley .com.]

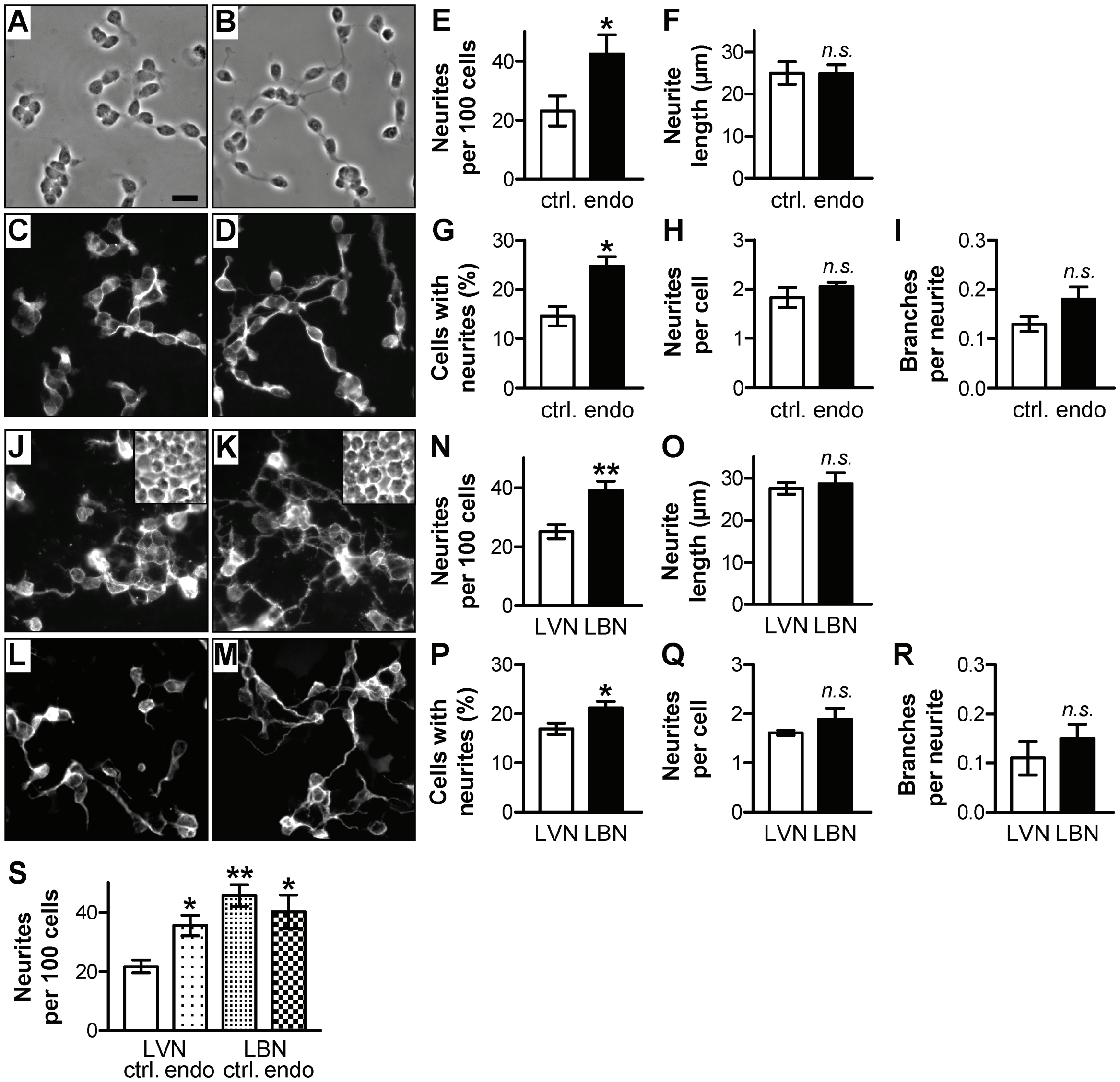

Figure 4 Effect of polySia removal and substrate NCAM on neurite formation. A–I: Phase contrast images (A, B), corresponding β-III-tubulin staining (C, D), and evaluation of neuritogenesis of neuroblasts seeded in parallel and cultured for 2 days under control conditions (A, C; ctrl. in E–I) or in the presence of 60 ng/mL endoN (B, D; endo in E–I). Scale bar, 20 µm. Mean numbers of neurites per 100 neuroblasts (E) and mean lengths of neurites (F) are shown ± s.e.m. (n = 8 cultures each). In four out of the eight experiments performed, the percentage of cells with one or more neurites (G), the number of neurites per cell (H) and the number of branches per neurite (I) were determined. (J–R) Neuroblasts in co-cultures with NCAM-negative (LVN, J, L) and NCAM-positive fibroblasts (LBN, K, M) were identified by polySia-immunofluorescence (J, K) or β-III-tubulin staining (L, M). Phase contrast images illustrate the confluent fibroblast monolayer (inserts). Neuritogenesis was evaluated as in (E–I) from n = 4 (N, O, P) or n = 3 cultures (Q, R). *, **, significant difference (t test), $p < 0.05$ or 0.01, respectively; n.s., not significant ($p > 0.1$). (S) Number of neurites per 100 neuroblasts cultured in the presence or absence of 60 ng/mL endoN on NCAM-negative (LVN) or NCAM-positive fibroblasts (LBN). Means ± s.e.m. of n = 3 cultures, each. One way ANOVA, $p < 0.0001$; *, ** significant difference versus LVN control ($p < 0.05$, $p < 0.01$, Newmann-Keuls post hoc analysis).

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2005). After 1 h of *in vitro* culture, 9.5% of the *β*-III-tubulin-positive neuroblasts were also immunopositive for Pax6. This number decreased to 5.8% after 2 days and Pax6 was no longer detectable after 6 days *in vitro*. Treatment with endoN for 2 days did not alter the relative amount of Pax6-positive cells [Fig. 5(A,B)] indicating that removal of polySia had no effect on dopaminergic commitment.

In contrast to Pax6, the percentage of calbindinpositive cells increased from 0.9% after 1 h to 8% after 2 days *in vitro*. Likewise, the number of calretinin-positive cells developed from 1.3 to 9% during the first 2 days in culture. Strikingly, removal of poly-Sia by endoN treatment had no effect on the percentage of calbindin-positive neurons [Fig. 5(C,D)], but caused a significant increase of cells expressing calretinin [Fig. 6(A–C)]. A similar increase of calretinin expression could be induced by incubation with soluble, nonpolysialylated NCAM presented in form of an NCAM-Fc chimera [Fig. 6(C), right graph] or by co-culture with NCAM-positive, polySia-negative fibroblasts [Fig. 6(F–H)]. To test, whether the effect of endoN treatment relates to differentiation induced by NCAM exposure, we used the dendrimeric C3d peptide, a synthetic ligand, which specifically binds to the first Ig-like domain of NCAM but has no NCAM-derived sequence (Ronn et al., 1999). As demonstrated previously this peptide is a potent inhibitor of NCAM interactions initiated by polySia removal (Seidenfaden et al., 2003). Figure 6(D) shows that the C3d peptide had no effect on calretinin expression of untreated neuroblasts but, in contrast to the control peptide C3d2ala (Ronn et al., 1999), abolished the response following endoN treatment. Similarly, C3d but not C3d2ala reduced the number of calretinin-positive cells if added to neuroblasts cultured on NCAM-positive monolayers [LBN, Fig. 6(H), right graph] Thus, effects of polySia removal and NCAM exposure could be equally blocked by the NCAM-binding peptide indicating that they are mediated by NCAM interactions.

To address the question, if the effect of polySia removal requires cell–cell contact, calretinin-positive cells without contacts were analyzed separately. Under the standard conditions of this study (100,000 cells/mm^2) most of the SVZa-derived neuroblasts aggregated into clusters. By reducing the

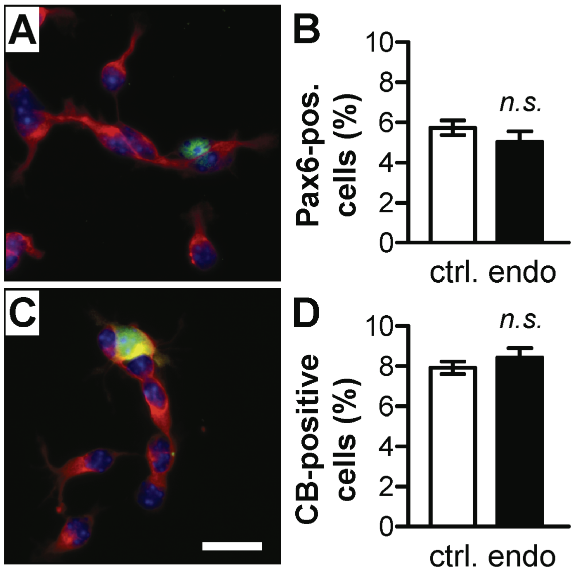

Figure 5 Pax6 and calbindin expression is not affected by polySia removal. A, C: Double-labeling of neuroblasts with antibodies against *β*-III-tubulin (red) and Pax 6 (A, green) or calbindin (C, green). Nuclei are counterstained with DAPI. Scale bar: 20 µm. (B, D) Evaluation of Pax6 (B) or calbindin expression (CB, D) of neuroblasts cultured for 2 days under control conditions (ctrl.) or in the presence of 60 ng/mL endoN (endo). Means ± s.e.m. from n = 6 cultures, each. n.s. difference not significant (t test, $p > 0.1$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

plating density to 50,000 cells/mm^2, the amount of isolated cells was enhanced and ~25% of all *β*-III-tubulin-positive neuroblasts were devoid of cell–cell contacts. Treatment with endoN had no significant effect on the relative amount of isolated neuroblasts (means ± s.e.m.: 25.1 ± 5.8% for controls and 27.4 ± 2.4% after endoN treatment; n = 4, each). Comparable to the situation at the higher cell density [Fig. 6(A)], calretinin was detected in about 9% of all neuroblasts with contact to at least one other cell and this number increased significantly after removing polySia with endoN [Fig. 6(E), “contact”]. In contrast, the frequency of calretinin-positive cells among isolated neuroblasts in the same cultures was not altered by endoN treatment [Fig. 6(E), “isolated”].

To further corroborate the differentiation-promoting effect of polySia-free NCAM, calretinin expression was comparatively analyzed in neuroblasts derived from mice lacking polySia due to NCAM deficiency (NCAM knock-out mice, $N^{-/-}$; Cremer et al., 1994) and from mice that are devoid of

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polySia but maintain normal expression levels of NCAM due to genetic ablation of the key enzymes for polysialylation (*St8sia-II*, *St8sia-IV* double knock-out mice, $II^{-/-}IV^{-/-}$; Weinhold et al., 2005). Compared with wildtype controls, the neuroblasts isolated from $II^{-/-}IV^{-/-}$ mice (carrying nonpolysialylated NCAM), but not the neuroblasts isolated from NCAM knockout mice displayed elevated calretinin expression (Fig. 7, left graph). Most important, the enhanced calretinin expression in $II^{-/-}IV^{-/-}$ cultures could be completely reversed by the addition of C3d peptide (Fig. 7, right graph). Together, these data provide strong evidence that the loss of polySia initiates NCAM trans-interactions, which promote the differentiation of neuroblasts towards a calretinin-positive phenotype.

PolySia-Free NCAM Enforces Differentiation of NCAM-Negative Neuroblasts

Since homophilic NCAM binding is abrogated by polySia (Johnson et al., 2005), it seems unlikely that homophilic trans-interactions account for neuritogenesis and biochemical

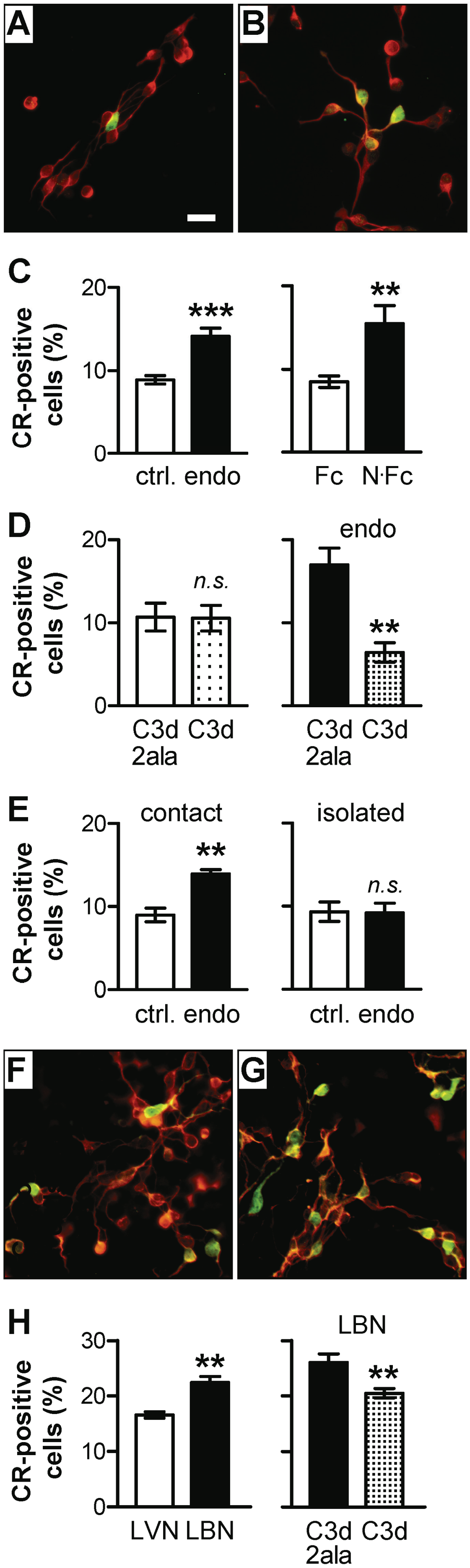

Figure 6 Effect of polySia removal, trans-interacting NCAM, and NCAM-specific peptide on calretinin expression. A–D: Double-labeling of neuroblasts with antibodies against β-III-tubulin (red) and calretinin (green) and evaluation of calretinin (CR)-positive cells in cultures under control conditions (A, ctrl. in C), after 2 days incubation with 60 ng/mL endoN (B, endo in C), Fc fragment or chimeric NCAM-Fc (1 µg/mL each; C), 1 µ*M* of control peptide C3d2ala or NCAM-binding peptide C3d in otherwise untreated cultures (D, left graph) or applied together with 60 ng/mL endoN (D, right graph). E: Separate evaluation of CR-expression among β-III-tubulin-positive cells with contact to at least one other cell (left graph) or among isolated neuroblasts (right graph). Incubation with endoN was performed as described for (C), but cells were plated at a lower density (see text for details). (F–H) Double-labeling of neuroblasts with β -III-tubulin (red) and calretinin (green) in co-cultures with NCAM-negative (LVN, F) and NCAM-positive fibroblasts (LBN, G) and evaluation of calretinin (CR)-positive cells (H). Means ± s.e.m. from n = 18, 10, or 4 cultures, each in C (left graph), C (right graph), or D, E, and H, respectively. n.s. difference not significant (t test, $p > 0.1$). *, **, ***, significant difference (t test, $p < 0.05, 0.01$ or 0.001, respectively). Scale bar, 20 µm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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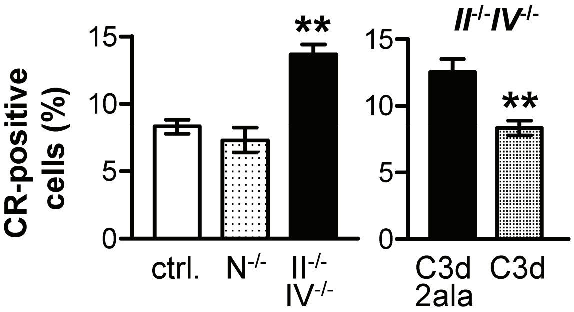

Figure 7 Calretinin expression in neuroblast cultures from NCAM- and polysialyltransferase-deficient mice. Percent calretinin-positive cells in neuroblast cultures from control animals (ctrl.), from mice lacking NCAM and polySia due to NCAM-deficiency ($N^{-/-}$), or from mice with normal NCAM expression but deficient in polySia due to genetic ablation of the polysialyltransferases *ST8SiaII* and *ST8SiaIV* ($II^{-/-}IV^{-/-}$; left graph). Comparison of $II^{-/-}IV^{-/-}$ neuroblast cultures incubated with C3d2ala or C3d-peptide (1 μ*M* each, right graph). The control group represents pooled data from cultures obtained from wildtype littermates of NCAM-deficient animals and from double-heterozygous $II^{+/-}IV^{+/-}$ neuroblasts with normal expression levels of NCAM and polySia. C3d had no effect on neuroblast cultures from double-heterozygous $II^{+/-}IV^{+/-}$ or $N^{-/-}$ animals (not shown). Means ± s.e.m. from n = 6, 6, or 3 cultures for ctrl., $N^{-/-}$ or $II^{-/-}IV^{-/-}$ and n = 7 cultures for peptide treatments. **, significant difference against all other groups, $p < 0.01$, Newmann-Keuls post hoc analysis of one way ANOVA with $p < 0.001$ (left graph) or t test, $p < 0.01$ (right graph).

differentiation of polySia-positive neuroblasts in response to NCAM exposure. Therefore, we asked, whether differentiation can be triggered by heterophilic NCAM interactions. To address this point, neuroblasts derived from NCAM-negative mice ($N^{-/-}$) were either exposed to an NCAM-positive cellular substrate or to soluble NCAM-Fc. As evident from the data presented in Figure 8, both treatments induced the same neuritogenic response and the same increase in the amount of calretinin-positive cells as in polySia-NCAM positive wildtype cultures (see Figs. 4 and 6). This experiment demonstrates that differentiation of NCAM-negative SVZ-derived neuroblasts can be triggered by heterophilic NCAM binding and strongly suggests that the response of polySia-NCAM positive wildtype neuroblasts to polySia free-NCAM is also induced by heterophilic NCAM trans- interactions.

DISCUSSION

In the mouse OB, calretinin-, calbindin-, and tyrosine hydroxylase-positive interneurons coexist as nonoverlapping populations, which are continuously produced from SVZ-derived neuroblasts (Kosaka et al., 1995; Brinon et al., 1999; Kohwi et al., 2007; Parrish- Aungst et al., 2007). Here, we demonstrate that polysialylation directs differentiation of SVZ-derived neuroblasts by controlling NCAM interactions. Downregulation of polySia promoted the appearance of calretinin, but had no effect on the relative amount of calbindin-positive cells and the subset of Pax6-expressing neuroblasts destined to become dopaminergic OB interneurons (Hack et al., 2005; Kohwi et al., 2005). By using cultures of isolated neuroblasts we could dissect this function of polySia from its firmly established role in neuroblast migration.

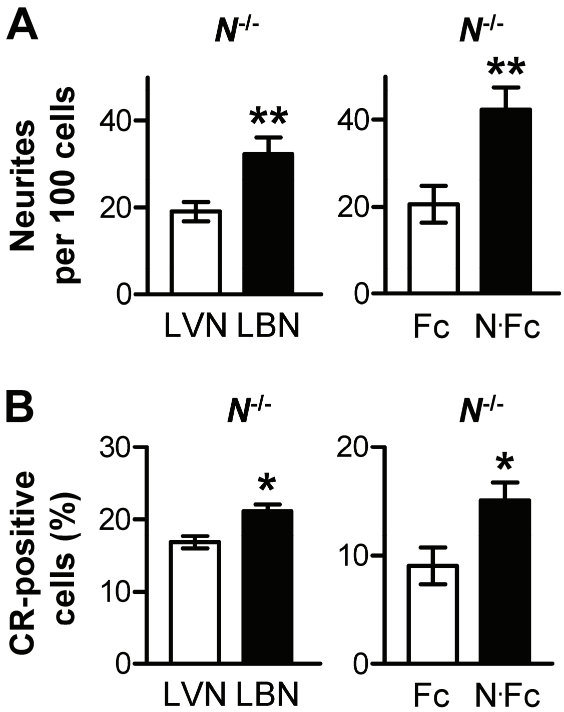

Figure 8 Effects of trans-interacting NCAM on NCAM-negative neuroblasts. NCAM-negative neuroblasts ($N^{-/-}$) were cultured on NCAM-negative (LVN) or NCAM-positive fibroblasts (LBN, left graphs), or incubated with soluble Fc fragment and chimeric NCAM-Fc (N.Fc, right raphs). A: Evaluation of neurite formation. B: Percent calretinin expressing cells. Means ± s.e.m. from n = 6 cultures, each, in A and B (right graph) and n = 4 cultures, each, in B, left graph. *, ** significant difference (t test, $p < 0.05$ or 0.01, respectively).

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Enhanced differentiation and neurite induction after endoN treatment were reproduced by exposure to NCAM. The uniform neuritogenic response, together with the absence of additive effects of polySia-removal and exposure to NCAM, points towards a shared mechanism. Compatible with the accumulation of polySia-NCAM at contact sites between the neuroblasts, the increase of calretinin-positive cells after enzymatic removal of polySia was cell contactdependent and could be abrogated by incubation with the C3d peptide. As shown by others, the synthetic NCAM ligand C3d evokes a dose- and incubation time-dependent increase of neurite length in cellular models lacking NCAM interactions, but inhibits neurite growth caused by exposure to NCAM in other experimental settings (Ronn et al., 1999; Ronn et al., 2000; Kiryushko et al., 2003). Under the conditions of the current study, incubation with 1 μ*M* C3d for 48h had no effect on polySia-positive or NCAM-negative neuroblast cultures. In contrast, the peptide prevented differentiation due to the loss of polySia as efficient as the response of polySia-positive neuroblasts to an NCAM-positive substrate. These findings are consistent with previous studies demonstrating that C3d abolishes the response of neuroblastoma cells to endoN treatment (Seidenfaden et al., 2003; Seidenfaden et al., 2006b) and strongly suggest that polySia removal initiates NCAM interactions between neuroblasts, which than can be blocked by the C3d peptide.

Similar to the present results, increased neuritogenesis was observed after endoN treatment of SVZ explants grown in collagen matrix (Petridis et al., 2004). As reported previously, chain migration is maintained in this *in vitro* system and polySia removal results in migration defects similar to those observed in the *in vivo* environment (Hu et al., 1996). Preserving the diversity of cell contacts, the explants culture system is close to the situation *in vivo*. At the same time, the interpretation of the data by Petridis et al. (2004) is hampered by this diversity as well as by the inability to separate the neuritogenic response from the concomitant disruption of neuroblast migration, which in all likelihood perturbs the dynamics of many cell surface interactions. In contrast, the present study demonstrates endoN-induced neuritogenesis and biochemical maturation in a controlled setting using neuroblast cultures, in which chain migration was a priori absent and cell motility not affected by endoN treatment.

As in the RMS *in vivo*, polySia was uniformly expressed on all neuroblasts of the SVZ-derived cultures. Its removal enhanced the generation of only the calretinin-, but not the calbindin-or tyrosine hydroxylase-positive cell type. This divergent responsiveness indicates heterogeneity in the differentiation potential, which is in line with increasing evidence that SVZ progenitors are intrinsically directed towards specific lineages characterized by distinct genetic determinants (Hack et al., 2005; Kohwi et al., 2005; Waclaw et al., 2006; for review, see Ninkovic and Götz, 2007). In contrast to the induction of calretinin expression, endoN treatment did not affect the decline of Pax6 and the absence of tyrosine hydroxylase immunoreactivity indicating the inability to maintain the precursor population with dopaminergic potential in neuroblast cultures from early postnatal mice. This outcome corresponds to the lack of tyrosine hydroxylase expression after applying endoN to SVZ explant cultures from 7-day-old mice (Petridis et al., 2004) but contrasts with the induction of this marker observed in the same study after removal of polySia from the SVZ of adult animals *in vivo*. On the one hand, the different response *in vivo* may be caused by altered interactions of neuroblasts with their stationary environment that are not reproduced *in vitro*. On the other hand, the generation of tyrosine hydroxylase-positive OB interneurons is considerably lower in neonates than in the adult, demonstrating age-dependent differences of either extrinsic cues or autonomous commitment of SVZ progenitors (De Marchis et al., 2007).

Taken together, the available data indicate that polySia expression postpones neuronal differentiation of SVZ-derived precursors, while its downregulation coordinates maturation of OB interneurons (Petridis et al., 2004 and current study). Beyond this, the present study demonstrates responsiveness of polySia-NCAM positive neuroblasts to nonpolysialylated NCAM suggesting that polySia-free NCAM on target structures could serve as an instructive signal for neuroblasts arriving in the OB. This possibility is intriguing, since NCAM, but not polySia, is heavily expressed by the axons of the olfactory neurons that form the glomeruli, while the

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newly arriving prospective periglomerular cells maintain polySia expression (Miragall and Dermietzel, 1992; Bonfanti and Theodosis, 1994). Attempts to comparatively analyze SVZ-derived precursor differentiation in NCAM- or polysialyltransferase-deficient mice revealed that both genetic mouse models exhibit small OBs in conjunction with an accumulation of precursors in the proximal parts of the RMS and massive astrogliosis (Chazal et al., 2000; Weinhold et al., 2005; Hildebrandt and Röckle, unpublished observation). Thus, neuroblast migration into the OB is impaired in both mouse models, preventing an appropriate evaluation of interneuron differentiation in the OB of these mice.

In vivo, the relevance of polySia as a specific control element of NCAM functions has been unequivocally documented by showing that malformations of major brain fiber tracts in polysialyltransferase-deficient mice were selectively rescued by additional deletion of NCAM (Weinhold et al., 2005; Hildebrandt et al., 2007). In tumor cell lines, enzymatic removal of polySia initiates NCAM signals leading to differentiation and improved survival (Seidenfaden et al., 2003, 2006b). In accordance with these mouse and tumor cell models, the response of SVZ-derived neuroblasts to endoN treatment is best explained by a gain of NCAM functions. In contrast, the congruent effects of polySia removal and NCAM exposure can not be explained by altered responsiveness to neurotrophins, as described after endoN treatment of cortical, septal or SVZ-derived neurons (Vutskits et al., 2001; Burgess and Aubert, 2006; Gascon et al., 2007). In particular, the survival of immature SVZ-derived neurons in response to neurotrophins was reduced after enzymatic removal of polySia as well as by using cells lacking polySia due to NCAM-deficiency, indicating that this effect is independent from specific NCAM functions (Gascon et al., 2007). Similarly, PDGF-induced glial differentiation is enhanced in neurospheres derived from either polysialyltransferase- or NCAM-deficient animals (Angata et al., 2007) and therefore not caused by a gain of polySiafree NCAM. Accelerated glial differentiation after endoN treatment has also been observed in oligospheres in vitro and after experimentally induced demyelination *in vivo* (Decker et al., 2000; Decker et al., 2002). Although the mode of polySia activity in relation to NCAM functions was not explored in these studies, increased adhesion to compounds of the extracellular matrix was discussed as a possible mechanism. Indeed, it has been shown recently that substrate interactions can direct fate and specification of neural precursors derived from embryonic stem cells (Goetz et al., 2006). For SVZ-derived neuroblasts, however, our data show that polySia removal and trans-interacting NCAM (soluble or cell-bound) cause equal responses, which strongly argues against a direct modulation of cell-substrate interactions.

The identical cellular response of NCAM-negative and polySia-NCAM positive neuroblasts to NCAM cues presented in trans demonstrates potent heterophilic NCAM interactions. In agreement with these observations, heterophilic NCAM binding has been shown to promote differentiation of neuroblastoma cells (Seidenfaden et al., 2003) and hippocampal progenitors from the embryonic brain (Amoureux et al., 2000). The influence of polySia was not addressed in the latter study, but it is known that polySia is abundantly expressed on neuroblasts in the hippocampal neurogenic region (Seki, 2002; Seki et al., 2007). Together with differentiation, the reduction of proliferation was a major effect of heterophilic NCAM binding in hippocampal progenitors and neuroblastoma cells (Amoureux et al., 2000; Seidenfaden et al., 2003). In both systems, therefore, the relationship between the inhibition of proliferation and the increase in differentiation remained open. In contrast, proliferation was completely absent in the SVZ-derived neuroblasts cultures used in the current study, demonstrating that NCAM is an instructive signal able to induce neural progenitor differentiation independent of its effect on proliferation.

As for hippocampal progenitors and neuroblastoma cells (Amoureux et al., 2000; Seidenfaden et al., 2003), the putative heterophilic NCAM receptor involved in the differentiation of SVZ-derived neuroblasts remains unknown. Among the numerous NCAM interaction partners described so far, some, like the fibroblast growth factor receptor or the cell adhesion molecule L1, bind to NCAM in cis, while others, like heparan and chondroitin sulfates, are either components of the extracellular matrix or have a merely modulatory impact on NCAM interactions (for an overview, see Hinsby et al., 2004). As recently described, the glial cell line-derived neurotrophic factor (GDNF) and its GPI-anchored receptor GFRα1 interact with NCAM and in the RMS, GDNF functions as a chemoattractant for SVZ-derived precursors (Paratcha et al., 2003; Paratcha et al., 2006). These interactions, however, affect functions of NCAM as receptor and not as a ligand. Moreover, GDNF binding to NCAM occurs independent of the presence of polySia (Paratcha et al., 2003).

In conclusion, the current data demonstrate that loss of polySia initiates NCAM trans-interactions, which promote survival as well as neurite induction and biochemical maturation of SVZ-derived precursors *in vitro*. The possibility to control timing of neuroblast differentiation and eventually increase neuron yields with the help of polySia- and NCAM-specific tools may prove valuable for therapeutic strategies aiming at neuron replacement. Under pathological conditions such as stroke or Huntington's disease, neuroblasts from the subependymal layer appear involved in brain repair (Arvidsson et al., 2002; Curtis et al., 2003), while their production is impaired in Parkinsonism (Hoglinger et al., 2004). In this context, it will be challenging to test if polySia removal, the inhibition of polySia synthesis, and/or the use of NCAM mimetics (Berezin and Bock, 2004) have the potential to manipulate neurogenesis from SVZderived stem cells and support endogenous brain repair processes.

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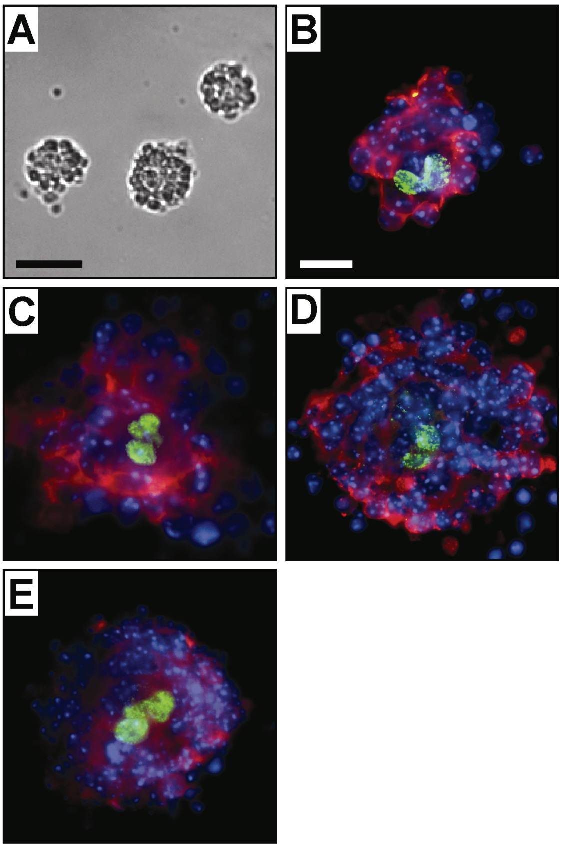

Supplementary figure 1 Neurospheres formed by SVZ-derived cells seeded in uncoated 12 well plates, i.e. under non adherent conditions. For immunofluorescence staining, neurospheres were transferred after one day in vitro (d.i.v.) to poly-D-lysine coated glass surface, where they attached. (A) Phase contrast image of neurospheres after 1 d.i.v. (B-E) Representative examples of neurospheres stained for beta-III-tubulin (B), polySia (C), A2B5 (D) and GFAP (E, all shown in red) and the proliferation marker BrdU (B-E, green) added 2h before fixation. DAPI stain was used to visualize nuclei (blue). Scale bars: 50 µm in A, 10 µm in B (for B-E).

Supplementary video 1 Representative time-lapse movies of neuroblasts recorded simultaneously under control conditions. 10 images /h were acquired over a 48h period.

Supplementary video 2 Representative time-lapse movies of neuroblasts recorded simultaneously in the presence of 200 ng/ml endoN. 10 images /h were acquired over a 48h period.

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Chapter 3- Changes of GABAergic interneuron populations in the forebrain of mice deficient for polysialic acid or NCAM

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Running title: Polysialic acid in interneuron development

Preface – About this manuscript

Aberrant NCAM expression or altered polysialylation have been linked to schizophrenia and mice with altered NCAM levels or unbalanced polysialylation of NCAM show several parallels to pathophysiological findings in schizophrenic patients. Numerous studies indicate that dysfunction in schizophrenia includes alterations of specific GABAergic interneurons in the prefrontal cortex (PFC) and hippocampus.

On this background, the second study of my thesis aimed at analyzing pathological changes of interneuron populations in mice with deficiencies of either polysialylation of NCAM or of NCAM itself. Densities of major interneuron subtypes were comparatively analyzed in brain regions relevant to the pathophysiology of schizophrenia in polysialyltransferase (*St8siaII*, *St8siaIV)* and *Ncam1* single-, double-, and triple-knockout mice.

My contributions to this manuscript comprised the preparation of the brains, immunofluorescent staining, microscopy, cell counting and statistical evaluation. Prof. H. Hildebrandt and I designed the experiments and wrote the paper.

Abstract

The neural cell adhesion molecule NCAM and its modification with polysialic acid (polySia) are major determinants of cellular interactions during brain development and plasticity. Variations in the genes for NCAM and one of the two polysialyltransferases, ST8SiaII, have been linked to schizophrenia. In mice, polySia deficiency impairs migration of subventricular zone-derived interneuron precursors towards the olfactory bulb and of undefined progenitors during neocortex development. Here, we analyzed how loss of polySia affects selected interneuron populations in brain regions relevant to the pathophysiology of schizophrenia. A panel of polySia-deficient mouse lines with differently combined *Ncam1* and polysialyltransferase deletions was used to dissect, whether effects were caused by loss of NCAM, loss of polySia, or reduced polysialylation of either NCAM or additional polySia carriers. Densities of cells immuno-positive for major interneuron markers (parvalbumin, calbindin, calretinin, tyrosine hydroxylase) were assessed in prefrontal cortex, hippocampus, and the glomerular layer of the olfactory bulb. Pronounced reductions of parvalbumin-positive, calbindin-negative cells in the prefrontal cortex and calbindin-positive cells in the olfactory bulb were detected in all NCAM- or polySia-deficient lines, while parvalbumin-positive cell densities were increased in the hippocampus. Together, these data demonstrate that attenuation of NCAM-bound polySia causes pathological changes of specific GABAergic interneuron subtypes.

Keywords:
brain pathology, calcium-binding proteins, mouse model, prefrontal cortex, schizophrenia

The neural cell adhesion molecule NCAM controls diverse aspects of brain development (Ronn et al. 1998; Hildebrandt et al. 2007). A unique feature of NCAM is its posttranslational modification by the addition of a linear homopolymer of α2,8-linked sialic acid (polysialic acid, polySia). Dynamic changes of NCAM isoform patterns and polySia levels during development have been shown for rodent brain (Chuong and Edelman 1984; Gennarini et al. 1986; Oltmann-Norden et al. 2008) as well as for human prefrontal cortex (PFC; Cox et al. 2009). PolySia synthesis is implemented by the polysialyltransferases ST8SiaII and ST8SiaIV. Together with polysialyltransferase mRNA, levels of polySia-NCAM are high during embryonal and early postnatal development before declining rapidly and becoming restricted to mainly sites of ongoing neurogenesis or plasticity (for review, see Bonfanti 2006; Mühlenhoff et al. 2009). Consistent with these expression patterns, polySia-NCAM is a prominent regulator of migration, axon outgrowth and synaptic plasticity (Bonfanti 2006; Gascon et al. 2007; Hildebrandt et al. 2007; Maness and Schachner 2007; Rutishauser 2008).

Nevertheless, mice lacking all forms of NCAM ($N^{-/-}$) and, as a consequence, are almost completely devoid of polySia, show an overall mild phenotype (Cremer et al. 1994). Mild but distinct phenotypes were also observed in mice with partial reductions of polysialylation due to ablation of ST8SiaII ($II^{-/-}$) or ST8SiaIV ($IV^{-/-}$; Eckhardt et al. 2000; Angata et al. 2004). In contrast, simultaneous ablation of the two polysialyltransferases ST8SiaII and ST8SiaIV ($II^{-/-}IV^{-/-}$) yielded mice that are entirely negative for polySia but positive for NCAM. These animals combine two categories of defects (Weinhold et al. 2005; Hildebrandt et al. 2009). First, defects which are unique to the $II^{-/-}IV^{-/-}$ mice and not observed in NCAM knockout animals, like postnatal growth retardation and precocious death, a high incidence of hydrocephalus as well as malformation of major brain axon tracts. These defects establish due to a gain of polySia-free NCAM as they are fully reversed by the additional deletion of NCAM in $II^{-/-}IV^{-/-}N^{-/-}$ triple knockout mice (Weinhold et al. 2005). Moreover, the axon tracts deficiencies correlate specifically with the amount of erroneously non-polysialylated NCAM during development (Hildebrandt et al. 2009). The second category comprises defects in brain morphology that are shared by the polysialyltransferase- and the NCAM-depleted mice. This includes a size reduction of the OB, which is caused by a migration deficit of subventricular zone-derived interneuron precursors (for review, see Hildebrandt et al. 2007). In addition, Angata et al. (2007) provided evidence of impaired migration of precursors during cortical development of *II*$^{-/-}$*IV*$^{-/-}$ mice.

Several lines of evidence link aberrant NCAM expression or altered polysialylation to schizophrenia. Elevated levels of a soluble NCAM fragment have been detected in the PFC, in the hippocampus, and in the cerebrospinal fluid of schizophrenic patients, and fragment concentrations were found to correlate with severity and duration of the disease (Poltorak et al. 1995; van Kammen et al. 1998; Vawter 2000; Vawter et al. 2001). By contrast, reduced polySia expression was observed in the hilus region of the hippocampus in schizophrenics (Barbeau et al. 1995). *NCAM1* and both polysialyltransferase genes map to chromosomal regions that harbour susceptibility loci for schizophrenia (11q23.1, 15q26, and 5q21 for *NCAM1*, *ST8SIA2* and *ST8SIA4*, respectively; Lewis et al. 2003; Lindholm et al. 2004; Maziade et al. 2005). Single nucleotide polymorphisms (SNPs) in *NCAM1* as well as in the promoter region of *ST8SIA2* (but not *ST8SIA4*) have been associated with schizophrenia (Arai et al. 2006; Atz et al. 2007; Sullivan et al. 2007; Tao et al. 2007).

Moreover, there are striking parallels between the phenotype of NCAM- or polySia-deficient mice and pathophysiological findings in schizophrenia. Ventricular enlargement, one of the most abundant abnormalities in schizophrenia (Shenton et al. 2001), has been reported for mice with specific deletion of NCAM-180 and variable degrees of ventricular dilatations including cases of severe hydrocephalus were observed in *II*$^{-/-}$*IV*$^{-/-}$ mice (Wood et al. 1998; Weinhold et al. 2005). In addition, a decreased size of the corpus callosum and the internal capsule as has been reported in schizophrenic patients (Innocenti et al. 2003; Hulshoff Pol et al. 2004; Douaud et al. 2007; Mitelman et al. 2007; Begre and Koenig 2008). This correlates with the fiber tract deficits observed in polysialylation compromised mice (Hildebrandt et al. 2009). A further remarkable similarity is the reduced size of the olfactory bulb (OB) both in patients with schizophrenia (Turetsky et al. 2000) and *N*$^{-/-}$ or *II*$^{-/-}$*IV*$^{-/-}$ mice (Cremer et al. 1994; Weinhold et al. 2005). Reminiscent to cognitive impairment in schizophrenia (Heinrichs and Zakzanis 1998), *N*$^{-/-}$ as well as polysialyltransferase-deficient *IV*$^{-/-}$ mice display deficits in learning or memory formation as well as in hippocampal long-term potentiation (Cremer et al. 1994; Cremer et al. 1998; Eckhardt et al. 2000; Bukalo et al. 2004; Senkov et al. 2006) and one study reported reduced prepulse inhibition of acoustic startle in NCAM-180 knockout mice (Wood et al. 1998; but see Plappert et al. 2005).

Numerous studies indicate that dysfunction in schizophrenia includes alterations of GABAergic interneurons and in many of these studies, the immunohistochemical detection of the calcium-binding proteins parvalbumin (PV), calbindin (CB) and calretinin (CR) has proven a powerful tool for the identification and evaluation of GABAergic interneuron subtypes (for review, see Benes and Berretta 2001; Eyles et al. 2002; Lewis et al. 2005; Lewis and Sweet 2009). Here, we address the effect of polySia deficiency on selected interneuron populations of the mouse forebrain by comparatively analyzing *St8siaII*, *St8siaIV* and *Ncam1* single-, double-, and triple-knockout lines. Densities of major interneuron subtypes in the PFC, hippocampus and OB were assessed by immunofluorescence staining of PV, CB, CR, and, in the case of the OB, tyrosine hydroxylase (TH; Kosaka et al. 1995; DeFelipe 1997; Matyas et al. 2004; Kohwi et al. 2007). The results indicate that reduction of NCAM-based polySia differentially affects PV- and CB-positive interneuron populations in the PFC, hippocampus and OB.

Materials and Methods

Mice

C57BL/6J and transgenic mice were bred at the central animal facility at Hannover Medical School. All protocols for animal use were in accordance with the guidelines established by the European Union regarding the use and care of laboratory animals and approved by the local authorities. *St8siaII*, *St8siaIV* and *Ncam1* single knockout strains, which have been backcrossed with C57BL/6J mice for six generations, were intercrossed to obtain double knockout ($St8siaII^{-/-}$ $St8siaIV^{-/-}$, $II^{-/-}IV^{-/-}$) or triple knockout ($St8siaII^{-/-}$ $St8siaIV^{-/-}$ $Ncam1^{-/-}$; $II^{-/-}IV^{-/-}N^{-/-}$) animals (Weinhold et al. 2005). Genotyping was performed by PCR as previously described (Weinhold et al. 2005).

Sectioning

One month old mice were deeply anesthetized with a mixture of 200mg/kg Ketamin (Gräub AG, Bern) and 8mg/kg Xylazin (Rompun, Bayer Health Care, Leverkusen) in 0.9% NaCl. Animals were perfused transcardially with 4%

paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After dissection, the brains were postfixed over night. 50µm coronal sections were obtained with a vibrating microtome (Leica Microsystems, Wetzlar, Germany). For each genotype n=3 mice were used. For $II^{-/-}IV^{-/-}$ mice, which have a high incidence of hydrocephalus (Weinhold et al. 2005), only specimen with moderate ventricular dilatation and no cortical thinning were processed and used for analysis. As $St8siaII^{+/-}$ $St8siaIV^{+/-}$ ($II^{+/-}IV^{+/-}$) animals were indistinguishable from wildtype animals, one $II^{+/-}IV^{+/-}$ mouse was included into the control group.

Immunofluorescence

Sections were permeabilized for 15 min with 0.4% Triton X-100 in phosphate buffered saline (PBS), pH7.4 before blocking for 1h with 10% FCS in PBS with 0.4% Triton X-100. Free floating sections were incubated with primary antibodies for 3 days at 4°C. The following monoclonal (mAb) or polyclonal antibodies (pAb) were applied according to the manufacturers' instructions: Calretinin- and calbindin D-28k-specific rabbit pAb (Swant, Bellinzona, Switzerland), tyrosine hydroxylase-specific rabbit pAb, and parvalbumin-specific mouse mAb (IgG_1, Swant). Rabbit and mouse IgG-specific Cy3- (Chemicon, Temecula, CA) and Alexa488 (Invitrogen/Molecular Probes, Karlsruhe, Germany) conjugated secondary antibodies were used as suggested by the suppliers. As first layer controls, cells were incubated in blocking solution lacking primary antibody. In double stained immunofluorescence samples, cross-reactivity of secondary antibodies was controlled by omitting either of the two primary antibodies. Stained sections were mounted on glass object slides (SuperFrost®Plus, Menzel, Braunschweig, Germany) and coverslipped using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

Microscopy, Area measurements, Cell Counting and Statistics

Microscopy was performed using a Zeiss Axiovert 200 M equipped with an ApoTome device for near confocal imaging, AxioCam MRm digital camera and AxioVison software (Carl Zeiss Microimaging, Göttingen, Germany). Near confocal optical sections of 5.1 µm thickness located approximately 10 µm above the bottom (caudal level) of each 50 µm vibratome section were obtained by ApoTome technology using a 10x Plan-Apochromat objective with 0.45 numerical aperture

(Zeiss). Micrographs covering the area of one entire hemisphere were acquired using the MosaiX module of the AxioVision software. AxioVison software was also used for area measurements and cell counting. For evaluation micrographs were coded and randomized to ensure that the observer was blind to experimental conditions. On each optical slice the regions of interest, glomerular layer (Gl) of the olfactory bulb (OB), prefrontal cortex (PFC), Ammon's horn (cornu ammonis, CA) and dentate gyrus (DG) of the hippocampus, were lined out, areas were measured and the total numbers of cells positive for the particular marker of interest were counted. Thus, counting covered 100% of the sample area within each section and therefore there was no need to make use of a counting frame, which is typically employed in the optical dissector method. Examination of shape and areas of randomly selected labelled cells revealed no difference between the different genotypes. Therefore, and because the aim of this study was not the determination of absolute cell numbers or densities, but a comparison between polySia-positive and polySia-deficient animals, there was no need to correct for the overcount produced by counting rather big objects in relatively thin optical sections (as discussed by e.g. Guillery 2002).

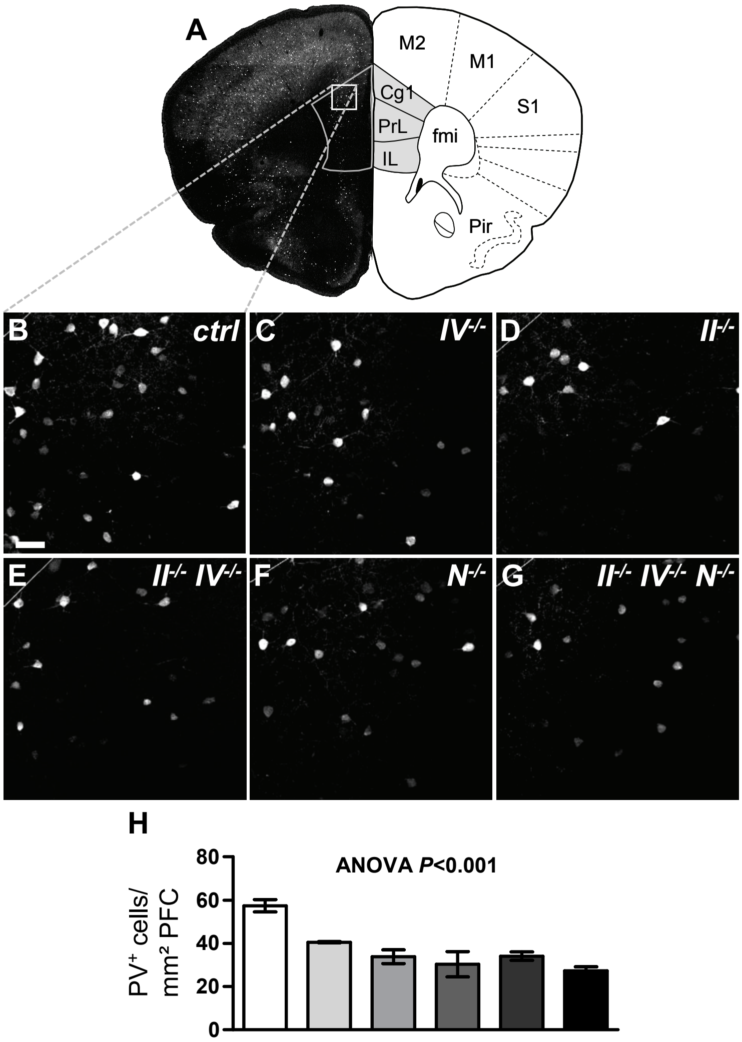
For each marker to be analysed, immuno-positive cells were quantified on MosaiX images obtained from three (for CR, CB and TH) or six (for PV) sections per animal and brain region. Three pairs of consecutive sections equally spaced between bregma level 4.05 mm and 3.7 mm (according to Paxinos and Franklin 2001) for OB, between bregma level 1.9 mm and 1.65 mm for PFC and between bregma level -1.2 mm and -1.85 mm for hippocampus were selected. For prefrontal cortex and hippocampus, these pairs of consecutive sections were labelled for parvalbumin together with either calretinin or calbindin and both hemispheres were evaluated. For the glomerular layer of the olfactory bulb, one OB from the first section out of each pair was stained for calretinin, the other for calbindin. On the second section, one OB was stained for tyrosine hydroxylase.

Results

Densities of PV-, CB-, and CR-immunoreactive cells in the PFC of polysialyltransferase- and NCAM-deficient mice

Due to the high mortality of $II^{-/-}IV^{-/-}$ mice after 4 weeks of age (Weinhold et al. 2005), all analyses were restricted to young, one month old animals. Consistent with previous observations that brains of polysialyltransferase-negative mice are smaller (Weinhold et al. 2005; Schiff et al. 2009) the area of the PFC as well as the area of the entire brain section at the respective cross-sectional level were reduced in $II^{-/-}IV^{-/-}$ mice (12% and 16% reduction, respectively; see suppl. Table 1). To compensate for the differences in overall brain size, cell counts for each PFC were normalized to the respective PFC area. Compared to the control group, the resulting densities of PV-positive cells in the PFC were significantly lower in both polysialyltransferase single knockout lines ($II^{-/-}$ and $IV^{-/-}$) as well as in all other polysialyltransferase- or NCAM-deficient genotypes (ANOVA, P<0.001, Fig. 1; for numbers of evaluated sections and listing of cell counts, see suppl. Table 1). By double immunofluorescence staining, PV-positive but CB-negative (PV^+CB^-), PV and CB double-positive (PV^+CB^+) and PV-negative but CB-positive, interneurons (PV^-CB^+) could be distinguished. Compared to the control group, the densities of PV^+CB^- cells were significantly reduced in all polysialyltransferase- or NCAM-deficient lines (ANOVA P <0.0001; Fig.2C). Although not statistically significant, this reduction was less pronounced in the $IV^{-/-}$ animals.

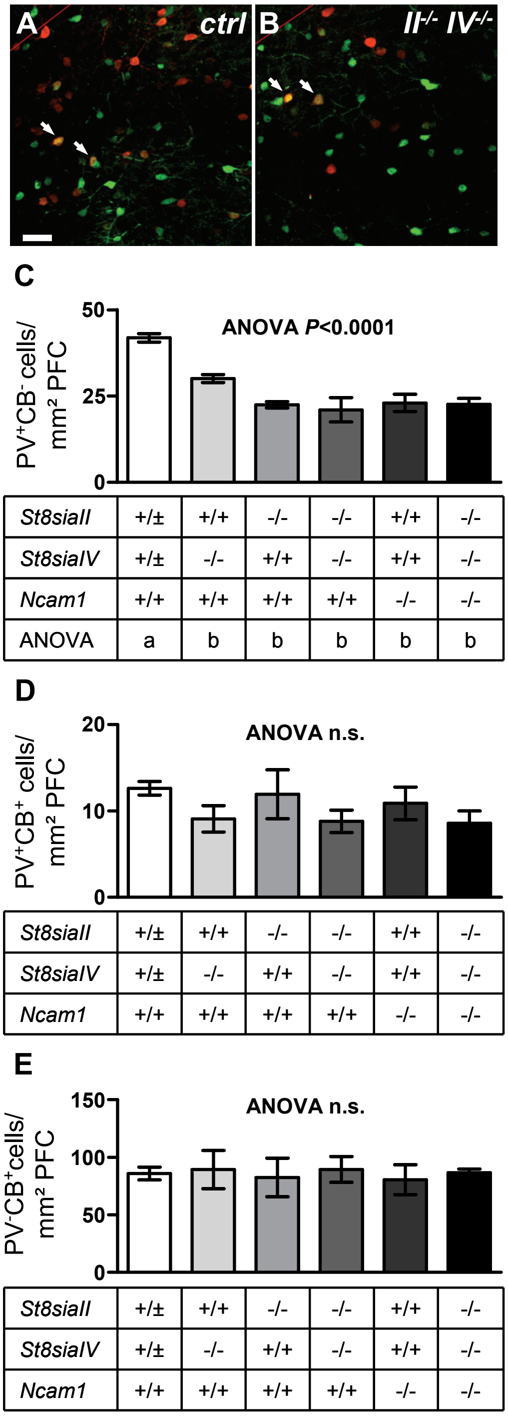
No significant differences were found by comparing the PV^+CB^+ subpopulation between the different genotypes (ANOVA P >0.05; Fig.2D). However, a comparison of the mean values of all ST8SiaIV-deficient genotypes ($IV^{-/-}$, $II^{-/-}IV^{-/-}$, $II^{-/-}IV^{-/-}N^{-/-}$) with those of lines with uncompromised ST8SiaIV levels (control, $II^{-/-}$, $N^{-/-}$) revealed a significant difference [mean values +/- s.e.m. for the ST8SiaIV-positive and -negative group are 11.82 +/-0.51 and 8.82 +/- 0.15, respectively (n=3, each); P<0.005, *t* test]. In contrast, densities of PV^-CB^+ interneurons were unchanged in the different genotypes (Fig.2 E; ANOVA P >0.1). Likewise, no significant differences in the expression of CR were observed (Fig. 3A-C; ANOVA P >0.05).



St8siaII	+/±	+/+	-/-	-/-	+/+	-/-
St8siaIV	+/±	-/-	+/+	-/-	+/+	-/-
Ncam1	+/+	+/+	+/+	+/+	-/-	-/-
ANOVA	a	b	b	b	b	b

Figure 1

Parvalbumin (PV) expression in the prefrontal cortex (PFC). (***A***) ApoTome MosaiX image showing the distribution of PV-postive cells in an overview of the left hemisphere at the level of the PFC and schematic illustration of the corresponding right hemisphere (modified from Paxinos and Franklin 2001). The area of the PFC, consisting of Cg1, PrL and IL, is outlined (left) or highlighted in grey (right). The position of the micrographs depicted in (*B-G*) is marked (white square). Abbreviations: Cg1: cingulate cortex, area 1; fmi: forceps minor of the corpus callosum; IL: infralimbic cortex; M1: primary motor cortex; M2: secondary motor cortex; Pir: piriform cortex; PrL: prelimbic cortex; S1: primary somatosensory cortex. (***B-G***) Representative details illustrating PV-positive cells in the dorsal PFC of different genotypes as indicated. Scale bar: 50µm. (***H***) Densities of PV-positive cells in the PFC. Per group, mean values ±SEM from n=3 animals are plotted. One-way ANOVA indicated significant differences (*P*<0.001) and Newman-Keuls *post hoc* test was applied. Means not marked with the same letter differ significantly (*P*<0.01).



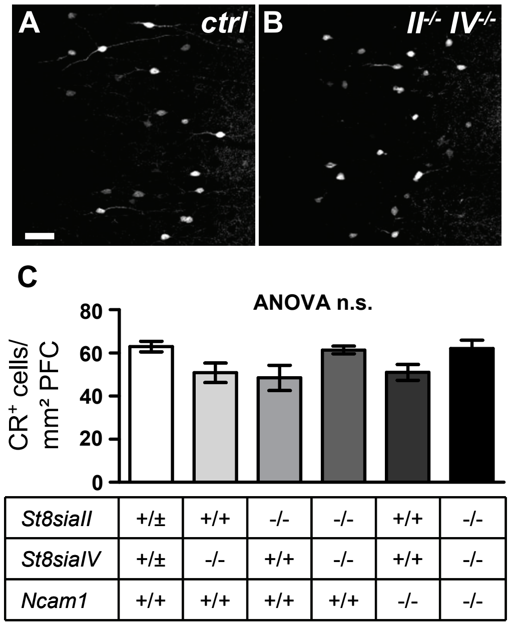
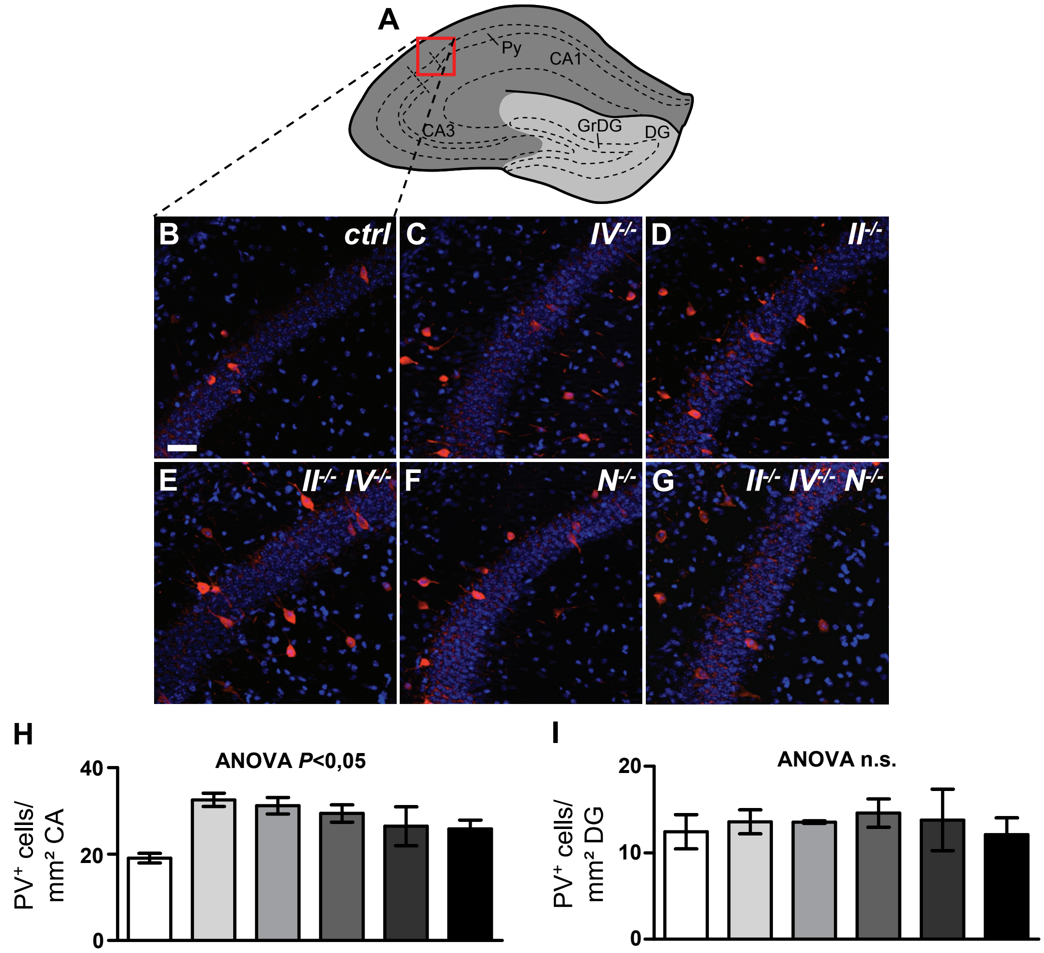


Figure 3:

Calretinin (CR) expression in the PFC. (***A, B***) Representative details illustrating CR-positive cells in the PFC (PrL region) of control (*A*) and *II*$^{-/-}$ *IV*$^{-/-}$ mice (*B*). Scale bar: 50µm. (***C***) Densities of the CR$^+$ cells in the PFC. Per group, mean values ±SEM from n=3 animals are plotted. Differences were not significant (ANOVA, *P*>0.05)

Figure 2

Evaluation of parvalbumin- (PV) and calbindin- (CB) positive cells in the PFC. (***A, B***) Representative details illustrating double immunofluorescence staining for PV (red) and CB (green) in the PFC (Cg1 region) of control (*A*) and *II*$^{-/-}$*IV*$^{-/-}$ mice (*B*). Double-positive cells appear yellow (arrows). Scale bar: 50µm. (***C-E***) Densities of PV$^+$CB$^-$ (*C*), PV$^+$CB$^+$ (*D*) and PV$^-$CB$^+$ (*E*) cells in the PFC. Per group, mean values ±SEM from n=3 animals are plotted. One-way ANOVA indicated no significant differences (n.s., *P*>0.1; *D, E*) or highly significant differences (*P*<0.0001; C) and means not marked with the same letter differ significantly if compared by Newman-Keuls *post hoc* test (*P*<0.01, C).



H

St8siaII	+/±	+/+	-/-	-/-	+/+	-/-
St8siaIV	+/±	-/-	+/+	-/-	+/+	-/-
Ncam1	+/+	+/+	+/+	+/+	-/-	-/-
ANOVA	a	b	b	b	ab	ab

I

St8siaII	+/±	+/+	-/-	-/-	+/+	-/-
St8siaIV	+/±	-/-	+/+	-/-	+/+	-/-
Ncam1	+/+	+/+	+/+	+/+	-/-	-/-

Figure 4:

Parvalbumin (PV) expression in the hipppocampus. (***A***) Schematic drawing of a hippocampus (coronal section, modified from Paxinos and Franklin, 2001). For evaluation the hippocampus was divided into the cornu ammonis (CA), highlighted in dark grey) and the dentate gyrus (DG, highlighted in light grey). The position of the micrographs depicted in (*B-G*) is indicated (red square). Abbreviations: CA1: field CA1 of hippocampus, CA3: field CA3 of hippocampus, DG: dentate gyrus, GrDG: granular layer of the dentate gyrus, Py: pyramidal cell layer of the hippocampus. (***B-G***) Representative details illustrating PV-positive cells (red) with nuclear counterstain (DAPI, blue) in the CA region of different genotypes as indicated. Scale bar: 50µm. (***H, I***) Densities of PV^+ cells in the CA region (*H*) and the dentate gyrus (*I*). Per group, mean values ±SEM from n=3 animals are plotted. One-way ANOVA indicated significant differences ($P<0.05$; *H*) and means not marked with the same letter differ significantly if compared by Newman-Keuls *post hoc* test ($P<0.05$; *H*). n.s., no significant differences ($P>0.1$; *I*).

Densities of PV-, CB-, and CR-immunoreactive cells in the hippocampus

Immunopositive cells of the CA fields and the dentate gyrus were counted separately and respective areas were measured (Fig. 4A). Compared to the control group, mice lacking both (*II*$^{-/-}$*IV*$^{-/-}$) or either of the two polysialyltransferases (*II*$^{-/-}$, *IV*$^{-/-}$) had significantly increased densities of PV^{+} interneurons in the CA fields (ANOVA *P*<0.05, Fig. 4H). Both NCAM-negative groups (*N*$^{-/-}$ and *II*$^{-/-}$*IV*$^{-/-}$*N*$^{-/-}$) displayed a slight increase, which neither differed significantly from the control nor from the other polysialyltransferase-deficient genotypes (Fig. 4B-H; for numbers of evaluated sections and listing of cell counts, see suppl. Table 2). In contrast to the CA fields, the densities of PV^{+} cells in the DG were not affected (ANOVA *P*>0.05, Fig 4I). In the hippocampus interneurons containing both PV and CB are very rare (Jinno and Kosaka 2002). In line with that, hardly any PV^{+}CB^{+} could be detected. CB expression was only evaluated in Ammon's horn, because in the DG calbindin is expressed mainly by granule cells and not interneurons (Baimbridge 1992; Freund and Buzsaki 1996; Matyas et al. 2004). The evaluation of CB^{+} cells in the CA fields revealed a high variability but no statistically significant differences between the genotypes (ANOVA *P*>0.05, Fig. 5A). Similar to the findings in the PFC, the density of CR^{+} cells in the CA fields was not affected in any of the groups analyzed (Fig. 5B). In the DG, many faintly CR^{+} cells were observed in the granule cell layer, especially at the interface with the hilus. Most likely, these cells are newly generated, immature granule cells, which transiently express CR (Brandt et al. 2003). Counting these cells revealed no significant differences between the diverse genotypes (data not shown).

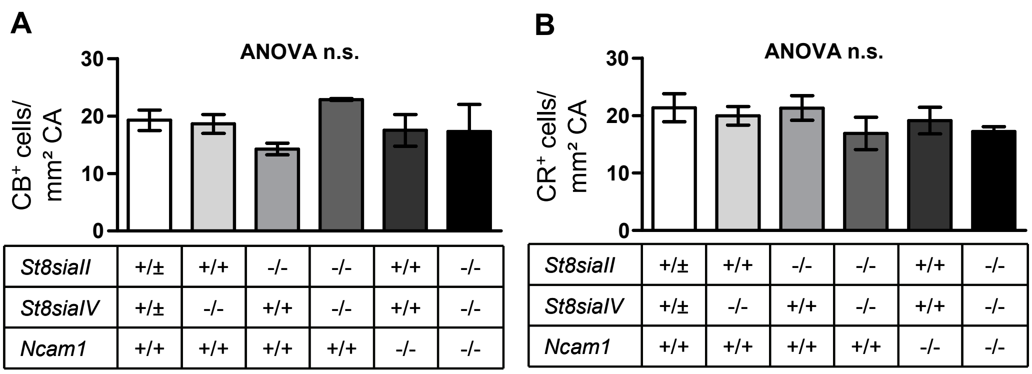
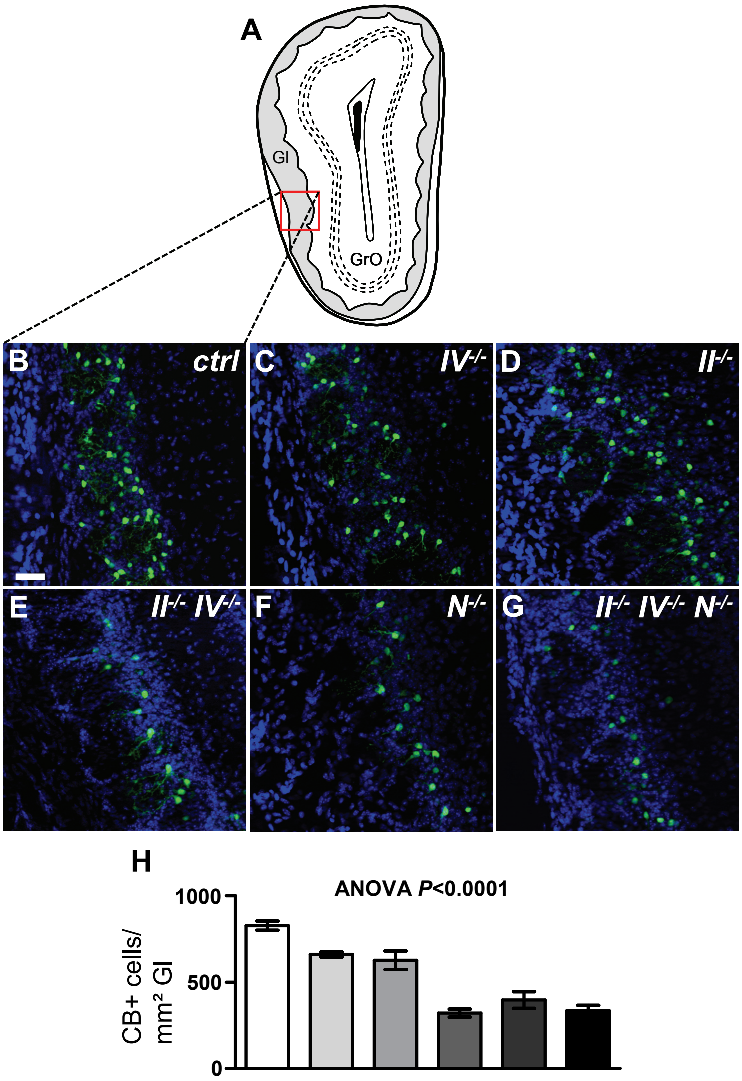


Figure 5

Densities of calbindin-positive cells (CB^{+}; ***A***) and calretinin-positive cells (CR^{+}; ***B***) in the CA area of the hippocampus. Per group, mean values ±SEM from n=3 animals are plotted. Differences were not significant (ANOVA, *P*>0.1)



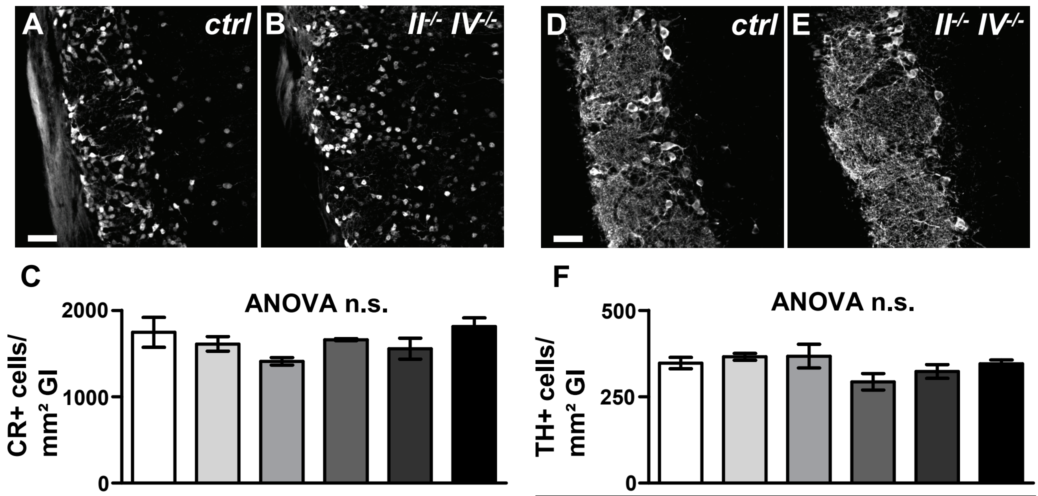
St8siaII	+/±	+/+	-/-	-/-	+/+	-/-
St8siaIV	+/±	-/-	+/+	-/-	+/+	-/-
Ncam1	+/+	+/+	+/+	+/+	-/-	-/-
ANOVA	a	b	b	c	c	c

Figure 6:

Calbindin (CB) expression in the glomerular layer of the olfactory bulb. (***A***) Schematic drawing of a coronal olfactory bulb section (according to Paxinos and Franklin, 2001). The glomerular layer (Gl) is highlighted in grey. The position of the micrographs depicted in (*B*-*G*) is indicated (red square). Abbreviation: GrO: granular cell layer of the olfactory bulb (***B-G***) Representative details illustrating CB-positive cells (green) with nuclear counterstain (DAPI, blue) in the Gl of different genotypes as indicated. Scale bar: 50µm. (***H***) Densities of CB^+ cells in the Gl of the olfactory bulb. Per group, mean values ±SEM from n=3 animals are plotted. One-way ANOVA indicated significant differences (P<0.0001) and Newman-Keuls post hoc test was applied. Means not marked with the same letter differ significantly (P<0.01).

Densities of CB-, CR-, and TH-immunoreactive cells in the OB

On each OB section the area of the glomerular layer (Gl) was determined and for each of the markers all immunopositive cells of the Gl were counted (Fig. 6A). Compared to the control group, a more than 50% reduction in the density of CB^+ cells was detected within the glomerular layer of $II^{-/-}IV^{-/-}$, $N^{-/-}$ and $II^{-/-}IV^{-/-}N^{-/-}$ mice (Fig. 6B, E-H; ANOVA P<0.0001; for numbers of evaluated sections and listing of cell counts, see suppl. Table 3). Both lines deficient for one of the two polysialyltransferases ($II^{-/-}$, $IV^{-/-}$) had an intermediate phenotype (Fig 6C, D, H). The expression of CR or TH was not altered in the glomerular layer of the OB (ANOVA P>0.1, Fig. 7 A-C and D-F, respectively).



St8siaII	+/±	+/+	-/-	-/-	+/+	-/-
St8siaIV	+/±	-/-	+/+	-/-	+/+	-/-
Ncam1	+/+	+/+	+/+	+/+	-/-	-/-

St8siaII	+/±	+/+	-/-	-/-	+/+	-/-
St8siaIV	+/±	-/-	+/+	-/-	+/+	-/-
Ncam1	+/+	+/+	+/+	+/+	-/-	-/-

Figure 7:

Calretinin (CR) and tyrosine hydroxylase (TH) expression in the glomerular layer (Gl) of the olfactory bulb. (***A, B***) Representative details illustrating CR-positive cells in the Gl of control (*A*) and $II^{-/-}$ $IV^{-/-}$ (*B*) mice. Scale bar: 50µm. (***C***) Densities of CR^+ cells in the glomerular layer of the olfactory bulb. Per animal three sections were evaluated. Per group mean values ±SEM determined from n=3 animals are plotted. Differences were not significant (ANOVA P>0.1). (***D, E***) Representative details illustrating TH-positive cells in the glomerular layer of control (*D*) and $II^{-/-}IV^{-/-}$ (*E*) mice. Scale bar: 50µm. (**F**) Densities of TH^+ cells in the glomerular layer of the olfactory bulb. Per group, mean values ±SEM determined from n=3 animals are plotted. Differences were not significant (ANOVA P>0.1)

Discussion

The influence of polySia deficiency on selected GABAergic interneuron populations of the mouse forebrain was analyzed in a panel of mouse lines with differently combined *Ncam1* and polysialyltransferase deletions. Together, the data of the current study reveal alterations of distinct GABAergic interneuron populations in the prefrontal cortex, the hippocampus and the olfactory bulb. The concurrent effects observed in polysialylation-deficient and NCAM-negative mice identify a lack of NCAM-bound polySia as the cause of these pathological changes. This is an important notion because, although NCAM is by far the major polySia carrier in the brain, context-dependent polysialylation of a limited number of other glycoproteins has been described (see Mühlenhoff et al. 2009 for a recent review).

PolySia deficiency inversely affects PV$^+$ interneurons in PFC and CA

The drastically reduced densities of PV$^+$ interneurons in the PFC of all mouse lines with partial or complete deficiencies of polySia clearly contrasts with the increase of PV$^+$ cells observed in the CA fields of mice negative for either one or both polysialyltransferases but positive for NCAM ($II^{-/-}$, $IV^{-/-}$, $II^{-/-}IV^{-/-}$). In the neocortex as well as in the hippocampus, PV$^+$ cells comprise two major types of cortical interneurons, basket and chandelier cells (Conde et al. 1994; Freund and Buzsaki 1996; DeFelipe 1997; Gabbott et al. 1997). The vast majority of PV$^+$ cells, at least in the somatosensory cortex, are PV$^+$CB$^-$ basket cells (Kawaguchi and Kubota 1997; Markram et al. 2004). It is, therefore, reasonable to assume that the almost 50% loss of PV$^+$CB$^-$ cells in the PFC observed in all polySia-deficient mouse lines includes alterations of basket cells. Most likely, the increase of PV$^+$ cells in the hippocampal CA region involves basket cells, too. As outlined below, the inverse relationship between these changes in the PFC and the hippocampus may reflect a causal link.

Changes in basket cells have been found in other mouse models with altered polySia or NCAM levels. Mice over-expressing a soluble extracellular domain fragment of NCAM (NCAM-EC) under the neuron-specific enolase promoter displayed a dramatic reduction of PV$^+$ puncta, but no reduction of PV$^+$ cell somata in the cingulate cortex indicating a decrease in the number of synaptic terminals of basket cells (Pillai-Nair et al. 2005). Further investigations of these mice revealed perturbed arborization of basket cells in the PFC during early postnatal stages, when endogenous polysialylated NCAM is replaced by polySia-negative NCAM (Brennaman and Maness 2008). Within the same time window, premature removal of polySia in the visual cortex results in precocious maturation of perisomatic innervation by basket interneurons leading to enhanced inhibitory synaptic transmission (Di Cristo et al. 2007). Together with the current findings, these data reveal that the balanced regulation of polySia and NCAM is essential for proper development of PV^+ basket cells.

In addition to altered basket cell counts, the densities of the PV^+CB^+ cells, indicative for a subpopulation of chandelier cells (DeFelipe 1997; del Rio and DeFelipe 1997), were significantly reduced in the $IV^{-/-}$ lines, if opposed to the $IV^{+/+}$ genotypes investigated. This result is remarkable as it points towards a specific role of ST8SiaIV in the development or the maintenance of PV^+CB^+ interneurons, which may be independent from the synthesis of polySia on NCAM. Although a direct comparison of PV^+CB^+ cells in the PFC of e.g. ST8SiaIV-positive NCAM knockout mice with ST8SiaIV- and NCAM-negative mice was statistically not significant this possibility warrants further investigation.

CB^+ interneurons of the OB are reduced in mice with defective tangential migration

Periglomerular and granular interneurons of the OB are replaced throughout life (Alvarez-Buylla and Garcia-Verdugo 2002). They are born in the subventricular zone and migrate towards the OB in the rostral migratory stream (RMS). Three non-overlapping subtypes of periglomerular interneurons are characterized by the expression of CR, CB, and TH (Kosaka et al. 1995) and, as shown recently, all three subtypes are GABAergic in the mouse (Kohwi et al. 2007). The prominent reduction of CB^+ cells in the glomerular layer as found here in the NCAM- or polySia-negative mice ($N^{-/-}$, $II^{-/-}IV^{-/-}$, $II^{-/-}IV^{-/-}N^{-/-}$) is clearly linked to the well-described deficits of the tangential migration of the interneuron precursors due to altered cell surface interactions in the absence of polySia (Ono et al. 1994; Hu et al. 1996; Chazal et al. 2000). A causal link between impaired rostral migration and reduced numbers of CB^+ periglomerular interneurons is supported by the striking similarity to the phenotype observed in doublecortin (DCX) knockout mice (Koizumi et al.

2006). In these animals, a cell-intrinsic block of neuroblast migration results in a significant reduction of CB^+ neurons in the glomerular layer of the OB. In both cases, however, it remains enigmatic, why the migration deficit specifically affects the CB^+ population of OB interneurons.

Taken together, impaired tangential migration is the most likely cause for the deficits of CB^+ interneurons in the NCAM or polySia-negative mice. In addition, a small but significant reduction of the CB^+ subpopulation of periglomerular cells was observed in both polysialyltransferase single knockout lines ($II^{-/-}$ and $IV^{-/-}$). This is unexpected, because migrating cells in the RMS express polySia in the absence of either ST8SiaII or ST8SiaIV, and a normal morphology of the rostral migratory stream and the OB has been reported for both lines (Eckhardt et al. 2000; Angata et al. 2004). On the other hand, the complete absence of polySia in the RMS of $II^{-/-}IV^{-/-}$ animals indicates that both polysialyltransferases contribute to polySia synthesis in this system and therefore minor, yet undetected reductions of polySia levels may account for the mild phenotype in the OB of $II^{-/-}$ and $IV^{-/-}$ mice.

Are cortical PV^+ interneurons affected by disturbed tangential migration?

Clearly, further studies, which are beyond the scope of the current phenotype analyses, are needed to unravel the mechanisms that account for the observed alterations of cortical interneurons. As shown in the current study, deletion of either one or both polysialyltransferases affects PV^+ interneuron populations. In contrast, CB^+ but not PV- or CR-positive interneurons have been shown to co-express polySia in the PFC of adult rats (Varea et al. 2005) and expression of polySia in the PFC of adult mice is exclusively affected by ST8SiaIV-deficiency (Nacher, Röckle and Hildebrandt, submitted). It therefore seems likely that the changes of PV^+ cells are caused by a lack of polySia during development and not by altered polySia expression in the mature cortex.

In keeping with the prominent role of polySia in tangential migration of the subventricular zone-derived interneuron precursors, it is attractive to speculate that dysfunctional migration may cause the observed alterations of PV^+ interneuron densities within the cortex. In rodents, most, if not all, GABAergic interneurons of the cortex originate within the subpallium and migrate tangentially to the developing pallium. Interneurons expressing PV appear to derive primarily from the medial ganglionic eminence (MGE), whereas CR^+ cells seem to emerge exclusively from the dorsal aspect of the caudal ganglionic eminence (CGE; Xu et al. 2004; Metin et al. 2006; Gelman et al. 2009). In contrast to CGE cells that migrate predominantly towards the caudal telencephalon, MGE cells tend to migrate laterally before they spread throughout the cortex. While some of the mechanisms that shape the early decisions used by interneurons to reach the cortex are at the beginning to be elucidated (for review, see Metin et al. 2006), the determinants of their intracortical migration, their spreading into the different cortical areas, and their subsequent differentiation into each particular type of interneuron remain to be revealed. Nevertheless, the inverse relationship between PV^+ cells being reduced in the PFC but increased in the hippocampus of polySia-deficient mice raises the intriguing possibility that polySia is involved in the regulation of cell surface interactions that shape decisions of directional migration of a distinct class of interneuron precursors.

Indeed, impaired migration of yet unidentified precursor cells during cortical development has been detected in $II^{-/-}IV^{-/-}$ mice (Angata et al. 2007). This study also reports on a substantial, approximately 20% reduction in the number of CB^+ cells in the cerebral cortex of adult $II^{-/-}IV^{-/-}$ animals. As no other neuronal markers where assessed, the specificity of this effect remains unresolved. More important, the apparent discrepancy with the specific reduction of PV^+ cells observed in the current study may be explained by the fact that the other study evaluated animals with drastic cortical thinning due to hydrocephalus formation. As shown in human fetal hydrocephalus this involves the loss of CB^+ and PV^+ cells (Ulfig et al. 2001). In contrast, only specimen with moderate ventricular dilatation and no cortical thinning were considered for the current analyses.

In addition to impaired migration, loss of polySia causes premature differentiation of neuronal precursors *in vitro* and *in vivo* (Petridis et al. 2004; Burgess et al. 2008; Röckle et al. 2008), defective development of brain axon tracts (Weinhold et al. 2005; Hildebrandt et al. 2009) as well as reduced proliferation, enhanced survival and improved differentiation of neuroblastoma cells (Seidenfaden et al. 2003; Seidenfaden et al. 2006). These effects, however, are induced by a gain of polySia-free NCAM. In contrast, the altered interneuron densities described in the current study are caused by reductions of polySia irrespective of the presence or absence of NCAM, as they were equally found in polysialylation- and NCAM-deficient mice. This distribution is compatible with the observation of migration deficits in mice with a specific depletion of polySia as well as in mice lacking polySia due to NCAM deficiency (Ono et al. 1994; Hu et al. 1996; Chazal et al. 2000) and therefore supports the idea that impaired migration is the cause for the altered cortical interneuron densities observed in all polySia-deficient lines.

Relation to pathological findings in schizophrenia

Aberrant GABAergic circuits have been implicated in various neurodevelopmental and psychiatric disorders such as schizophrenia, bipolar disorder, autism and Tourette syndrome (Benes and Berretta 2001; Belmonte et al. 2004; Kalanithi et al. 2005). Numerous pathological reports demonstrate alterations of calcium-binding protein containing interneurons in particularly the PFC of schizophrenic patients (reviewed in Reynolds et al. 2001; Eyles et al. 2002; Lewis et al. 2005; Lewis and Sweet 2009). Despite considerable inconsistencies, some of these studies demonstrate reduced densities of PV^+ and, to a lesser extent, CB^+ interneurons. In contrast, the CR^+ subtype seems to be consistently unaltered (Beasley et al. 2002; Reynolds et al. 2002). These data, therefore, are comparable with the alterations of specifically PV^+ but not CR^+ interneurons observed in the PFC of polySia-deficient mice.

In addition to pathological changes in the PFC, hippocampal dysfunction is considered to play a major role in the pathophysiology of schizophrenia (Gothelf et al. 2000; Schmajuk 2001; Harrison 2004; Hall et al. 2009) and decreased density of PV^+ interneurons in the hippocampus is one of the most consistent postmortem findings in the brain of schizophrenic patients (Zhang and Reynolds 2002; Reynolds et al. 2004; Torrey et al. 2005). This clearly contrasts with the increase of PV^+ interneurons observed in the CA region of mice with compromised polySia-levels. However, as in the PFC, polySia-deficiency seems to cause a significant imbalance between inhibitory interneurons and excitatory transmission in the hippocampus.

In conclusion, we therefore propose that dysregulated interneuron development caused by a lack of NCAM-bound polySia is a candidate mechanism for pathological alterations of GABAergic interneuron subtypes, which might be involved in the pathogenesis of schizophrenia and other neuropsychiatric disorders.

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Supplementary tables

Supplementary Table 1: Prefrontal cortex areas and cell counts

		mean area [mm²]		relative area	mean cell counts/PFC [a]					
	animal (line, mating, litter)	PFC [b]	total brain section [c]	PFC (% of brain section)	PV^+ [b]	CB^+ [d]	PV^+ CB^- [d]	PV^+ CB^+ [d]	PV^- CB^+ [d]	CR^+ [d]
ctrl.	bl/6 #27 /8-21 (*II*$^{+/+}$*IV*$^{+/+}$)	1.32	31.83	4.15	70.08	159.00	58.17	16.50	142.50	77.67
	tg1 #62/3-2 (*II*$^{+/+}$*IV*$^{+/+}$)	1.32	30.28	4.36	74.17	123.67	57.83	18.50	105.17	76.00
	tg3 #151/3-10 (*II*$^{+/-}$*IV*$^{+/-}$)	1.11	26.57	4.16	70.50	103.33	46.67	14.00	89.33	72.60
IV$^{-/-}$	tg1 #76/3-4	1.27	30.35	4.18	51.83	149.67	34.83	13.33	136.33	76.33
	tg1 #76/3-5	1.36	34.38	3.96	54.50	82.67	42.00	8.00	74.67	63.33
	tg1 #76/3-6	1.18	29.22	4.04	47.83	128.67	34.83	12.00	116.67	57.83
II$^{-/-}$	tg2 #142/1-33	1.05	27.03	3.88	40.67	113.33	24.00	14.83	89.50	60.00
	tg2 #142/1-34	1.32	31.13	4.24	36.67	76.50	28.33	8.67	67.83	48.83
	tg2 #142/1-35	1.14	26.18	4.35	40.00	137.17	27.33	17.67	119.50	56.83
II$^{-/-}$ *IV*$^{-/-}$	tg4 #141/3-1	1.01	24.92	4.05	38.05	120.17	25.50	11.00	109.17	58.33
	tg4 #141/3-5	1.07	23.52	4.55	36.75	80.50	27.33	6.83	73.67	62.17
	tg4 #103/6-1 [e]	1.14	25.75	4.43	21.50	138.00	18.00	12.50	125.50	65.00
N$^{-/-}$	tg5 #98/5-1	1.04	26.51	3.92	31.33	102.67	18.33	13.83	88.83	56.83
	tg5 #98/5-2	1.31	34.45	3.80	47.75	83.67	36.00	9.83	73.83	56.33
	tg5 #98/5-3	1.13	27.62	4.09	40.75	123.67	27.00	13.00	110.67	63.83
II$^{-/-}$ *IV*$^{-/-}$ *N*$^{-/-}$	tg4 #134/3-11	1.20	29.42	4.08	37.25	119.67	28.67	10.50	109.17	85.83
	tg4 #134/3-12	1.20	28.16	4.26	31.00	110.83	22.50	12.67	98.17	71.50
	tg4 #103/55-1 [e]	1.26	26.62	4.73	31.75	104.50	28.50	7.00	97.50	79.00

[a] Abbreviations: CB, calbindin, CR, calretinin; PFC, prefrontal cortex; PV, parvalbumin
[b] if not noted otherwise, mean values for left and right PFC from six sections per brain were determined for each animal
[c] per brain, mean areas from the evaluated sections at the level of the PFC were determined
[d] if not noted otherwise, mean values for left and right PFC from three sections per brain were determined for each animal
[e] areas and numbers of PV^+ cells were determined from two sections per brain, numbers of CB^+ and CR^+ cells from one section, each

Supplementary Table 2: Hippocampus areas and cell counts

	animal (line, mating, litter)	mean area [mm²]				relative area hip (% of brain section)	mean cell counts/hip [a]			
		hippocampus [b]			total brain section [c]		PV+ [b]		CB+ [d]	CR+ [d]
		CA	DG	total			CA	DG	CA	CA
ctrl. [e]	bl/6 #27/8-21 ($II^{+/+}IV^{+/+}$)	1.88	0.70	2.58	54.24	4.76	32.13	6.75	38.83	56.00
	tg1 #62/3-2 ($II^{+/+}IV^{+/+}$)	1.69	0.65	2.35	52.07	4.51	35.38	10.63	33.83	36.00
	tg3 #151/3-10 ($II^{+/-}IV^{+/-}$)	1.63	0.54	2.17	48.27	4.50	31.25	6.25	25.67	29.00
$IV^{-/-}$	tg1 #76/3-4	1.61	0.54	2.15	50.68	4.24	52.42	7.92	34.83	36.83
	tg1 #76/3-5	1.55	0.50	2.04	52.21	3.91	54.50	7.58	25.67	30.33
	tg1 #76/3-6	1.62	0.53	2.15	52.97	4.06	48.42	5.75	29.67	27.00
$II^{-/-}$	tg2 #142/1-33	1.44	0.51	1.95	48.20	4.05	50.33	6.92	22.67	35.83
	tg2 #142/1-34	1.87	0.66	2.53	51.78	4.89	53.67	9.08	28.67	38.17
	tg2 #142/1-35	1.74	0.64	2.38	55.51	4.29	52.17	8.50	22.17	29.83
$II^{-/-}$ $IV^{-/-}$ [e]	tg4 #141/3-1	1.89	0.55	2.45	43.70	5.61	48.13	7.13	43.00	25.50
	tg4 #141/3-5	1.88	0.48	2.37	43.47	5.45	58.75	6.25	41.83	29.50
	tg4 #103/6-1	1.13	0.31	1.44	47.46	3.03	35.63	5.63	23.67	32.50
$N^{-/-}$	tg5 #98/5-1	1.62	0.54	2.16	48.96	4.41	56.50	10.83	34.83	36.33
	tg5 #98/5-2	1.78	0.65	2.42	53.50	4.52	44.42	8.58	30.17	37.33
	tg5 #98/5-3	2.17	0.85	3.03	64.24	4.72	42.42	6.75	29.00	31.50
$II^{-/-}$ $IV^{-/-}$ $N^{-/-}$ [e]	tg4 #134/3-11	2.06	0.60	2.67	70.29	3.80	46.38	5.00	31.33	40.50
	tg4 #134/3-12	1.83	0.51	2.20	53.94	4.08	47.00	6.83	16.33	44.00
	tg4 #103/55-1	1.64	0.49	2.13	43.41	4.91	48.25	7.13	42.83	26.00

[a] Abbreviations: CA, Cornu ammonis; CB, calbindin, CR, calretinin; DG, dentate gyrus; hip, hippocampus; PV, parvalbumin
[b] if not noted otherwise, mean values for left and right hip from six sections per brain were determined for each animal
[c] per brain, mean areas from the evaluated sections at the level of the hip were determined
[d] if not noted otherwise, mean values for left and right hip from three sections per brain were determined for each animal
[e] areas and numbers of PV^+ cell were determined from four sections per brain, CR^+ from one section, each

Supplementary Table 3: Olfactory bulb areas and cell counts

	animal (line, mating, litter)	mean area [mm²] Gl (evaluated area) [b]	mean area [mm²] total OB section [c]	relative area Gl (% of brain section)	mean cell counts/Gl [a] CB+ [d]	mean cell counts/Gl [a] CR+ [d]	mean cell counts/Gl [a] TH+ [d]
ctrl.	bl/6 #27 /8-21 ($II^{+/+}IV^{+/+}$)	0.73	4.81	15.18	454.00	1279.33	250.33
	tg1 #62/3-2 ($II^{+/+}IV^{+/+}$)	0.61	4.03	15.14	504.67	1286.00	231.33
	tg3 #151/3-10 ($II^{+/-}IV^{+/-}$)	0.73	4.12	17.72	565.33	1264.33	259.67
$IV^{-/-}$	tg1 #76/3-4	0.72	3.86	18.65	491.00	1102.33	255.33
	tg1 #76/3-5	0.77	4.90	15.71	460.00	1266.33	268.00
	tg1 #76/3-6	0.61	3.71	16.44	403.33	1093.00	234.00
$II^{-/-}$	tg2 #142/1-33	0.67	3.84	17.45	393.67	987.33	250.33
	tg2 #142/1-34	0.81	4.74	17.09	538.67	1142.00	245.00
	tg2 #142/1-35	0.67	3.78	17.72	386.00	1096.67	235.67
$II^{-/-}$ $IV^{-/-}$	tg4 #103/6-1	0.55	1.96	28.06	161.33	999.00	120.00
	tg3 #151/3-11	0.46	2.29	20.09	132.33	850.67	145.33
	tg3 #151/3-14	0.44	1.92	22.92	135.33	735.33	158.67
$N^{-/-}$	tg5 #98/5-1	0.53	2.46	21.54	164.00	760.00	179.67
	tg5 #98/5-2	0.61	3.37	18.10	269.67	930.33	160.33
	tg5 #98/5-3	0.23	2.86	8.04	71.00	635.33	58.33
$II^{-/-}$ $IV^{-/-}$ $N^{-/-}$	tg4 #134/3-11	0.43	2.06	20.87	126.33	770.67	155.33
	tg4 #134/3-12	0.43	2.03	21.18	104.33	782.67	176.33
	tg4 #103/55-1	0.50	2.04	24.51	220.33	1019.00	134.67

[a] Abbreviations: CB, calbindin, CR, calretinin; Gl, glomerular layer; TH, tyrosine hydroxylase
[b] per brain, mean values for the Gl from nine OB sections were determined
[c] per brain, mean areas from the evaluated OB sections were determined
[d] per brain, mean values from three OB sections were determined

Chapter 4 – General Discussion

The neural networks in the neocortex of higher vertebrates consist of two broad classes of neurons: principal or projection neurons and local circuit neurons or interneurons. While projecting neurons are excitatory, interneurons are mostly inhibitory and use GABA (γ-aminobutyric acid) as transmitter. Interneurons are crucial for the functional balance, complexity and computational architecture of neural circuits (Huang et al. 2007). Aberrant development and function of the cortical GABAergic system have been implicated in various neurodevelopmental and psychiatric disorders, for example, schizophrenia (Lewis et al. 2005), autism (Belmonte et al. 2004) and Tourette syndrome (Kalanithi et al. 2005). Understanding the mechanisms that underlie the construction and plasticity of the GABAergic system will be a prerequisite for the development of new therapeutic approaches.

NCAM and its unique sugar moiety polySia are tightly associated with nervous system development and plasticity (Hinsby et al. 2004a; Hildebrandt et al. 2007; Rutishauser 2008). So far, however, their influence on the development of specific GABAergic interneuron subtypes in the cerebral cortex and olfactory bulb (OB) has been elusive and the specific impact of the polySia modification on the one hand and the NCAM protein backbone on the other has not been dissected. Therefore, the two studies of this thesis addressed the role of NCAM and polySia in interneuron development *in vitro* and *in vivo*. The obtained data indicate that loss of polySia affects the development of GABAergic interneurons of the mouse forebrain. However, it seems that different mechanisms are involved *in vitro* and *in vivo*.

The subventricular zone (SVZ) is the largest neurogenic region in the adult brain. *In vivo*, neuroblasts born in the SVZ migrate as chains along the rostral migratory stream (RMS) into the olfactory bulb (OB), where they differentiate into interneurons (Doetsch and Alvarez-Buylla 1996; Lois et al. 1996; Doetsch et al. 1997; Doetsch et al. 1999). In the mouse OB, three non-overlapping subtypes of periglomerular interneurons can be characterized by the expression of calbindin (CB), calretinin (CR) and tyrosine hydroxylase (TH; Kosaka et al. 1995). As demonstrated in the first study of this thesis, removal of polySia from cultured SVZ-derived neuroblasts with endosialidase (endoN) induced neuritogenesis and enhanced specifically the differentiation of these precursors towards the CR-positive interneuron subtype. In contrast, as shown in the second study no increase of the CR^+ interneuron population of the glomerular layer was observed in the OB of polySia-deficient mice. Instead, the evaluation of the different markers revealed a prominent reduction of calbindin (CB)-positive cells in NCAM- or polySia-negative mice ($N^{-/-}$, $II^{-/-}IV^{-/-}$, $II^{-/-}IV^{-/-}N^{-/-}$). This indicates that two different functions of polySia are involved in the generation of interneurons *in vitro* and *in vivo*. Previous studies have shown that acute removal of polySia causes premature differentiation of neuronal precursors (Petridis et al. 2004; Burgess et al. 2008), as well as enhanced differentiation of neuroblastoma cells (Seidenfaden et al. 2003; Seidenfaden et al. 2006). This is consistent with the increased neuritogenesis and maturation of SVZ-derived precursors into CR^+ interneurons after endoN treatment *in vitro* as observed in the first study of this thesis. Since endoN-induced differentiation could be prevented by incubation with the synthetic NCAM-binding protein C3d, this effect is most likely caused by a gain of polySia-free NCAM. On the contrary, the reduced densities of CB^+ interneurons in the glomerular layer were equally developed in polysialylation- and NCAM-deficient mice. Thus, the observed alterations *in vivo* are not caused by a gain of polySia-free NCAM but by a reduction of polySia irrespective of the presence or absence of NCAM. This is compatible with the assumption that the loss of CB^+ cells is a consequence of the prominent defect of tangential neuroblast migration, which is also observed in the absence of polySia or NCAM (Tomasiewicz et al. 1993; Cremer et al. 1994; Ono et al. 1994; Hu et al. 1996; Chazal et al. 2000; Weinhold et al. 2005; Angata et al. 2007). This assumption is further supported by the striking similarity to the phenotype observed in doublecortin (DCX) knockout mice (Koizumi et al. 2006), in which a cell-intrinsic block of neuroblast migration results in a significant reduction of CB^+ neurons in the glomerular layer of the OB. Taken together, these findings indicate that in the absence of chain migration *in vitro*, loss of polySia induces differentiation of SVZ-derived precursors, whereas *in vivo* this effect may be concealed by the more severe consequences of impaired migration.

Loss of polySia affects not only the density of CB-positive OB interneurons but also specific subpopulations of GABAergic interneurons in other brain regions. In contrast to the reduced density of CB^+ interneurons in the OB, parvalbumin (PV)-expressing cells were affected in the prefrontal cortex (PFC) and hippocampus. In the cortex as well as in the hippocampus, PV^+ cells comprise two major types of interneurons, chandelier and basket cells (Conde et al. 1994; Freund and Buzsaki 1996; DeFelipe 1997; Gabbott et al. 1997). As the vast majority of PV^+ interneurons, at least in the somatosensory cortex, are PV^+CB^- basket cells (del Rio and DeFelipe 1997; Kawaguchi and Kubota 1997; Markram et al. 2004), it is reasonable to assume that the primarily affected interneurons in the PFC and hippocampus include basket cells. PV^+ cells were drastically decreased in the PFC of all mouse lines with partial or complete deficiencies of polySia, whereas an increase of PV^+ interneurons was observed in the CA fields of the hippocampus. Increased densities of PV^+ cells were also described in the CA1 field of the hippocampus of mice deficient for the cell adhesion molecule close homologue of L1 (CHL1; Nikonenko et al. 2006). Interestingly, like CHL1-mutants, $IV^{-/-}$ mice exhibit impaired long-term potentiation indicating a possible link of this phenotype to enhanced GABAergic inhibition.

Concerning the implications of NCAM polysialylation in schizophrenia, it is remarkable that the loss of poySia affects particularly PV^+ cortical interneurons. Although disputed, several studies found alterations of PV^+ but not CR^+ interneurons in schizophrenics (Beasley et al. 2002; Reynolds et al. 2002; reviewed in: Reynolds et al. 2001; Eyles et al. 2002; Lewis et al. 2005; Lewis and Sweet 2009). In this regard, the reduced densities of PV^+ interneurons in the PFC of polySia-deficient mice correspond to pathological findings (Beasley et al. 2002; Reynolds et al. 2002), whereas the increase in the hippocampus is reciprocal to the consistently observed decrease of PV expression in the hippocampus of schizophrenic patients (Zhang and Reynolds 2002; Reynolds et al. 2004; Torrey et al. 2005).

In analogy to the changes of OB interneurons discussed above, interneuron alterations observed in the PFC and hippocampus may be caused by either altered differentiation or disturbed migration. Since these changes are not due to a gain of NCAM functions other mechanisms must be responsible. Although highly speculative, a possible mechanism relates to the ability of polySia to modify cellular responses to brain-derived neurotrophic factor (BDNF; Vutskits et al. 2001; Glaser et al. 2007). As BDNF has been shown to bind to polySia (Kanato et al. 2008), one possible function of polySia may be the enrichment of BDNF. Therefore, reduced BDNF-TrkB signaling may account for the altered PV expression in the PFC of polySia-deficient mice. Indeed, signalling of BDNF through its receptor TrkB has been reported to influence the development of cortical GABAergic neurons and TrkB is predominantly expressed by PV-positive cortical interneurons (Cellerino et al. 1996; Huang et al. 1999; Yamada et al. 2002; Patz et al. 2004). In contrast to mice with decreased TrkB expression, however, a conditional BDNF-knock out mouse revealed no differences in mRNA expression levels of GAD67 (the 67kD isoform of the GABA-synthesizing enzyme glutamic acid decarboxylase) or PV (Hashimoto et al. 2005). It therefore has been concluded that changes in TrkB but not a lack of BDNF cause the altered expression of interneuron markers. Thus, it appears not likely that polySia affects interneuron densities by functioning as a BDNF scavenger factor.

Another growth factor essential for differentiation and migration of neuronal precursors is GDNF (glial cell line-derived neurotrophic factor; Pozas and Ibanez 2005; Paratcha et al. 2006). It has been shown that NCAM directly binds GDNF as well as the GPI-anchored GDNF family receptor α1 (GFRα1) and thus can function as an alternative signaling receptor for members of the GDNF ligand family (Paratcha et al. 2003). It therefore would be attractive to speculate that loss of polySia either alters interactions of NCAM with GFRα1 to induce NCAM-dependent progenitor differentiation, or affects GDNF signaling to cause the observed alterations of interneuron densities. However, signaling via the NCAM-GFRα1 complex is unlikely to be influenced by polySia depletion, because both, polysialylated and non-polysialylated forms of NCAM are equally able to bind GFRα1 and GDNF (Paratcha et al. 2003; Nielsen et al. 2009). Moreover, although migration and differentiation of cortical GABAergic neurons requires GFRα1 signaling, the impact of GDNF on these processes is independent from NCAM (Pozas and Ibanez 2005).

Similar to the tangential migration of neuroblasts within the RMS, interneuron precursors from the ganglionic eminence (GE) migrate tangentially towards the cortex. Thus, dysfunctional migration may not only account for reduced interneuron densities in the OB but also for the observed alterations within the PFC and hippocampus. The GE is the main source of interneurons in the developing rodent brain and at least three progenitor domains, the lateral (LGE), medial (MGE) and caudal ganglionic eminence (CGE), can be distinguished. Most of the PV and CB-expressing interneurons derive from the MGE, while CR-containing cells arise predominantly in the CGE, whereas LGE cells contribute to interneurons in the olfactory bulb (Flames and Marin 2005; Wonders and Anderson 2005; Metin et al. 2006; Rakic 2009). Some of the mechanisms of interneuron precursor migration are at the beginning to be elucidated (Metin et al. 2006), but mostly, the factors controlling their spreading into the different cortical areas, their intracortical migration, and their subsequent differentiation into the different interneuron subtypes remain to be revealed. In this respect, the fact that PV+ cells are reduced in the PFC but increased in the hippocampus of polySia-deficient mice raises the interesting possibility that polySia is involved in the regulation of directional migration of a distinct class of interneuron precursors, thus shaping their cortical distribution. Indeed, Angata et al. (2007) described impaired migration and altered distribution of precursor cells during cortical development of polySia-deficient *II*$^{-/-}$*IV*$^{-/-}$ mice. Additionally, this study found a decrease of CB^{+} cells in the cerebral cortex of adult *II*$^{-/-}$*IV*$^{-/-}$ mice. This is inconsistent with the outcome of our study where the CB^{+} interneuron subtype was unaffected but the density of PV^{+} cells was decreased. However, since only CB and no other neuronal markers were used by Angata et al. (2007), the specificity of the observed defect remains questionable. More important, mice with drastic cortical thinning due to hydrocephalus formation were evaluated in their study. Hydrocephalus formation, however, has been shown to involve the loss of CB^{+} and PV^{+} neurons in the cortex of rats and humans (Tashiro et al. 1997; Ulfig et al. 2001). For these reasons, and in clear contrast to the study by Angata and colleagues, only animals with moderate ventricular dilatation and no cortical thinning were considered in our study.

Albeit there is some indication that dysregulated migration may not only account for the interneuron alterations observed in the OB but also for those in the cortex, it is evident that further studies are needed to unravel the underlying mechanisms. Above all, it is open, if the reduced density of PV^{+} interneurons in the PFC of polySia-deficient mice is due to a lack of entire cells or just due to a lack of protein expression. The same issue is also heavily debated in schizophrenia. Some studies suggest that numbers of PV-expressing interneurons in the dorsolateral PFC of schizophrenics are not reduced but that these cells have decreased expression levels of PV and other GABAergic markers and might therefore be functionally impaired (Lewis et al. 2005). One possibility to address this question in mice may be the use of GAD67-GFP (glutamic acid decarboxylase67-green fluorescence protein) knock-in mice (Tamamaki et al. 2003). In these mice, all GABAergic interneurons are labeled and cross-breeding with polySia-deficient lines will allow to determine, if loss of polySia causes changes of GABAergic interneuron numbers. Another strategy will be the investigation of other markers of PV^+ interneurons. For example, staining with the *Wisteria floribunda* lectin visualizes extracellular matrix structures, so-called perineuronal nets, specifically surrounding PV^+ interneurons of e.g. the PFC (Hartig et al. 1992; Brauer et al. 1993; Dityatev et al. 2007). A normal distribution of these perineuronal nets in the PFC of polySia-deficient mice would point towards an unaltered interneuron number but decreased PV expression, whereas a decrease of perineuronal nets would indicate a reduction in cell number.

Instead of altered migration, the loss or gain of specific interneuron populations could be caused by altered proliferation during embryogenesis. Future studies may address this possibility by BrdU (5-bromo-2-deoxyuridine)-labeling experiments to mark and trace proliferating cells. Alternatively, the loss of PV^+ interneurons in the PFC could be due to degeneration. Thus, apoptotic cell death should be studied in the different polySia-deficient mouse lines. In addition, in the case of a degenerative process a progressive cell loss should be detectable. This could be addressed by simply analyzing polySia-deficient mice at different ages. Due to the precocious lethality of $II^{-/-}IV^{-/-}$ mice, the current study was restricted to young, one month old animals. After revealing that animals deficient for either of the two polysialyltransferases or NCAM exhibit the same reduction of PFC interneurons, these mice can be now be traced over time. Finally, the reduction of interneuron density in the PFC as well as the increase in the hippocampus observed in four week old animals could be caused by a delay or an acceleration of differentiation. If this would be the case, the respective defect should diminish with age.

Although NCAM is by far the major polySia carrier in the brain, it is important to keep in mind that context-dependent polysialylation of a limited number of other glycoproteins has been described (for a recent review see: Mühlenhoff et al. 2009). In the brain, so far only the α-subunit of the voltage-gated sodium channel has been discussed as a possible alternative carrier of polySia (Zuber et al. 1992). Most recently, SynCAM 1 was identified as a novel polysialylated protein in brains from NCAM-deficient and wildtype mice (Galuska et al. 2009). In this context it is remarkable that in addition to altered basket cell counts in the PFC, the densities of the PV^+CB^+ cells, indicative for a subpopulation of chandelier cells (DeFelipe 1997), were significantly reduced in the $IV^{-/-}$ lines, if opposed to the $IV^{+/+}$ genotypes investigated. This observation points towards a specific role of ST8SiaIV in the development or the maintenance of PV^+CB^+ interneurons, which may be independent from the synthesis of polySia on NCAM. Although a direct comparison of PV^+CB^+ cells in the PFC of e.g. ST8SiaIV-positive NCAM knockout mice ($N^{-/-}$) with ST8SiaIV- and NCAM-negative mice ($II^{-/-}IV^{-/-}N^{-/-}$) was statistically not significant this possibility warrants further investigation. Reduced chandelier cartridges have also been reported in schizophrenia (Woo et al. 1998; Pierri et al. 1999). Therefore, a more detailed analysis of chandelier cells in the different polysialyltransferase-deficient mice would be of general interest.

Perspectives

The results of my thesis indicate that *in vitro*, in the absence of migration, downregulation of polySia initiates NCAM trans-interactions which promote differentiation of SVZ-derived interneuron precursors, whereas chronical loss of polySia *in vivo* during development impairs precursor migration and results in altered interneuron densities in the forebrain.

The SVZ is a major source for adult neural stem cells and neuronal precursors, which could be used in cell-based brain repair approaches, e.g. in Parkinson's disease or after stroke (Lindvall et al. 2004). Therefore, the possibility to control timing of neuroblast differentiation and eventually increase neuron yields *in vitro* with the help of polySia- and NCAM-specific tools is intriguing, as it may be applicable in therapeutic strategies aiming at neuron replacement. In addition, enzymatic degradation of polySia by target-oriented application of endoN, manipulation of endogenous polysialyltransferase activity and/or the use of NCAM-mimetic peptides like C3d may have the potential to trigger endogenous brain repair processes. Thus, both approaches warrant further investigation.

In contrast to the enhanced differentiation observed *in vitro*, reduced polySia expression during brain development leads to altered interneuron composition in the forebrain and thereby to a modified network function. Aberrant GABAergic circuits have been linked to diverse neurodevelopmental and psychiatric disorders such as schizophrenia, bipolar disorder, autism and Tourette syndrome (Benes and Berretta 2001; Belmonte et al. 2004; Kalanithi et al. 2005). Thus, it suggests itself to analyze the different polySia-deficient mice in behavioral tests of sensory gating or working memory, which are indicative for cognitive deficits as observed in e.g. schizophrenia. Previous studies with $N^{-/-}$ mice indicate deficits in learning or memory formation (Cremer et al. 1994; Cremer et al. 1998; Eckhardt et al. 2000; Bukalo et al. 2004; Senkov et al. 2006) and one study reported reduced prepulse inhibition of acoustic startle (PPI) in NCAM-180 knockout mice (Wood et al. 1998). In contrast, a study on NCAM-null mice was not able to recapitulate this defect indicating that a deficit in prepulse inhibition may become apparent only after treatement with a 'second hit' (Plappert et al. 2005). Likewise, a first study on $II^{-/-}$ mice found no deficits of prepulse inhibition as tested in a large behavioural test battery (Angata et al. 2004). Thus, refined testing of the different polySia-deficient lines under more challenging conditions is needed. Studies along these lines will help to further explore, if interference with NCAM polysialylation holds the potential to contribute to a neurodevelopmental predisposition to cognitive impairment and neuropsychiatric disease.

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Abbreviations

II$^{-/-}$	*St8siaII*-knock out
IV$^{-/-}$	*St8siaIV*-knock out
BDNF	brain-derived neurotrophic factor
BrdU	5-bromo-2-deoxyuridine
CA	cornu ammonis (Ammon's horn)
CAM	cell adhesion molecule
CB	calbindin
cc	corpus callosum
Cg1	cingulate cortex, area 1
CGE	caudal ganglionic eminence
CHL1	close homologue of L1
CNTF	ciliary neurotrophic factor
CR	calretinin
CSPG	chondroitin sulfate proteoglycan
E	embryonic day
ECM	extracellular matrix
endoN	endosialidase (endo-N-acetylneuraminidase)
ERK-kinase	extracellular signal-related-kinase
FAK	focal adhesion kinase
FGFR	fibroblast growth factor receptor
FnIII	fibronectin type III
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GDNF	glial cell line-derived neurotrophic factor
GE	ganglionic eminence
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GFRα1	GDNF family receptor α1
Gl	glomerular layer of the olfactory bulb
GPI	glycosylphosphatidylinositol
HSPG	heparan sulfate proteoglycan
Ig	immunoglobulin
IL	infralimbic cortex
kDa	kilo Dalton

LGE	lateral ganglionic eminence
LTD	long term depression
LTP	long term potentiation
lv	lateral ventricle
MAP-kinase	mitogen activated protein-kinase
MGE	medial ganglionic eminence
$N^{-/-}$	NCAM-knock out
NCAM	neural cell adhesion molecule
Neu5Ac	N-acetylneuraminic acid
NSC	neural stem cell
OB	olfactory bulb
P	postnatal day
PDGF	platelet-derived growth factor
PFC	prefrontal cortex
polySia	polysialic acid
polyST	polysialyltransferase
PrL	prelimbic cortex
PSTD	polysialyltransferase domain
PV	parvalbumin
RMS	rostral migratory stream
SGZ	subgranular zone
SNP	single nucleotide polymorphism
SVZ	subventricular zone
TH	tyrosin hydroxylase

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Publications

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- Weinhold B., Seidenfaden R., **Röckle I.**, Mühlenhoff M., Schertzinger F., Conzelmann S., Marth J.D., Gerardy-Schahn R., Hildebrandt H. (2005) Genetic ablation of polysialic acid causes severe neurodevelopmental defects rescued by deletion of the neural cell adhesion molecule. J. Biol. Chem. 280:42971-77.

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