Role of polysialic acid in the genesis of GABAergic neurons of the cerebral cortex and for thalamocortical connectivity

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Role of polysialic acid in the genesis of GABAergic neurons of the cerebral cortex and for thalamocortical connectivity

THESIS
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by

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<tr>
<td>AMPA</td>
<td>2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-indolyl-phosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>CA</td>
<td>Cornu ammonis</td>
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<tr>
<td>CB</td>
<td>calbindin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>Cg1</td>
<td>cingulate cortex area 1</td>
</tr>
<tr>
<td>CGE</td>
<td>caudal ganglionic eminence</td>
</tr>
<tr>
<td>CHL1</td>
<td>close homolog of L1</td>
</tr>
<tr>
<td>CR</td>
<td>calretinin</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCX</td>
<td>Doublecortin</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>DIG-UTP</td>
<td>digoxigenin conjugated uridine-5′-triphosphate</td>
</tr>
<tr>
<td>DISC1</td>
<td>Disrupted-in-schizophrenia 1</td>
</tr>
<tr>
<td>div</td>
<td>days <em>in vitro</em></td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>endo</td>
<td>endosialidase</td>
</tr>
<tr>
<td>ERBB4</td>
<td>v-erb-a erythroblastic leukemia viral oncogene homolog 4</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamate decarboxylase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GE</td>
<td>ganglionic eminence</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>Gl</td>
<td>glomerular layer</td>
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<td>Glc</td>
<td>glucose</td>
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HEPES  2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HGF  hepatocyte growth factor
IgG  immunoglobulin
IL  infralimbic cortex
i.p.  intraperitoneally
IZ  intermediate zone
LGE  lateral ganglionic eminence
LIS1  Lissencephaly-1
LTP  long term potentiation
mAb  monoclonal antibody
MGE  ganglionic eminence
mRNA  messenger RNA
mPFC  medial prefrontal cortex
NBT  nitro blue tetrazolium chloride
NCAM  neural cell adhesion molecule
NF  neurofilament
NMDA  N-methyl-D-aspartate
NRG1  neuregulin 1
NT4  neurotrophin 4
OB  olfactory bulb
P  postnatal day
pAb  polyclonal antibody
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PDGF  platelet-derived growth factor
PFA  paraformaldehyde
PFC  prefrontal cortex
PNN  perineuronal net
polySia  polysialic acid
polyST  polysialyltransferase
PPI  prepulse inhibition
PrL  prelimbic cortex
PV  parvalbumin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>RMS</td>
<td>rostral migratory stream</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>Rt</td>
<td>reticular thalamic nucleus</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SEMA</td>
<td>semaphorin</td>
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<tr>
<td>Sst</td>
<td>somatostatin</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>SynCAM</td>
<td>synaptic cell adhesion molecule</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TrkB</td>
<td>tyrosine-related kinase B</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick end labeling</td>
</tr>
<tr>
<td>u-PAR</td>
<td>urokinase-type plasminogen activator receptor</td>
</tr>
<tr>
<td>VGLUT</td>
<td>vesicular glutamate transporter</td>
</tr>
<tr>
<td>WFA</td>
<td><em>Wisteria floribunda</em> agglutinin</td>
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Abstract - Role of polysialic acid in the genesis of GABAergic neurons of the cerebral cortex and for thalamocortical connectivity

Posttranslational addition of polysialic acid (polySia) by the polysialyltransferases (polySTs) ST8SIA2 and ST8SIA4 is a unique and prominent modification of the neural cell adhesion molecule NCAM and a major determinant of nervous system development. Dysregulation of this system has been linked to schizophrenia and mice with aberrant polysialylation or NCAM expression show neuroanatomical defects reminiscent to observations in patients. The present work consists of three parts dealing with (i) the impact of polySia on selected interneuron populations in brain regions relevant to the pathophysiology of schizophrenia, (ii) the role of polySia for the migration of cortical interneurons and (iii) the development of a schizophrenia-like phenotype in polySia-deficient mice.

In the first part, interneuron densities were analyzed in the medial prefrontal cortex (PFC), the hippocampus and the olfactory bulb of mice with differently combined Ncam1 and polyST deletions. Pronounced reductions of specifically the perineuronal net- and parvalbumin (PV)-positive, calbindin-negative and the somatostatin (Sst)-positive interneurons were detected in the medial PFC of all NCAM- or polySia-deficient lines, whereas other types of interneurons were unaffected. Disturbed patterns of tangentially migrating cortical interneuron precursors were observed in polyST-negative lines indicating that migration deficits may cause these alterations. Corresponding to the well-described migration deficits of olfactory interneuron precursors, reductions of calbindin-positive cells were found in the olfactory bulb of all polySia-deficient lines. In contrast, PV-positive cell densities were increased in the hippocampus. Together, these findings demonstrate that attenuation of NCAM-bound polySia causes pathological changes of specific interneuron subtypes.

The second part deals with the mechanisms leading to reduced densities of PV- and Sst-positive interneurons in the PFC. The assumed loss of interneurons was corroborated by analyzing polyST-negative mice with genetically labeled interneurons. In ST8SIA2- or ST8SIA4-deficient mouse embryos, reduced levels of polySia were detected in the area of interneuron migration from the medial ganglionic eminence (MGE) into the cortex. Time lapse recordings revealed significantly reduced velocities in slice cultures that have been treated with endosialidase to remove polySia. Compromised migration of interneurons from
ST8SIA2-deficient MGE explants into co-cultured wildtype pallium indicated that levels of polySia on the migrating interneurons themselves are critical. Furthermore shorter leading processes of migratory interneurons were observed after enzymatic removal of polySia in slices. The same effect was observed in primary cultures of isolated interneurons suggesting a cell-autonomous mechanism of polySia-dependent interneuron migration. Thus, reduced polySia levels impede the migration of cortical interneurons. This provides a possible link between genetic variation of polyST genes, neurodevelopmental alterations and interneuron dysfunction as observed in neuropsychiatric disease.

In the third part of my thesis, mice deficient for ST8SIA2 (St8sia2−/−) or ST8SIA4 (St8sia4−/−) were assessed neuroanatomically and, in a collaborative effort with the laboratory of A. Zharkovsky (Univ. Tartu, Estonia), subjected to tests of cognition and sensory functions. St8sia2−/− but not St8sia4−/− mice displayed enlarged lateral ventricles, a size reduction of the thalamus accompanied by a smaller internal capsule and a highly disorganized pattern of fibers connecting thalamus and cortex. Reduced levels of the vesicular glutamate transporter VGLUT2 were detected in St8sia2−/− mice, implicating a compromised glutamatergic thalamocortical input into the frontal cortex. Recognition memory was impaired in St8sia2−/− and in St8sia4−/− mice, but only St8sia2−/− mice displayed impaired working memory, deficits in prepulse inhibition, which could be attenuated by clozapine treatment, as well as anhedonic behavior and increased sensitivity to amphetamine-induced hyperlocomotion. These data indicate that reduced polysialylation in St8sia2−/− mice causes deficits in thalamocortical connectivity and a schizophrenia-like behavior. We therefore propose that ST8SIA2 deficiency has the potential to cause a neurodevelopmental predisposition to schizophrenia.
**Zusammenfassung - Die Rolle von Polysialinsäure in der Entstehung von GABAergen Neuronen der Großhirnrinde und für die thalamokortikale Konnektivität**


Der zweite Teil beschäftigt sich mit möglichen Mechanismen, die zu reduzierten Zelldichten von Parvalbumin- und Somatostatin-positiven Interneuronen im PFC polySia-defizienter Mäuse führen könnten. Durch Analyse von PolyST-negativen Mäusen mit genetisch

1 - General Introduction

1.1 NCAM and its posttranslational modification with polysialic acid

Cell adhesion molecules play important roles in interactions of cells with other cells or matrix. A prominent example for this group of proteins is the neural cell adhesion molecule (NCAM) (Edelman, 1987; Rutishauser et al., 1988). Genes for NCAM (NCAM1 in humans and Ncam1 in mice) encode 25 exons (Murray et al., 1986; Cunningham et al., 1987; Walsh and Dickson, 1989) that could theoretically lead to a great variety of different transcripts. In the brain, three major isoforms are expressed (Fig. 1). According to their apparent molecular weight, these are termed NCAM-180, NCAM-140 and NCAM-120. The extracellular part of all isoforms consists of five immunoglobulin (IgG)-like and two fibronectin type three domains. While NCAM-120 is linked to the cell membrane via a glycophasphatidylinositol anchor (He et al., 1986, 1987), NCAM-140 and NCAM-180 are transmembrane proteins with a short and a long cytosolic domain (Gennarini et al., 1984; Murray et al., 1986). Other splice variants have been reported to result from expression of special exons: MSD1a-c (muscle specific domain), the triplet AAG, and π (VASE in humans) (Ronn et al., 1998). A secreted NCAM isoform is generated when utilizing the SEC-exon, resulting in a truncated form of the extracellular part of the protein (Bock et al., 1987; Gower et al., 1988). Moreover, release of the NCAM extracellular region (NCAM-EC) was observed after ectodomain shedding from membrane bound isoforms by a disintegrin and metalloprotease (ADAM) family metalloprotease (Vawter et al., 2001; Hinkle et al., 2006; Kalus et al., 2006; Brennaman and Maness, 2008).
Fig. 1 Major NCAM isoforms and their modification with polysialic acid. NCAM-120, NCAM-140 and NCAM-180 are named according to their apparent molecular weight. Further variation by alternatively spliced exons can occur as exemplified for NCAM-140. All isoforms can be modified with polysialic acid (polySia) at N-glycosylation sites in the fifth IgG-like domain which leads to an increase of the hydrodynamic radius of the protein (see text for details). GPI, glycophasphatidyl-inositol

Posttranslational modification can occur on all NCAM isoforms. Studies reported on phosphorylation and palmitoylation of the cytoplasmic domain (Sorkin et al., 1984; Little et al., 1998; Niethammer et al., 2002; Ponimaskin et al., 2008) and on glycosylation of extracellular parts of the protein (Geyer et al., 2001; Liedtke et al., 2001; von der Ohe et al., 2002). The IgG-like domains contain six N-glycosylation sites (Albach et al., 2004) that can be modified with variable glycans, leading to high structural diversity. The most prominent modification of NCAM is the glycosylation with polysialic acid (polySia) (Fig. 1). This unique glycan structure comprises polymers of sialic acids, i.e. derivates of 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 or 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid). Over 50 naturally occurring derivatives of sialic acid have been identified so far (Angata and Varki, 2002). On NCAM, polySia is composed of up to 90 α2,8-glycosidically linked N-acetylneuraminic acid (Neu5Ac) residues (Inoue et al., 2000; Galuska et al., 2006; Galuska et al., 2008) that are attached to a highly variable, di-, tri-, or tetranatennary core glycan at the 5th and 6th N-glycosylation site in the fifth IgG-like domain (Nelson et al., 1995; Liedtke et al.,
Polysialylation is accomplished by two Golgi-associated polysialyltransferases (polySTs), ST8SIA2 (also named ST8Siall or STX) and ST8SIA4 (also named ST8SialIV or PST) (Eckhardt et al., 1995; Nakayama et al., 1995; Kojima et al., 1995; Scheidegger et al., 1995), that can act independently from each other. Both enzymes show a high sequence homology and are members of the mammalian sialyltransferase family with type II transmembrane topology, a short N-terminal cytoplasmic tail and a stem region (Harduin-Lepers et al., 2001, 2005). The catalytic domain is situated in the Golgi lumen and contains the sialylmotifs L, S, and VS that are conserved in all mammalian sialyltransferases and are involved in substrate binding (Datta and Paulson, 1995; Datta et al., 1998). Moreover polySTs contain two additional structurally unique polybasic motifs, the polysialyltransferase domain in the catalytic domain (Nakata et al., 2006) and the polybasic region in the stem region (Foley et al., 2009; Zapater and Colley, 2012).

In the perinatal brain, NCAM-180 and NCAM-140 are polysialylated, while NCAM-120 is devoid of any polySia \textit{in vivo} (Seki and Arai, 1991a; Probstmeier et al., 1994; Oltmann-Norden et al., 2008). Besides NCAM, other polySia carriers have been identified. In the rat brain, Zuber et al. (1992) demonstrated polysialylation of sodium channel alpha subunits. In human milk, CD36 is a target of this modification (Yabe et al., 2003). Also the polySTs themselves have been identified as polySia carriers (Mühlenhoff et al., 1996; Close and Colley, 1998; Close et al., 2000, 2001). More recently, it has been shown that neuropilin-2 on human dendritic cells (Curreli et al., 2007; Rey-Gallardo et al., 2010; Rey-Gallardo et al., 2011) and the synaptic cell adhesion molecule SynCAM 1 in early postnatal mouse brain (Galuska et al., 2010) can be modified by polySia.

1.2 Expression of polySia during brain development

In the mouse brain, expression of both polySTs starts at embryonic day (E) 8.5 and polySia can be detected from E9 onwards (Probstmeier et al., 1994; Ong et al., 1998). Together with an increase of NCAM expression, levels of mRNA of both enzymes rise after E10.5 and reach a plateau before E14.5 that is maintained until birth (Schiff et al., 2009). In the perinatal phase the entire pool of NCAM is polysialylated (Galuska et al., 2006). While the expression of NCAM stays fairly constant during the first three weeks of postnatal development, mRNA levels of both polySTs and, as a consequence the levels of polySia are dropping rapidly resulting in the appearance of polySia-free NCAM (Oltmann-Norden et al., 2008).
Most neurons are polySia-positive at some stage of their differentiation (Bonfanti, 2006). Thus, polySia has been detected on radial glia (Bartsch et al., 1990; Hekmat et al., 1990; Li et al., 2004; Schiff et al., 2009) and on developing axons, e.g. in the corticospinal tract and thalamocortical fibers (Daston et al., 1996; Schiff et al., 2011). In the postnatal brain, when overall levels of polySia are reduced, expression is restricted to sites of ongoing neurogenesis and plasticity. Prominent examples are neuroblasts from neurogenic niches of the anterior subventricular zone (SVZ) that migrate to the olfactory bulbs to constantly replace periglomerular and granule interneurons (Bonfanti and Theodosis, 1994; Rousselot and Nottebohm, 1995; Kornack and Rakic, 2001; Ponti et al., 2006). In the hippocampus, granular cell precursors of the dentate gyrus have been reported to be positive for polySia (Seki and Arai, 1991b, 1993). Besides precursor cells, also few mature neurons express polySia in the prefrontal cortex, the piriform cortex, the hippocampus, and the amygdala (Varea et al., 2005, 2007; Nacher et al., 2002a, b; Gilabert-Juan et al., 2011). These cells are most likely interneurons (Gomez-Climent et al., 2011).

PolySia is also implicated in synapse formation und GABAergic maturation (Dityatev et al., 2000, 2004). In the visual cortex of mice, polySia is involved in the functional maturation of GABAergic inhibition during the so-called critical period of plasticity (Di Cristo et al., 2007), that is the activity-dependent development of neuronal circuits, triggered by the functional maturation of inhibitory synapses at a time when synaptic connections ultimately consolidate into their final wiring patterns (reviewed by Hensch, 2005). In the mouse visual cortex, this period starts about one week after eye opening at postnatal day 13 and peaks one month after birth. In the visual cortex, but also in the prefrontal cortex, polySia levels decline before the third postnatal week, preceded by down regulation of ST8SIA2 and ST8SIA4 expression (Di Cristo et al., 2007; Brenneman and Maness, 2008; Bélanger and Di Cristo, 2011).

### 1.3 PolySia-deficient mouse models

To examine the roles of polySia and NCAM for brain development, various mouse models have been established. Regarding the numerous developmental functions NCAM is accredited to, mice deficient for all NCAM isoforms (Ncam1−/−, Cremer et al., 1994) or with a NCAM-180 specific exon deletion (Tomasiewicz et al., 1993) display a surprisingly mild phenotype. They are viable, fertile and show an overall normal brain morphology. Nevertheless, two prominent neuroanatomical defects have been reported for those animals (reviewed by Hildebrandt et al., 2007). These are a size reduction of the olfactory bulbs,
caused by migration deficit of SVZ-derived interneuron precursors and a defective lamination of mossy fibers projecting from the hippocampal dentate gyrus to the CA3 subfield of Ammon’s horn. Since both defects can be phenocopied by enzymatic removal of polySia, they are considered to be polySia-dependent (Ono et al., 1994; Seki and Rutishauser, 1998).

So far, no neuroanatomical defects have been detected in mice deficient for ST8SIA4 (St8sia4−/−, Eckhardt et al., 2000). This is consistent with the more prominent impact of ST8SIA2 on polySia synthesis during brain development and the observations that ST8SIA4 is the predominant enzyme in the adult brain (Hildebrandt et al., 1998; Ong et al., 1998; Galuska et al., 2006; Oltmann-Norden et al., 2008; Schiff et al., 2009; Nacher et al., 2010). In contrast, mice deficient for ST8SIA2 (St8sia2−/−, Angata et al., 2004) show a malformation of the hippocampal mossy fiber tract, reminiscent to the phenotype of Ncam1−/− animals. Interestingly, both polySTs can compensate for each other. During the perinatal phase, polySia synthesis in ST8SIA4-deficient mice is completely counterbalanced by ST8SIA2. Reversely, more than 50% of the available NCAM are still fully polysialylated in St8sia2−/− mice (Galuska et al., 2006; Oltmann-Norden et al., 2008). This was, however, not due to a compensatory increase in transcription of the second polyST gene (Galuska et al., 2006) but is possibly based on the limited availability of NCAM as the major acceptor. Whether a similar compensation also takes place at earlier developmental time points remains to be resolved.

By combining single polyST deletion strains, mice deficient for both polySTs (St8sia2−/− St8sia4−/−) were generated. Those animals lack any polySia and displayed a much more severe phenotype than Ncam1−/−, St8sia4−/−, or St8sia2−/− single knock-out mice (Weinhold et al., 2005; Angata et al., 2007; Hildebrandt et al., 2009). Comparative analysis of St8sia2−/− St8sia4−/− mice and Ncam1−/− mice revealed two types of defects, (i) those that develop in absence of polySia independent of the presence of NCAM and (ii) those that appear only in St8sia2−/− St8sia4−/− mice, caused by appearance of polySia-free NCAM (Weinhold et al., 2005). Defects of the first category include smaller olfactory bulbs, caused by defective migration of SVZ precursors, and delamination of hippocampal mossy fibers. Defects of the second category comprise postnatal growth retardation, precocious death, high incidence of progressive hydrocephalus and anomalies of major brain fiber tracts, like agenesis of the anterior commissure, hypoplasia of the mammillothalamic tract and the internal capsule, the major gateway of fibers from and to the cerebral cortex. Importantly, all defects of the second
category could be rescued by additional deletion of NCAM in $\text{St8sia}^2$ $\text{St8sia}^4$ $\text{Ncam}^1$ mice (Weinhold et al., 2005) and the severity of the axon tract deficits correlated with the amount of polySia-free NCAM untimely expressed during brain development (Hildebrandt et al., 2009).

1.4 The function of polySia

Studies using polySia- or NCAM-deficient mice as well as in vitro techniques demonstrated several functions of polySia and different models of action have been proposed (reviewed by Hildebrandt et al., 2007; Rutishauser, 2008). Due to its unusual physical properties, forming a long, negatively charged glycopolymer, polySia leads to steric inhibition of cell-cell contacts (Rutishauser and Landmesser, 1996). NCAM-mediated cell adhesion and other adhesive cell surface interactions can be regulated by the appearance of polySia. In this way, polySia may prevent inappropriate cell contacts, explaining the impact of polySia on fasciculation and neurite outgrowth. The developmental defects in $\text{St8sia}^2$ $\text{St8sia}^4$ mice that establish due to a gain of NCAM function further demonstrate that polySia is needed to shield NCAM and is implicated in the organization and timing of NCAM interactions.

Besides its role in cell adhesion, polySia is also involved in the regulation of NCAM signaling. A well established model of NCAM signaling involves association of NCAM with fibroblast growth factor (FGF) receptors and their activation, leading to downstream signaling through the mitogen-activated protein kinase ERK1/2 pathway (Kolkova et al., 2000; Cavallaro et al., 2001; Niethammer et al., 2002; Hinsby et al., 2004; Francavilla et al., 2009). NCAM interactions are hereby heterophilic and do therefore not depend on homophilic NCAM binding. PolySia-regulated and ERK1/2-dependent heterophilic NCAM signaling was demonstrated in neuroblastoma cells and SVZ-derived interneuron precursors, leading to differentiation, growth arrest and increased survival (Seidenfaden et al., 2003, 2006; Röckle et al., 2008). These findings meet in vivo evidence that removal of polySia by endosialidase promotes neuronal differentiation of postnatally generated olfactory bulb interneurons (Petridis et al., 2004) and dentate granule cells (Burgess et al., 2008). Increased NCAM-mediated signaling upon loss of polySia was also observed in tumor cells, resulting in reduced motility and enhanced focal adhesion at the cell-substrate interface (Eggers et al., 2011). In this case, signaling was independent from FGF receptor and ERK1/2 activation but involved association of the src-family kinase p59$^{fyn}$ with paxilin.
Another potential polySia-specific function is the sensing of growth factors. Loss of polySia was shown to promote platelet-derived growth factor (PDGF)-induced differentiation of glial cells (Angata et al., 2007) and maturation of oligodendrocyte preprogenitors (Decker et al., 2000, 2002). Furthermore, hippocampal and hypothalamic neurons are more sensitive for brain-derived neurotrophic factor (BDNF) or ciliary-derived neurotrophic factor in the presence of polySia (Muller et al., 2000; Vutskits et al., 2001, 2003). Similarly, PDGF-induced chemotaxis of oligodendrocyte precursors is reduced in the absence of polySia (Zhang et al., 2004; Glaser et al., 2007). In addition, direct binding of BDNF and FGF 2 to polySia has been suggested, while binding of BDNF depends on the chain length of polySia (Kanato et al., 2008; Ono et al., 2012).

### 1.5 Interneurons

Two main classes of neurons are situated in the cerebral cortex. Pyramidal cells are in general excitatory and use glutamate as main neurotransmitter. Locally projecting inhibitory interneurons instead utilize γ-aminobutyric acid (GABA) and comprise 20 to 30 percent of the cortical neurons. All GABAergic cells express glutamic acid decarboxylase (GAD) (Tamamaki et al., 2003), the key enzyme in GABA-synthesis that occurs in two different forms according to the apparent molecular weight of 67 kDa (GAD67) and 65 kDa (GAD65), resulting from distinct genes in humans (GAD1 and GAD2) and mice (Gad1 and Gad2), respectively. Distinct types of interneurons are defined by their neurochemical, anatomical and electrophysiological characteristics and several classes of interneurons have been identified (reviewed by Markram et al., 2004; Ascoli et al., 2008; Corbin and Butt, 2011). In the mouse, well-defined interneuron types include (i) fast spiking parvalbumin-expressing basket and chandelier cells, (ii) somatostatin-containing cells with intrinsic burst spiking or adapting non-fast-spiking properties, (iii) calretinin- and / or vasointestinal peptide-immunoreactive interneurons that are rapidly adapting and bipolar- or double-bouquet-like, and (iv) rapidly adapting cells with multipolar morphologies expressing neuropeptide Y and / or reelin, but not somatostatin.

Interneurons synchronize the output of pyramidal neurons in dependence of the subcellular localization of their input. In the example of the primary somatosensory cortex, a typical flow of information within a cortical column originates at thalamocortical synapses onto neurons in mid-layers, predominantly layer 4. Neurons transmit signals by excitatory forward connections to more superficial layers before they are relayed to deep layers and onward-
transmitted to other areas of the cortex or subcortical regions (Thomson and Bannister, 2003). Interneurons modulate activity of projecting pyramidal cells at all levels of this column circuitry by receiving excitatory input, not only from the respective cortical column, but also other regions. For example, layer 4 interneurons of the primary somatosensory cortex are targeted directly by the thalamus (Gibson et al., 1999; Porter et al., 2001). In general, interneurons participate in column-specific feed-forward inhibition, as shown for fast spiking cells in layer 4, in feed-back inhibition, as in the case of Martinotti cells (Silberberg et al., 2002; Wang et al., 2004; Berger et al., 2010), but also in lateral inhibition of other cortical columns, as shown for wide arbor basket cells or Martinotti cells (reviewed by Corbin and Butt, 2011). Targets of inhibitory contacts are often restricted to specific parts of pyramidal cells. Parvalbumin-positive basket cells synapse on soma and proximal dendrites and somatostatin-positive Martinotti cells often target apical dendrites (Corbin and Butt, 2011).

Interneurons originate in the subpallial SVZ. During embryonic development, ganglionic eminences (GEs) emerge shortly after neural tube closure at around E11.5 and are subdivided into three distinct domains according to their relative position: medial (MGE), lateral (LGE) and caudal (CGE) ganglionic eminence (Fig. 2). The MGE is the source for cortical parvalbumin- and somatostatin-positive interneurons, whereas the CGE gives rise to a distinct subset of interneurons expressing combinations of reelin, calretinin and vasointestinal peptide (reviewed by Gelman and Marin, 2010). The LGE is the source of striatal GABAergic projection neurons as well as of olfactory bulb and amygdala interneurons (Kohwi et al., 2007; Young et al., 2007; Cocas et al., 2011). At least 20 different subtypes of interneurons originate in the ganglionic eminences (Parra et al., 1998; Klausberger and Somogyi, 2008) and their genesis and postmitotic differentiation depends on the expression of homeodomain transcription factors, for example Nkx2.1, Lhx6, Gsh2, and Dlx1/2 and bHLH transcription factors, like Olig1/2 or Mash1 (Flames et al., 2007). Spatially restricted expression of these and other factors further subdivides the ganglionic eminences. For example, Lhx6 and Nkx2.1 are expressed throughout the whole MGE, but the dorsal part, giving rise to parvalbumin-positive interneurons, also expresses transcriptional activator Gli1 and Nk family member Nkx6.2 (Corbin et al., 2008; Welagen and Anderson, 2011). In the ventral MGE that lacks expression of these additional factors, somatostatin-positive cells originate (Fogarty et al., 2007; Wonders et al., 2008). Another origin of different types of interneurons is the preoptic area (Gelman et al., 2009), but it still remains unclear whether this structure gives rise to distinct and specialized classes of cells.
Fig. 2 Tangential migration of interneurons from the ganglionic eminences to the cortex. (a) Schematic illustration of an E13.5 mouse brain, sagittal view. Cereb, cerebellum; Dienceph, diencephalon; Telenceph, telencephalon. (b) Schematic coronal sections at indicated positions in (a). Medial (MGE), lateral (LGE) and caudal (CGE) ganglionic eminences give rise to distinct populations of interneurons. Migration into the cortex (ctx) is influenced by chemoattractants and motogenic factors (+), as well as by chemorepellents (-). See text for details. str, striatum; PV, parvalbumin; Sst, somatostatin; VIP, vasointestinal peptide; NPY, neuropeptide Y; CR, calretinin. Figure based on Marín and Rubenstein (2003).

Migration of interneurons from their origin of genesis to the respective brain region has been well described for the generation of olfactory bulb interneurons. Precursors derived from the embryonic LGE and its remnants in the postnatal brain, the anterior SVZ of the lateral ventricle, migrate tangentially in the rostral migratory stream (RMS) that emerges around mid-gestation and persists throughout adulthood (Alvarez-Buylla et al., 2000). In this way, these precursors provide a constant source for periglomerular and granule interneurons of the olfactory bulbs in mice and possibly also in humans (Curtis et al., 2007). Although different in detail, cortical interneurons also undergo a tangential migration. During brain development, interneuron precursors enter the pallium on two migratory routes. An early stream of cells invades the preplate in the marginal zone at E12. A second, more prominent cohort of cells enters the intermediate zone at E13. At later stages of cortical development
and after development of the cortical plate, the first migratory stream splits and cells migrate in the marginal zone and the subplate (reviewed by Métin et al., 2006). Migration of interneurons is greatly influenced by external guidance cues (Fig. 2). Interneurons sense GABA and glutamate via GABA<sub>A</sub> and AMPA/NMDA receptors while migrating into the cortex (reviewed by Luján et al., 2005). Guidance cues also include repulsive factors like semaphorins (SEMA3A and SEMA3F) in the striatum (reviewed by Marín and Rubenstein, 2003) and attractors like neuregulin 1 (NRG1) in the cortex (Flames et al., 2004). Furthermore, motogenic factors like hepatocyte growth factor (HGF) (Powell et al., 2001), BDNF and neurotrophin 4 (NT4) (Polleux et al., 2002) influence the migration of cortical interneurons. Migratory behavior can also be regulated by intrinsic programs, involving cytoplasmic calcium- and cAMP-levels, as shown for cerebellar granule cells that exhibit spontaneous and periodical turning without cell–cell contact and in the absence of external guidance cues (Kumada et al., 2009).

The mode of interneuron migration from the embryonic MGE differs from the persistent migration of olfactory bulb interneurons in the postnatal RMS (Marín and Rubenstein, 2003). Cortical interneurons disperse and migrate rather individually, independent from specific cellular substrates, whereas postnatal olfactory bulb interneurons migrate in chains of cells that move in close contact with each other. During embryonic development, migration of olfactory bulb interneurons is also independent from cellular contacts.
1.6 PolySia and schizophrenia

Schizophrenia is a devastating neuropsychiatric disorder affecting about 1% of the population. It is characterized by positive symptoms, such as delusions and hallucinations, as well as by negative symptoms, including apathy and social withdrawal. Schizophrenia also includes cognitive symptoms, like deficits in attention and working memory. Several models of schizophrenia are currently discussed. Since the risk of developing this disorder is directly associated with the degree of biological relatedness to an affected individual, one model assumes that genetic risk factors contribute to the susceptibility of developing this disorder (reviewed by Lewis and Levitt, 2002). Another paradigm, the neurodevelopmental model of schizophrenia, states that the illness is the end state of series of defective neurodevelopmental processes, starting years before the onset of the symptoms (reviewed by Rapoport et al., 2012).

There are several links of aberrant NCAM expression and altered polysialylation to schizophrenia. Studies reported on elevated levels of soluble NCAM in the prefrontal cortex, hippocampus, and cerebrospinal fluid of schizophrenic patients that correlate with the severity and duration of the disease (Poltorak et al., 1995; van Kammen et al., 1998; Vawter, 2000; Vawter et al., 2001). In addition, reduced polySia levels have been demonstrated in the hilus region of the hippocampus (Barbeau et al., 1995) and, most recently, in layers 4 and 5 of the dorsolateral prefrontal cortex of schizophrenics (Gilabert-Juan et al., 2012). Genes for NCAM and both polySTs (NCAM1, ST8SIA2 and ST8SIA4) map to chromosomal regions that harbor susceptibility loci for schizophrenia (11q23.1, 15q26, and 5q21 for NCAM1, ST8SIA2, and ST8SIA4) (Lewis et al., 2003; Lindholm et al., 2004; Maziaide et al., 2005). Furthermore, single nucleotide polymorphisms in NCAM1 and ST8SIA2, as well as a risk haplotype within ST8SIA2 have been linked to schizophrenia (Arai et al., 2006; Atz et al., 2007; Sullivan et al., 2007; Tao et al., 2007; McAuley et al., 2012). Similarly, associations with autism spectrum disorders and bipolar disorders have been reported (Anney et al., 2010; McAuley et al., 2012) indicating that variation in the ST8SIA2 gene is linked to increased risk to mental illness.

Some of the clinical parameters in schizophrenia match observed deficits in NCAM- and polySia-deficient mice. Ventricular enlargement has been reported in humans (Shenton et al., 2001) and in mice, deficient for the specific NCAM-180 isoform (Wood et al., 1998). In St8sia2+/− St8sia4+/− mice, ventricular dilatations and as well as a progressive hydrocephalus
were observed (Weinhold et al., 2005). Decreased sizes of the corpus callosum and internal capsule in humans (Innocenti et al., 2003; Hulshoff Pol et al., 2004; Douaud et al., 2007; Mitelman et al., 2007; Begré and Koenig, 2008) are reminiscent to fiber tract deficits in polySia-compromised mice (Hildebrandt et al., 2009; Schiff et al., 2011). Reduced sizes of olfactory bulbs were reported in schizophrenic patients (Turetsky et al., 2000), as well as in Ncam1<sup>−/−</sup> (Cremer et al., 1994) and St8sia2<sup>−/−</sup> St8sia4<sup>−/−</sup> mice (Weinhold et al., 2005). There are also similarities between cognitive impairments in patients (Heinrichs and Zakzanis, 1998) and deficits in learning, memory formation and long term potentiation in Ncam1<sup>−/−</sup> and St8sia4<sup>−/−</sup> mice (Cremer et al., 1994; Cremer et al., 1998; Eckhardt et al., 2000; Bukalo et al., 2004; Senkov et al., 2006).

Dysfunction in schizophrenia involves alterations of interneurons (reviewed by Benes and Berretta, 2001; Reynolds et al., 2001; Eyles et al., 2002; Lewis et al., 2005; Lewis and Sweet, 2009; Marín, 2012). Although results from different studies are conflicting, reduced densities of parvalbumin-positive cells, as well as reduced mRNA levels of parvalbumin and somatostatin have been described in the prefrontal cortex of schizophrenic patients (Beasley et al., 2002; Reynolds et al., 2002; Hashimoto et al., 2003; Morris et al., 2008; Fung et al., 2010).
1.7 Objectives

PolySia and NCAM play important roles during nervous system development and seem to be implicated in the pathophysiology of schizophrenia. Some of the defects observed in mice with aberrant polysialylation are reminiscent to the neuroanatomical defects in patients. Since polySia and NCAM are involved in the migration of olfactory bulb interneuron precursors and alterations of GABAergic interneurons are frequently observed in schizophrenia, one important question is, if any of the major interneuron populations is affected by altered NCAM polysialylation. The first study of my thesis addressed this question by evaluating selected interneuron populations of the olfactory bulb, prefrontal cortex and hippocampus in mouse models with impaired polysialylation or NCAM deficiency.

As mentioned above, polySia plays an important role for the migration of olfactory bulb interneuron precursors and the results from the first study presented in this thesis indicated that tangential migration of a specific population of MGE-derived cortical interneurons may be compromised in polySia-deficient mouse embryos, which could lead to altered interneuron densities in the adult. Therefore, the second study of this thesis addressed the question, if cortical interneuron migration directly depends on polySia and investigated mechanisms that may cause defective precursor migration. PolyST-deficient mice with genetically labeled interneurons were used to assess a possible loss of interneurons in the prefrontal cortex of 3-month-old mice and different in vitro culture techniques of embryonic brains were applied to evaluate changes of interneuron migration upon polySia deficiency in vitro.

Aberrant polysialylation in mice leads to neuroanatomical defects reminiscent to clinical parameters of schizophrenia. The third study addressed the question whether polyST deficiency might cause a schizophrenia-like phenotype in mice. For this purpose, St8sia2<sup>−/−</sup> mice, St8sia4<sup>−/−</sup> mice and their wildtype littermates were assessed neuroanatomically and subjected to tests of cognition and sensory functions.
2 - Results

Chapter 1 - Altered densities and compromised migration of interneurons in the forebrain of mice deficient for polysialic acid or NCAM

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Preface - About the manuscript

In the first study of this thesis, densities of selected interneuron populations were analyzed in the medial prefrontal cortex, the hippocampus and the olfactory bulb of mice with differently combined \textit{Ncam1} and polyST deletions. Furthermore, a potential impact of polySia-deficiency on developmental interneuron migration was assessed.

My contributions to this manuscript comprised the organization of breeding, dissection and analysis of 3-month-old and embryonic mice, including immunofluorescent stainings, microscopy and respective evaluations. Prof. H. Hildebrandt, Dr. I. Röckle and I designed the experiments and wrote the manuscript.
Abstract

The neural cell adhesion molecule NCAM and its modification with polysialic acid (polySia) are major determinants of brain development and dysregulation of this system has been linked to schizophrenia. Here, we analyzed how loss of polySia in mice affects selected interneuron populations in brain regions relevant to the pathophysiology of schizophrenia. A panel of mouse lines with differently combined \textit{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. Pronounced reductions of perineuronal net- and parvalbumin-positive, calbindin-negative cells and of somatostatin-positive cells were detected in the medial prefrontal cortex of all NCAM- or polySia-deficient lines, while calbindin- and calretinin-positive cells were unaffected. Searching for mechanisms that may cause these alterations disturbed tangential migration of cortical interneuron precursors was observed in polysialyltransferase-deficient lines. Similarly, the well-known migration deficits of olfactory interneuron precursors coincide with reductions of calbindin-positive cells in the olfactory bulb of polySia-deficient mice. In the hippocampus, however, the different lines displayed disparate patterns of increased parvalbumin-positive cell densities. Together, these findings demonstrate that attenuation of NCAM-bound polySia interferes with interneuron precursor migration and causes pathological changes of specific interneuron subtypes.
Introduction

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 (polySTs) ST8SIA2 and ST8SIA4. Together with polyST 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 that lack 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 ST8SIA2 (2−/−) or ST8SIA4 (4−/−) (Eckhardt et al., 2000; Angata et al., 2004). In contrast, simultaneous ablation of the two polySTs ST8SIA2 and ST8SIA4 (2−/−4−/−) 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 2−/−4−/− 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 2−/−4−/−N−/− triple knockout mice (Weinhold et al., 2005). Moreover, the axon tract 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 polyST- and the NCAM-depleted mice. This includes a size reduction of the olfactory bulb (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 2−4− 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 polyST genes map to chromosomal regions that harbor 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 2−4− mice (Wood et al., 1998; Weinhold et al., 2005). In addition, a decreased size of the corpus callosum and the internal capsule has been reported in schizophrenic patients (Innocenti et al., 2003; Hulshoff Pol et al., 2004; Douaud et al., 2007; Mitelman et al., 2007; Begré and Koenig, 2008). This correlates with the fiber tract deficits observed in polysialylation compromised mice (Hildebrandt et al., 2009; Schiff et al., 2011). A further remarkable similarity is the reduced size of the OB both in patients with schizophrenia (Turetsky et al., 2000) and N− or 2−4− mice (Cremer et al., 1994; Weinhold et al., 2005). Reminiscent to cognitive impairment in schizophrenia (Heinrichs and Zakzanis, 1998), N− as well as polyST-deficient 4− 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 St8sia2, St8sia4 and Ncam1 single-, double-, and triple-knockout lines. Densities of major interneuron subtypes in the medial PFC (mPFC), hippocampus and OB were assessed by immunofluorescence staining of PV, CB, and CR, as well as somatostatin (Sst) for the mPFC, or tyrosine hydroxylase (TH) for the OB (Kosaka et al., 1995; DeFelipe, 1997; Matyas et al., 2004; Xu et al., 2006; Kohwi et al., 2007). The results indicate that interference with NCAM-based polySia leads to reduced densities of PV- and Sst-positive interneuron populations in different subregions and layers of the mPFC. Moreover, CB-positive cells were consistently reduced in the glomerular layer of the OB, while PV-positive cells in the CA1/2 region of the hippocampus were variably increased in the different polySia-deficient lines. A developmental analysis in 2/− and 4/− mice revealed an aberrant pattern of interneuron precursors migrating from the ganglionic eminence to the dorsal telencephalon. Highly reminiscent of the well-known migration deficit of OB interneuron precursors these findings suggest that disturbed tangential migration accounts at least in part for the observed interneuron phenotype in polySia-deficient mice.
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. St8sia2, St8sia4 and Ncam1 single knockout strains, which have been backcrossed with C57BL/6J mice for six generations, were intercrossed to obtain double knockout (St8sia2^-/-, St8sia4^-/-, 2^-/-) or triple knockout (St8sia2^-/- St8sia4^-/- Ncam1^-/-; 2^-/- 4^-/- N^-/-) animals (Weinhold et al., 2005). Genotyping was performed by PCR as previously described (Weinhold et al., 2005). For staging of embryos, the morning of the vaginal plug was considered as embryonic day (E) 0.5.

Sectioning

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. One- or three-month-old animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After dissection, the brains were postfixed overnight. Embryonic brains were directly fixed by immersion of the head in 4% paraformaldehyde. 50-µm-thick coronal sections were obtained with a vibrating microtome (Leica Microsystems, Wetzlar, Germany). At least three mice were analyzed for each genotype and stage (E13.5, one and three months). As St8sia2^+/+ St8sia4^+/+ (2^+/+ 4^+/+) animals were indistinguishable from wildtype animals, one 2^-/+ 4^-/+ mouse was included into the one-month-old control group. To ensure precise staging of embryonic mice, St8sia4^+/+ (4^+/+) and St8sia2^+/+ (2^+/+) were analyzed in comparison to heterozygous littermates (4^-/+ and 2^-/+). For 2^-/+ 4^-/+ mice, which have a high incidence of hydrocephalus (Weinhold et al., 2005), only specimens with moderate ventricular dilatation and no cortical thinning were processed and used for analysis. However, in contrast to all other genotypes investigated in this study, the 2^-/+ 4^-/+ mice appeared weak and had significantly reduced body weights, as described before (Weinhold et al., 2005).

Immunofluorescence and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Sections were permeabilized for 15 min with 0.4% Triton X-100 in phosphate buffered saline (PBS), pH 7.4 before blocking for 1 h with 10% fetal calf serum (FCS; Gibco, Karlsruhe,
Germany) in PBS with 0.4% Triton X-100. Free floating sections were incubated with primary antibodies for 1-3 days at 4°C. The following monoclonal (mAb) or polyclonal antibodies (pAb) were applied according to the manufacturers’ instructions: CR- and CB D-28k-specific rabbit pAb (Swant, Bellinzona, Switzerland), TH-specific rabbit pAb, PV-specific mouse mAb (IgG1, Swant), and Sst-specific rat mAb (IgG2b, Chemicon, Temecula, CA). PolySia-specific mAb 735 (IgG2a) (Frosch et al., 1985) was used at 5 µg/ml. Rabbit and mouse IgG-specific Cy3- (Chemicon) 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 (SuperFrostPlus, Menzel, Braunschweig, Germany) and coverslipped using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Perineuronal nets were visualized using biotinylated *Wisteria floribunda* agglutinin, (WFA; Sigma-Aldrich, Munich, Germany) as described earlier by e.g., Härtig et al. (1992), and Cy3-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). Terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick end labeling (TUNEL) was performed as described before (Schiff et al., 2011).

**Microscopy, area measurements and cell counting**

Microscopy was performed using an Axiovert 200 M microscope equipped with an ApoTome device for near confocal imaging, AxioCam MRm digital camera and AxioVision 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-thick vibratome section were obtained by ApoTome technology using a 10x Plan-Apochromat objective with a numerical aperture of 0.45 (Zeiss). Micrographs covering the area of one entire hemisphere were acquired using the MosaiX module of the AxioVision software. AxioVision software was also used for area measurements. Cells were counted by visual inspection assisted by the interactive event counting tool of AxioVision software. 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), medial prefrontal cortex (mPFC), Ammon’s horn (cornu ammonis, CA) region CA1/2 and CA3, 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. mPFC and CA1/2 were further subdivided into infralimbic (IL), prelimbic (PrL) and cingulate cortex area 1 (Cg1), according to Paxinos and Franklin (2001), and into upper and deep layers corresponding to layers 1 to 3 and 5 to 6 of mPFC (lacking a distinct layer 4) or into stratum oriens, pyramidal layer, and the strata radiatum and lacunsum-moleculare of CA1/2, according to the Allen Mouse Brain Atlas (Lein et al., 2007), available from: http://mouse.brain-map.org, respectively. Examination of shape and areas of randomly selected labeled 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 analyzed, immuno-positive cells were quantified on MosaiX images obtained from three (for CR, CB, and TH) or six (for PV and Sst) 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 1.54 mm for mPFC and between bregma level -1.2 mm and -1.85 mm for hippocampus were selected. For mPFC and hippocampus, these pairs of consecutive sections were labeled for PV together with either CR or CB, or, in case of the mPFC, for PV together with WFA or Sst. Always 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 CR, the other for CB. On the second section, one OB was stained for TH.

Assessment of cortical interneuron precursor migration

Migrating cortical interneuron precursors were labeled by CB immunofluorescence (Pozas and Ibanez, 2005). As illustrated in Fig. 7G, tangential migration was assessed on coronal sections of E13.5 brains by measuring the length of the intermediate zone migratory stream and the extent of the dorsal telencephalon to the maximal dorsal expansion of the lateral ventricle beginning at the pallial/subpallial boundary. The length of the migratory stream was divided by the extent of the dorsal telencephalon to obtain a measure of relative interneuron migration. Per animal, four consecutive sections were evaluated and three animals were analyzed per group (genotype). To ensure that embryos were matched for developmental stage, heterozygous (+/-) and homozygous (-/-) littermates were used and compared by
external features according to Kaufman (1992). Patterns of migrating interneurons in $4^{+/+}$ and $2^{+/+}$ animals were indistinguishable and respective values were combined in the control group.

Results

Altered densities of PV-immunoreactive cells in the mPFC of polyST- and NCAM-deficient mice

Due to the high mortality of $2^{-/-}4^{-/-}$ mice after 4 weeks of age (Weinhold et al., 2005), all comparative analyses involving mice of this genotype were restricted to young, one-month-old animals. Consistent with previous observations that brains of polyST-negative mice are smaller (Weinhold et al., 2005; Schiff et al., 2009) the area of the mPFC as well as the area of the entire brain section at the respective cross-sectional level were reduced in $2^{-/-}4^{-/-}$ mice (12% and 16% reduction, respectively; see Supplementary Table 1). To compensate for the differences in overall brain size, cell counts for each evaluated region were normalized to the respective area. Compared to the control group, the resulting densities of PV-positive cells in the mPFC were significantly lower in both polyST single knockout lines ($2^{+/+}$ and $4^{+/+}$) as well as in all other polyST- or NCAM-deficient genotypes (Fig. 1A-H; for numbers of evaluated sections and listing of cell counts, see Supplementary Table 1). Subdividing the mPFC into upper and deep layers of cingulate cortex area 1 (Cg1), prelimbic cortex (PrL) and infralimbic cortex (IL) revealed that in all of these polySia-deficient lines the most prominent reductions were observed in the upper and deep layers of Cg1, which in the wildtype situation harbors the highest densities of PV-positive cells (Fig. 1I,J). The effect, however, was not confined to this specific region, but significant reductions or at least a trend, were also observed in the upper and deep layers of PrL and IL.
Fig. 1 Parvalbumin (PV) expression in the medial prefrontal cortex (mPFC) at P30. (A) Schematic illustration of one hemisphere at the level of the mPFC (left; modified from Paxinos and Franklin, 2001) and ApoTome MosaiX image showing the distribution of PV-positive cells in the mPFC (right). The area of the mPFC, consisting of cingulate cortex area 1 (Cg1), prelimbic cortex (PrL) and infralimbic cortex (IL), is highlighted in grey (left) or outlined and subdivided into upper and deep layers (right). The grey square indicates the position of the micrographs depicted in (B-G). (B-G) Representative details illustrating PV-positive cells in Cg1 of the different polyST- and NCAM-deficient mice with the indicated genotypes. Scale bar: 50 µm. (H-J) Densities of PV-positive cells (PV⁺) in the entire mPFC (H), or in the upper (I, cortical layers 1-3) or deep layers (J, cortical layers 5-6) of Cg1, PrL and IL, as indicated. Per group (genotype), mean values ±SEM from n=3 animals are plotted. Results from one-way ANOVA are indicated and for ANOVA with P<0.05, Newman-Keuls post hoc test was applied. *, **, *** significant difference against control with P<0.05, P<0.01, or P<0.001, respectively.
Fig. 2 Evaluation of parvalbumin (PV)- and calbindin (CB)-positive cells in the medial prefrontal cortex (mPFC) at P30. (A,B) Representative ApoTome images illustrating double immunofluorescence staining for PV (red) and CB (green) in the mPFC (cingulate cortex area 1) of control (ctrl, A) and $2^i4^i$ mice (B) at P30. Double-positive cells appear yellow (arrows). Scale bar: 50 µm. (C-E) Densities of PV-positive but CB-negative (PV+CB-) (C), PV and CB double-positive (PV+CB+) (D) and PV-negative but CB-positive (PV-CB+) (E) cells in the mPFC of mice with the indicated genotypes at P30. Per group (genotype), mean values ±SEM from n=3 animals are plotted. Results from one-way ANOVA are indicated and for ANOVA with $P<0.05$, Newman-Keuls post hoc test was applied. ***, significant difference against control with $P<0.01$ or $P<0.001$, respectively. n.s., not significant ($P>0.1$).
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 (Fig. 2A,B). Compared to the control group, the densities of PV⁺CB⁻ cells were significantly reduced in the mPFC of all polyST- or NCAM-deficient lines at postnatal day (P) 30 (Fig. 2C). Again, the effect was most pronounced in Cg1 (Supplementary Fig. 1). For the PV⁺CB⁺ subpopulation no significant differences between genotypes were found by evaluation of the entire mPFC area (Fig. 2D). However, in the upper layers of Cg1, but not in any other subdivision, a significant decrease of PV⁺CB⁺ cells was observed for all polyST- or NCAM-deficient mice (Supplementary Fig. 2). Abundant PV-negative cells with a weak expression of CB were detected in the upper layers. As shown in rat, many of these cells are non-GABAergic (Gonchar and Burkhalter, 1997). Therefore, they were excluded from the current analyses. For the remaining PV⁺CB⁺ cells, no significant differences between genotypes were found by evaluation of the entire mPFC (Fig. 2E) or any of its subdivisions (data not shown). Likewise, no significant differences in the expression of CR were observed (Fig. 3 and data not shown).

Expression of PV starts around P7 and throughout the next weeks these neurons slowly mature (for a recent review, see Powell et al., 2011). In order to assess, if the reduced densities of PV-positive cells could be due to a developmental delay we asked for persistence of the defects in older, three-month-old 4⁻/⁻ and 2⁻/⁻ mice. PV-positive cells were significantly reduced in upper and deep layers of the mPFC, as well as in all subdivisions, except for the IL (Supplementary Fig. 3). Developmental delay or transient reduction, therefore, can be excluded. Instead, a permanent downregulation of PV expression, or either loss or agenesis of the respective interneuron populations during development could cause the lower PV-positive cell densities in the mPFC of polySia-deficient mice.
Fig. 3 Calretinin (CR) expression in the medial prefrontal cortex (mPFC) at P30. (A-D) Representative ApoTome images illustrating CR-positive cells in the deep layers (layers 5-6) of cingulate cortex area 1 (Cg1) and prelimbic cortex (PrL) in control (ctrl) and 2^+/- 4^-/- mice, as indicated. Scale bar: 50 µm. (E) Densities of the CR-positive (CR^+) cells in the mPFC of mice with the indicated genotypes. Per group (genotype), mean values ±SEM from n=3 animals are plotted. Differences were not significant (n.s.; one-way ANOVA, P>0.1).
In a first attempt to distinguish between these possibilities, we asked for increased apoptotic cell death in the phase of interneuron maturation. However, at P10 only low numbers of apoptotic cells were detected by TUNEL staining in the mPFC of wildtype controls, $4^{-/-}$ and $2^{-/-}$ mice [mean numbers of TUNEL-positive cells per mm$^2$ mPFC (+/- SEM): 9.9 (+/- 1.0), 8.2 (+/- 0.9), and 9.5 (+/- 0.7) for ctrl, $4^{-/-}$, and $2^{-/-}$, respectively; n = 6 sections from two animals, each]. We therefore asked next, if the observed effects were confined to the expression of PV as a marker and analyzed the abundance of perineuronal nets (PNNs). PNNs are formed by mature basket cells and can be labeled with *Wisteria floribunda* agglutinin (WFA; Härtig et al., 1992; Ojima, 1993; Wegner et al., 2003). Indeed, PNN staining with WFA could be used as a marker for a subpopulation of PV-positive interneurons in the mPFC of three-month-old mice (Fig. 4A-C). In upper and deep layers, the density of PV-positive cells with PNNs was significantly reduced in $2^{-/-}$ and $4^{-/-}$ mice and, except for the deep layers of IL, all subdivisions were affected (Fig. 4D,E). For the remaining PV-positive cells without PNNs (PV’PNN’) significant reductions were detected in the upper mPFC layers of $4^{-/-}$ mice [ANOVA, $P<0.05$; mean numbers of PV’PNN’ cells per mm$^2$ mPFC area (+/- SEM): 11.2 (+/-2.3), 3.8 (+/-1.0), and 8.2 (+/-0.5) for ctrl, $4^{-/-}$, and $2^{-/-}$, n=3, respectively] and a trend towards reduced numbers was obtained for the deep layers [ANOVA, $P=0.08$; mean numbers of PV’PNN’ cells per mm$^2$ mPFC area (+/- SEM): 69.1 (+/- 6.5), 45.6 (+/- 2.6), and 59.1 (+/- 7.7) for ctrl, $4^{-/-}$, and $2^{-/-}$, n=3, respectively]. In contrast, numbers of PV-negative cells with PNNs (PV’PNN’) were unaffected [ANOVA, $P=0.5$; mean numbers of PV’PNN’ cells per mm$^2$ mPFC (+/- SEM): 16.4 (+/-1.6), 20.7 (+/-0.3), and 20.6 (+/-4.3) for ctrl, $4^{-/-}$, and $2^{-/-}$, n=3, respectively]. These results exclude the possibility that expression of specifically PV is lost by otherwise unaffected cells in the mPFC of polySia-deficient mice and speak in favor of loss or agenesis of the respective interneuron population. However, the simultaneous loss of PV and PNN staining not necessarily indicates a loss of neurons. Formation of PNNs in the rodent cortex occurs late in postnatal development and depends on activity (for review, see Rhodes and Fawcett, 2004), which provides a possible link to explain the loss of both markers.
Fig. 4 Colocalization of parvalbumin (PV) and perineuronal nets (PNNs) on cells of the medial prefrontal cortex (mPFC) of 3-month-old control (ctrl), 4−/− and 2−/− mice. (A-C) Representative ApoTome images illustrating the distribution of PV- (green) and PNN- (red) positive cells at the level of the deep layers (layers 5-6) of cingulate cortex area 1 (Cg1). Scale bar: 50 µm. (D,E) Densities of PV and PNN double positive cells (PV+PNN+) in the upper layers (D, layers 1-3) and deep layers (E, layers 5-6) of Cg1, prelimbic cortex (PrL) and infralimbic cortex (IL) of the mPFC. Per group (genotype), mean values ±SEM from n=3 animals are plotted. Results from one-way ANOVA are indicated and for ANOVA with P<0.05, Newman-Keuls post hoc test was applied. *, **, significant difference against control with P<0.05 or P<0.01, respectively. n.s., not significant (P>0.1).
Altered densities of Sst-immunoreactive cells in the mPFC of polyST-deficient mice

Similar to the PV-positive interneurons, the large majority of the somatostatin-positive population of cortical interneurons originates in the medial ganglionic eminence (MGE) and migrate tangentially from the subpallium into the developing neocortex (Gelman and Marin, 2010). In search for a possible developmental background of the observed changes of PV-positive cells, densities of Sst-positive cells were analyzed in the mPFC of three-month-old 2\(^{-}\) and 4\(^{-}\) mice (Fig. 5). In the total area of the upper mPFC layers, as well as in all of its subdivisions, densities of Sst-positive cells were significantly reduced (Fig. 5D). In the deep layers, a trend (\(P=0.08\)) towards lower numbers of Sst-positive cells was observed for the total mPFC, and significantly reduced densities were obtained in the PrL and IL of 4\(^{-}\) animals (Fig. 5E).

![Fig. 5 Somatostatin (Sst) expression in the medial prefrontal cortex (mPFC) of 3-month-old control (ctrl), 4\(^{-}\) and 2\(^{-}\) mice. (A-C) Representative ApoTome images illustrating the distribution of Sst-positive cells (Sst\(^{+}\)) at the level of deep layers (layers 5-6) of cingulate cortex area 1 (Cg1). Scale bar: 50 µm. (D,E) Densities of Sst\(^{+}\) cells in the upper layers (D, layers 1-3) and deep layers (E, layers 5-6) of Cg1, prelimbic cortex (PrL) and infralimbic cortex (IL) of the mPFC. Per group (genotype), mean values ±SEM from n=3 animals are plotted. Results from one-way ANOVA are indicated and for ANOVA with \(P<0.05\), Newman-Keuls post hoc test was applied. *, **, significant difference against control with \(P<0.05\) or \(P<0.01\), respectively. n.s., not significant (\(P>0.1\)).
Compromised cortical interneuron migration in polyST-deficient mice

Since densities of both PV- and Sst-positive cells were reduced in polySia-deficient mice, compromised migration of interneuron precursors from the MGE may account for the observed phenotype. In the mouse, interneuron migration from the MGE starts at E12.5 and migrating precursors transiently express CB while entering the dorsal telencephalon and during their tangential migration through the marginal and intermediate zone (Pozas and Ibanez, 2005 and references therein). As shown in Fig. 6, CB-immunoreactive cells with typical morphologies of migrating neurons were detected in sections of control, 4^+/−, and 2^+/− brains at E13.5. Double labeling with polySia-specific antibody revealed some overlap (Fig. 6A-C), but the abundance of polySia expression did not allow for a clear distinction, if polySia was present on the migratory neurons themselves or on structures in close contact. Moreover, no marked reductions of polySia immunoreactivity were detected on the sections from 4^+/− or 2^+/− mice. This is in good accordance with the coexpression of both polySTs in the embryonic brain and their capacity for mutual compensation (Ong et al., 1998; Galuska et al., 2006; Schiff et al., 2009). It also resembles the findings on polySia levels in the rostral migratory stream, which were not noticeably reduced in postnatal 4^+/− or 2^+/− single knockout mice (Eckhardt et al., 2000; Angata et al., 2004). Despite this inability to detect a loss of polySia, significant reductions in the absolute and relative lengths of the migratory streams formed by CB-positive cells in the intermediate zone of the dorsal telencephalon at E13.5 were observed in 4^+/− and 2^+/− mice, while the extent of the telencephalon itself was unaltered (Fig. 7). These results indicate that the reduced interneuron densities in polyST-deficient mice could be caused by deficits in tangential migration from the MGE into the dorsal telencephalon.

![Fig. 6 Colocalization of calbindin (CB) and polysialic acid (polySia) in the forebrain of E13.5 mice.](image)

Representative ApoTome images of coronal forebrain sections from control (ctrl, A), 4^+/− (B) and 2^+/− (C) mice, as indicated. CB, red; polySia, green. Scale bar: 20 µm.
Fig. 7 Assessment of interneuron migration from the ganglionic eminence (GE) to the dorsal telencephalon. (A-F) Representative ApoTome images of coronal sections from E13.5 control (ctrl), 4−/− and 2−/− animals, stained for calbindin to detect migrating interneuron precursors in subpallium and pallium (neocortex, NCx). The lateral ventricle (lv) is delineated by a dashed line. Cells at the very end of each migratory stream are indicated by arrows. (A-C) Overview, scale bar: 50 µm. (D-F) Detail of the end of the respective migratory stream, scale bar: 25 µm. (G) Schematic illustration of tangential interneuron migration from subpallial-localized GE into the pallium. The lengths of interneuron migratory streams (marked with “a”) were estimated and compared to the extent of the neocortex on the respective section (marked with “b”) starting at the pallial/subpallial boundary (indicated by a dashed line). (H-J) Relative interneuron migration (J), calculated as length of a migratory stream (H) divided by the extent of the neocortex (NCx; I). Mean values ±SEM from n=6 (ctrl) or n=3 animals (4−/−, 2−/−) are plotted. Results from one-way ANOVA are indicated and Newman-Keuls post hoc test was applied for ANOVA with P<0.05. ***, significant difference against control with P<0.001. n.s., not significant (P>0.1)
Altered densities of CB- but not CR- and TH-immunoreactive cells in the OB of polyST- and NCAM-deficient mice

The observed deficits of cortical interneuron migration in polyST-deficient mice were reminiscent to the well-known role of polySia in the migration of olfactory bulb interneurons. Several detailed studies demonstrated that the rostral migration of OB interneuron precursors depends on the presence of polySia (Ono et al., 1994; Hu et al., 1996; Chazal et al., 2000), and, as shown in NCAM-deficient mice, defective migration in the absence of polySia leads to smaller OBs and a massive loss of olfactory bulb granule cells (Tomasiewicz et al., 1993; Cremer et al., 1994; Gheusi et al., 2000). In addition to the homogenous population of the GABAergic granule cells, three non-overlapping subtypes of periglomerular interneurons can be distinguished by the expression of CR, CB and TH (Kosaka et al., 1995) and all three subtypes are also GABAergic in the mouse (Kohwi et al., 2007). To study possible consequences of a loss of polySia for the periglomerular interneurons, we analyzed the densities of CR-, CB- and TH-positive cells in the OB of polyST- and NCAM-deficient mice at P30. On each OB section the area of the Gl was determined and for each of the markers all immunopositive cells within the Gl were counted (Fig. 8A; for numbers of evaluated sections and listing of cell counts, see Supplementary Table 2). Compared to the control group, a more than 50% reduction in the density of CB-positive cells was detected within the glomerular layer of $2^{-/-}4^{-/-}$, $N^{-/-}$ and $2^{-/-}4^{-/-}N^{-/-}$ mice (Fig. 8B,E-H). Both lines deficient for one of the two polySTs ($2^{-/-}$, $4^{-/-}$) had an intermediate phenotype (Fig. 8C,D,H). The expression of CR or TH was not altered in the glomerular layer of the OB (Fig. 8I-K and L-N, respectively). Thus, the complete absence of polySia or NCAM as well as the lack of only one of the polySTs led to subtle differences in one particular type of periglomerular interneurons in the OB. This selectivity is remarkably similar to the specific reductions of only some interneuron populations in the mPFC.
Fig. 8 Expression of calbindin (CB), calretinin (CR) and tyrosine hydroxylase (TH) in the glomerular layer (Gl) of the olfactory bulb at P30. (A) Schematic drawing of a coronal olfactory bulb section (according to Paxinos and Franklin, 2001). The Gl is highlighted in grey. The position of the micrographs depicted in B-G, I-J and L-M is indicated (red square). GrO, granular cell layer of the olfactory bulb. (B-G) Representative ApoTome images illustrating CB-positive cells (CB⁺, green) in the Gl of the different polyST- and NCAM-deficient mice with the indicated genotypes. (H) Densities of CB⁺ cells in the Gl. (I,J) Representative details illustrating CR-positive cells (CR⁺ green) in the Gl of ctrl (I) and 2⁻⁻⁻⁻⁻⁻ mice (J). (K) Densities of CR⁺ cells in the Gl of mice with the indicated genotypes. (L,M) Representative details illustrating TH-positive cells (TH⁺, green) in the Gl of ctrl (L) and 2⁻⁻⁻⁻⁻⁻ mice (M). (N) Densities of TH⁺ cells in the Gl of the olfactory bulb. In all graphs (H, K, N) mean values ±SEM from n=3 animals are plotted per group (genotype). Results from one-way ANOVA are indicated and Newman-Keuls post hoc test was applied for ANOVA with P<0.05. **, ***, significant difference against control with P<0.01 or P<0.001, respectively. n.s., not significant (P>0.1). Scale bars: 50 µm. Nuclear counterstain (DAPI), blue.
Altered densities of PV-immunoreactive cells in the hippocampus of polyST- and NCAM-deficient mice

Immunopositive cells of the CA fields and the dentate gyrus of one-month-old mice were counted separately and respective areas were measured (Fig. 9A; for numbers of evaluated sections and listing of cell counts, see Supplementary Table 3). Compared to the control group, mice lacking both (2\textsuperscript{-/-}4\textsuperscript{-/-}) or either of the two polySTs (2\textsuperscript{-/-}, 4\textsuperscript{-/-}) had significantly increased densities of PV-positive interneurons in the CA fields (Fig. 9B-H). Both NCAM-negative groups (N\textsuperscript{-/-} and 2\textsuperscript{-/-}4\textsuperscript{-/-}N\textsuperscript{-/-}) displayed a slight increase, which neither differed significantly from the control nor from the other polyST-deficient genotypes (Fig. 9B-H). In contrast to the CA fields, the densities of PV-positive cells in the DG were not affected (Fig. 9I). Separate evaluations of CA1/2 and CA3 revealed a prominent increase in only the CA1/2 region of the 2\textsuperscript{-/-} and 4\textsuperscript{-/-} mice (Fig. 9J,K). Further subdivision of CA1/2 yielded an even more disparate pattern with significant increases of PV-positive cell densities in the strata radiatum and lacunosum-moleculare of all polySia-deficient groups except for the 4\textsuperscript{-/-} genotype as well as in the pyramidal layer of the 2\textsuperscript{-/-} and, clearly more pronounced, in the 4\textsuperscript{-/-} group (Fig. 9L-N).

The evaluation of CB-positive cells in the CA fields revealed a high variability but no statistically significant differences between the genotypes (Supplementary Fig. 4A). CB-positive cells in the DG were not evaluated, because CB is expressed mainly by DG granule cells and not interneurons (Baimbridge, 1992; Freund and Buzsaki, 1996; Matyas et al., 2004). In line with a previous report (Jinno and Kosaka, 2002) hardly any PV\textsuperscript{+}CB\textsuperscript{+} interneurons were detected in the hippocampus. Thus, double-positive cells could not be quantified. Similar to the findings in the mPFC, the density of CR-positive cells in the CA fields was not affected in any of the groups analyzed (Supplementary Fig. 4B). In the DG, many faintly CR-positive 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).

Taken together, all of the five polyST- or NCAM-deficient mouse lines displayed a significant increase of PV-positive cells in at least one of the hippocampal subfields analyzed. However, in contrast to the fairly consistent reductions of PV-positive cells in the mPFC, the CA1/2 layers of particularly the 2\textsuperscript{-/-} and 4\textsuperscript{-/-} mice were differentially affected.
Fig. 9 Parvalbumin (PV) expression in the hippocampus at P30. (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 CA region was further subdivided into CA1/2 and CA3 and into stratum oriens (Or), pyramidal layer (Py) and strata radiatum (Rad) and lacunosum-moleculare (LMol) of CA1/2. GrDG, granular layer of the DG. The position of the micrographs depicted in B-G is indicated (white square). (B-G) Representative ApoTome images illustrating PV-positive cells (PV⁺) in the CA region of the different polyST- and NCAM-deficient mice with the indicated genotypes. Scale bar: 50 µm. (H-N) Densities of PV⁺ cells in the entire CA region and the DG (H, I), in CA1/CA2 and CA3 (J, K), and in the strata Rad and LMol, Py, and Or of the CA1/2 region (L-N). Per group (genotype), mean values ±SEM from n=3 animals are plotted. Results from one-way ANOVA are indicated and for ANOVA with P<0.05, Newman-Keuls post hoc test was applied. *, **, ***; significant difference against control with P<0.05, P<0.01, or P<0.001, respectively. n.s., not significant (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 \textit{Ncam1} and polysialyltransferase deletions. Together, the data 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 Galuska et al., 2010 and references therein).

Basket cells of the mPFC are affected by polySia-deficiency

A major finding of the current study is the reduction of PV-positive cells in the mPFC of all five polyST- or NCAM-deficient lines comparatively analyzed at the age of four weeks. The loss of NCAM-bound polySia, therefore, affects the PV-positive interneurons comprising basket and chandelier cells (Markram et al., 2004; Gelman and Marin, 2010). Changes were detected in all three evaluated parts of the mPFC, Cg1, PrL, and IL, and in both the upper and deep layers. Further attempts to characterize the affected cell population identified the group of PV+CB- cells to be reduced in the entire mPFC with the Cg1 most prominently affected. Specifically in the upper layers of Cg1, however, there was also a major reduction of the PV+CB+ subpopulation. As shown for three-month-old specimen of 2-/- and 4-/- mice, the reduction of PV-expressing interneurons was persistent and includes but was not confined to PV-positive cells with WFA-positive PNNs. This special type of extracellular matrix is formed during late stages of cortical maturation at the end of the critical period of cortical plasticity (for review, see Rhodes and Fawcett, 2004). Among the PV-positive cells of the rat cortex, PNNs are characteristic for a subpopulation of basket cells (Ojima, 1993; Wegner et al., 2003). Importantly, PNNs of PV-negative cells were not affected by polySia deficiency. Thus, PV+PNN+ basket cells but also other subgroups of the PV-positive basket and chandelier cells are affected by disturbed polysialylation of NCAM.

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 under the neuron-specific enolase promoter display a dramatic reduction of PV-positive puncta, but
no reduction of PV-positive 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-positive basket cells.

Disturbed tangential migration is a likely cause of MGE-derived interneuron defects

As shown for the two polyST-deficient lines, densities of Sst-expressing interneurons were reduced concomitantly with PV-positive cells in particularly the upper layers of the mPFC. Sst-positive cells include several classes of interneurons but share a common feature with the PV-containing interneurons in that they are both derived from the MGE (Gelman and Marin, 2010). Together with the observed deficits in cortical interneuron migration these findings strongly imply that the reduced densities of PV- and Sst-positive interneurons in the mPFC of polyST-deficient mice are caused by dysfunctional migration from the MGE. Notably, in the mature mPFC polySia expression is abolished specifically in 4^-/^- but not in 2^-/^- mice (Nacher et al., 2010). In contrast, the loss of PV- or Sst-positive cells and the deficits in cortical interneuron migration were similar in both lines supporting that changes of interneuron densities are not caused by altered polySia expression in the mature cortex but rather by a lack of polySia during development.

Fate mapping studies indicate that PV-, Sst- and CB-expressing interneurons of the adult cortex are generated from precursors of the central and ventral MGE characterized by the Nkx2.1 and Lhx6 transcription factors, while the CR and Sst double-positive Martinotti cells and the bipolar CR-expressing interneurons are generated from the dorsal MGE or outside the MGE, respectively (Fogarty et al., 2007). Nkx2.1 knockout mice, which die at birth, lack Sst-positive cells at E16.5 (Anderson et al., 2001) and in the cortex of two-week-old Lhx6 null mice dramatic reductions of PV- and Sst-positive cells were observed (Liodis et al., 2007). The latter study also established that these reductions were not due to a failure of GABAergic specification in the MGE but associated with delayed tangential migration and defective differentiation of MGE-derived progenitors into PV- and Sst-expressing
interneurons. These earlier studies therefore support the assumption that the joint reduction of PV- and Sst-positive cells in the mPFC of polyST-deficient mice originates from deficits in tangential migration and altered subtype specification of MGE-derived cortical interneuron precursors.

Assuming that defects of migration from the central and ventral MGE are the primary cause of altered densities of PV- and Sst-positive cells, it is surprising that PV-CB+ interneurons were not affected in the mPFC or its subdivisions in any of the polySia-deficient genotypes studied. Counts for PV-CB+ cells in the upper layers may be confounded by omitting the weakly CB-positive, PV-negative cells of uncertain identity. In the deep layers a minor fraction (<30%) of CB-positive cells was immunoreactive for Sst (Kröcher, unpublished observation). This is distinct from the high overlap of CB and Sst described for layer V and VI of the rat frontal cortex (Kawaguchi and Kubota, 1997) but raises the question, why the reductions of Sst in the deep layers of PrL and IL in 4-/- mice were not reflected by respective changes of the PV-CB+ population. Either changes of an Sst and CB double-positive subpopulation occurred but were too small to be detected by counting all PV-CB+ cells or an Sst-positive but CB-negative subpopulation is affected. Taken together, the interneuron populations possibly affected by disturbed migration from the central and ventral MGE show only partial reductions. This leaves the possibility that lower numbers of CB-positive interneuron precursors reaching the mPFC or their delayed arrival affects mainly the maturation of these cells into PV- or Sst-expressing interneurons.

Impaired migration of yet unidentified precursor cells during cortical development has been described in 2-/-4-/- mice (Angata et al., 2007). In clear contrast with our results, this study also reports on a substantial, approximately 20% reduction in the number of CB-positive cells in the cerebral cortex of adult 2-/-4-/- animals. As no other neuronal markers where assessed, the specificity of this effect remains unresolved. More important, animals with drastic cortical thinning due to hydrocephalus formation were evaluated in the earlier study. As shown in human embryonic hydrocephalus, cortical thinning is associated with a loss of CB- and PV-positive cells (Ulfig et al., 2001). Thus, the results of Angata et al. may reflect a cell loss secondary to hydrocephalus formation. In contrast, only 2-/-4-/- specimen with moderate ventricular dilatation and no cortical thinning were considered for the current analyses. Effects of ventricular dilatation therefore are very unlikely to cause the defects described here for the 2-/-4-/- mice. This is corroborated by the almost identical reductions of PV-positive
but not CB-positive cells between the $2^{-/-}$ and the other polySia-deficient lines not prone to hydrocephalus.

**PolySia deficiency inversely affects PV-positive interneurons in mPFC and CA**

Some of the mechanisms that control the generation of cortical interneuron diversity are about to be elucidated but major 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. In Lhx6 null mice PV-positive interneurons were consistently reduced in the neocortex and hippocampus and this phenotype has been attributed to altered specification possibly caused by a delay in tangential migration from the MGE (Liodis et al., 2007). In contrast, all mouse lines with partial or complete lack of polySia display a reduction of PV-positive interneurons in the mPFC and an increase of PV-positive cells in either the strata radiatum and lacunsum-moleculare or in the pyramidal layer of the CA1/2 field of the hippocampus. Clearly, further studies are needed to unravel the mechanisms that account for this unexpected inverse relationship. However, there are indications that not only the decrease in the mPFC but also the increase of PV-positive interneurons in the hippocampus of polySia-deficient mice may be caused by altered migration and specification of MGE-derived precursors. Corresponding to the observed increase in specifically the CA1/2 region, the MGE is known to produce hippocampal interneurons that migrate preferably to CA1/2 and avoid the DG, while the caudal ganglionic eminence generates interneurons that migrate to both the CA and the DG regions (for review, see Danglot et al., 2006). Since MGE-derived interneurons tend to migrate laterally before they spread throughout the cortex (for review, see Métin et al., 2006; Gelman and Marin, 2010), the inverse distribution of PV-positive cells between mPFC and CA1/2 raises the intriguing possibility that polySia is involved in the regulation of cell surface interactions that shape decisions of directional migration.

Together, the observed migration deficit and the altered densities of MGE-derived interneuron populations in the mPFC and hippocampal CA1/2 region of polysialylation-deficient mice suggest a major role of polySia in a common neurodevelopmental mechanism that triggers the balanced distribution and/or subtype specification of MGE-derived cortical interneuron precursors. In addition, the layer specific differences between the $2^{-/-}$, $4^{-/-}$ and $N^{-/-}$ mice indicate specific contributions of the two polySTs in the fine tuning of this process and may contribute to distinct phenotypic features observed in these mouse lines. Impaired long
term potentiation (LTP) in CA1 has been detected in $N^{-/-}$ and $4^{-/-}$ but not in $2^{-/-}$ mice (Eckhardt et al., 2000; Angata et al., 2004). In contrast, densities of PV-positive cells differed mainly between $N^{-/-}$ and $4^{-/-}$ in the stratum pyramidale, while $N^{-/-}$ and $2^{-/-}$ mice displayed a comparable increase in the strata radiatum and lacunosum-moleculare. Therefore, and because LTP is also disturbed by acute enzymatic removal and rescued by application of polySia (Senkov et al., 2006 and references therein), a major contribution of the enhanced PV-positive cell densities to synaptic plasticity in CA1 appears unlikely. On the other hand, increased numbers of PV-positive cells and altered perisomatic inhibition in CA1 of CHL1-null mice or in the DG of mice deficient for tenascin-R have been linked to impaired LTP (Nikonenko et al., 2006; Morellini et al., 2010). Thus, compensatory mechanisms restoring normal CA1 LTP in $2^{-/-}$ but not $N^{-/-}$ mice with increased densities of PV-positive cells should be considered. In fact, the hippocampal network has an increasingly recognized capacity to reorganize inhibitory circuits in order to compensate for altered GABAergic functions (Arellano et al., 2004; Schneider Gasser et al., 2007).

**CB-positive 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). As shown in $N^{-/-}$ mice, defective migration in the absence of polySia leads to a massive loss of granule cells (Gheusisi et al., 2000). Likewise, the prominent reduction of CB-positive cells in the glomerular layer as found here in the NCAM- or polySia-negative mice ($N^{-/-}, 2^{-/-}4^{-/-}, 2^{-/-}4^{-/-}N^{-/-}$) is clearly linked to the well-described deficits of the tangential migration of 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-positive 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 interneuron precursor migration results in a significant reduction of CB-positive neurons in the glomerular layer of the OB. In both cases, however, it remains enigmatic, why the migration deficit specifically affects the CB-positive population of periglomerular interneurons.
Taken together, impaired tangential migration in the RMS is the most likely cause for the deficits of CB-positive interneurons in the OB of NCAM- or polySia-negative mice. In addition, a small but significant reduction of the CB-positive subpopulation of periglomerular cells was observed in both polyST single knockout lines. This is unexpected, because migrating cells in the postnatal RMS express polySia in the absence of either ST8SIA2 or ST8SIA4, and a normal morphology of the RMS 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 2−/−4−/− animals indicates that both polySTs contribute to polySia synthesis in this system. Therefore minor, yet undetected reductions of polySia levels in the postnatal RMS or deficits during the embryonic development of OB interneurons from the lateral ganglionic eminence may account for the moderate reductions of CB-positive periglomerular interneurons in 2−/− and 4−/− mice.

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, 2006). These effects, however, are induced by a gain of polySia-free NCAM. In contrast, all of the major interneuron changes 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 is highly compatible with the assumption that impaired tangential migration is indeed the common cause for the altered interneuron densities observed in the OB, mPFC and hippocampus of all polySia-deficient lines, although the mode of interneuron migration from the embryonic MGE differs from the persistent migration of OB interneurons (Marin and Rubenstein, 2003). Cortical interneuron precursors disperse and migrate rather individually, whereas the olfactory interneuron precursors migrate in chains of cells that move in close contact with each other.

**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 demonstrated reduced densities of PV-positive and, to a lesser extent, CB-positive interneurons (Beasley et al., 2002; Reynolds et al., 2002) while others revealed reductions of PV or Sst on the level of mRNA expression (Hashimoto et al., 2003; Morris et al., 2008; Fung et al., 2010). In contrast, the CR-positive subtype seems to be consistently unaltered. The patient data, therefore, are comparable with the findings of altered PV- and Sst-positive but not CR-positive interneuron densities in the mPFC of all the mouse lines with impaired polysialylation analyzed in the current study.

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

Altered levels of NCAM or polySia were found in schizophrenia but also in other neuropsychiatric disorders with a possible genetic and neurodevelopmental contribution (for review, see Brenneman and Maness, 2010). SNPs in NCAM1 and ST8SIA2 have been associated with schizophrenia and two recent genome wide association studies identified SNPs within or close to the ST8SIA2 gene that are linked to autism spectrum or bipolar I disorder, respectively (Anney et al., 2010; Lee et al., 2010). These disorders show a genetic overlap, share behavioral characteristics and cognitive deficits and may arise from a neurodevelopmental insult leading to adult imbalance between excitatory and inhibitory neurotransmission (Carroll and Owen, 2009). 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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References


Lewis DA, Hashimoto T, Volk DW. 2005. Cortical inhibitory neurons and schizophrenia. Nat Rev Neurosci. 6:312-324.


Thesis - 53 - Results

Tim Kröcher

Chapter 1


Pozas E, Ibanez CF. 2005. GDNF and GFRalpha1 promote differentiation and tangential migration of cortical GABAergic neurons. Neuron. 45:701-713.


Supplementary material

Suppl. Fig. 1 Densities of parvalbumin-positive, calbindin-negative cells (PV⁺CB⁻) in the upper (A) or deep layers (B) of Cg1, PrL and IL of the different polyST- and NCAM-deficient mice with the indicated genotypes at P30. Per group (genotype), mean values ±SEM from n=3 animals are plotted. Results from one-way ANOVA are indicated and for ANOVA with $P<0.05$, Newman-Keuls post hoc test was applied. *, significant difference against control with $P<0.05$. n.s., not significant ($P>0.1$).

Suppl. Fig. 2 Densities of parvalbumin- and calbindin-positive cells (PV⁺CB⁺) in the upper (A) or deep layers (B) of Cg1, PrL and IL of mice with the indicated genotypes at P30. Per group (genotype), mean values ±SEM from n=3 animals are plotted. Results from one-way ANOVA are indicated and for ANOVA with $P<0.05$, Newman-Keuls post hoc test was applied. **, significant difference against control with $P<0.01$. n.s., not significant ($P>0.1$).
Suppl. Fig. 3 Densities of parvalbumin-positive cells (PV⁺) in the upper (A) or deep layers (B) of Cg1, PrL and IL of 3-month-old control (ctrl), 4⁺⁻ and 2⁻⁻ mice, as indicated. Per group (genotype), mean values ±SEM from n=3 animals are plotted. Results from one-way ANOVA are indicated and for ANOVA with $P<0.05$, Newman-Keuls post hoc test was applied. *, **, significant difference against control with $P<0.05$ or $P<0.01$, respectively. n.s., not significant ($P>0.1$).

Suppl. Fig. 4 Densities of calbindin-positive cells (CB⁺; A) and calretinin-positive cells (CR⁺; B) in the CA area of the hippocampus of mice with the indicated genotypes at P30. Per group, mean values ±SEM from n=3 animals are plotted. One-way ANOVA revealed no significant differences (n.s., $P>0.1$).
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<td>b if not noted otherwise, mean values for left and right mPFC from six sections per brain were determined for each animal</td>
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<td>d calculations were performed with exact (non-rounded) values</td>
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### Röckle et al., Supplementary Table 2: Olfactory bulb areas and cell counts

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<td>tg4 #134/55-1</td>
<td>0.5</td>
<td>2.0</td>
<td>24.5</td>
<td>220</td>
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</table>

**a** Abbreviations: CB, calbindin; CR, calretinin; GI, glomerular layer; TH, tyrosine hydroxylase
**b** per brain, mean values for the GI from nine OB sections were determined
**c** per brain, mean areas from the evaluated OB sections were determined
**d** calculations were performed with exact (non-rounded) values
**e** per brain, mean values from three OB sections were determined
### Röckle et al., Supplementary Table 3: Hippocampus areas and cell counts

<table>
<thead>
<tr>
<th>Animal (line, mating, litter)</th>
<th>mean area [mm²]</th>
<th>relative area hippocampus (% of brain section)</th>
<th>mean cell counts/hip&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>hippocampus&lt;sup&gt;b&lt;/sup&gt;</td>
<td>total brain section&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>1.6</td>
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</table>

<sup>a</sup> Abbreviations: CA, Cornu ammonis; CB, calbindin; CR, caleteinin; DG, dentate gyrus; hip, hippocampus; PV, parvalbumin

<sup>1</sup> if not noted otherwise, mean values for left and right hip from six sections per brain were determined for each animal

<sup>c</sup> calculations were performed with exact (non-rounded) values

<sup>d</sup> per brain, mean areas from the evaluated sections at the level of the hip were determined

<sup>e</sup> if not noted otherwise, mean values for left and right hip from three sections per brain were determined for each animal

<sup>f</sup> areas and numbers of PV<sup>+</sup> cell were determined from four sections per brain, CR<sup>+</sup> from one section, each
Chapter 2 - A critical role of polysialic acid for migration of GABAergic interneurons derived from the medial ganglionic eminence

This manuscript has been prepared for submission

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Preface - About this manuscript

In the first study of this thesis, reduced densities of parvalbumin- and somatostatin-positive interneurons were observed in the PFC of polySia- or NCAM-deficient mice. Here, we focused on the mechanism that leads to these observations. To confirm the assumed loss of interneurons in the PFC, polyST-negative mice that express GFP driven by the promoter of the interneuron-specific enzyme glutamic acid decarboxylase (GAD67) were analyzed. The polySia-degrading enzyme endosialidase was used to investigate the effects of polySia-deficiency on migration of interneurons in vitro. Time lapse microscopy was performed to examine the migratory behavior of polySia-deficient interneurons and different culture techniques of embryonic GAD67-GFP brains were applied to investigate a potential mechanism of disturbed interneuron migration upon removal of polySia.

My contributions to this manuscript comprised the organization of mouse breeding, dissection and analysis of adult and embryonic mice, performance of slice, co-, explant and primary cultures, immunofluorescent stainings, Western blotting, in situ hybridization, microscopy, and respective evaluations. Prof. H. Hildebrandt and I designed the experiments and wrote the manuscript.
Abstract

Posttranslational addition of polysialic acid (polySia) is a unique and prominent modification of the neural cell adhesion molecule NCAM and a major determinant of brain development. Polysialylation is implemented by the two polysialyltransferases (polySTs) ST8SIA2 and ST8SIA4, both assigned with common but also distinct developmental functions. Dysregulation of polySia-NCAM expression and variations in ST8SIA2 have been linked to schizophrenia and other psychiatric disorders. Reminiscent to neuropathological findings in the prefrontal cortex (PFC) of schizophrenic patients, reduced densities of parvalbumin- and somatostatin-positive interneuron populations have been reported for polyST-deficient mice and altered embryonic patterns of migratory interneurons pointed towards a developmental cause. Here, we used genetic labeling of interneurons with GAD67-GFP to obtain evidence for cell loss in the PFC of polyST-deficient mice. We demonstrate reduced levels of polySia in the area of cortical interneuron migration in ST8SIA2- or ST8SIA4-deficient embryos. The impact of polySia on the migration process was monitored by applying endosialidase to remove polySia in embryonic slice cultures from GAD67-GFP transgenic mice. Time lapse recordings revealed significantly reduced velocities in endosialidase-treated slice cultures. Interneuron recruitment from the medial ganglionic eminence (MGE) into the pallium was attenuated, while laminar distribution within the cortex was maintained. Reduced entry of interneurons into the pallium was corroborated by comparatively analyzing co-cultures of ST8SIA2-deficient MGE with wildtype pallium and vice versa. In the absence of polySia shorter leading processes were observed in slices and isolated interneurons supporting a cell-autonomous mechanism of impaired motility after polySia reduction. Thus, cortical interneuron migration depends on polySia. This provides a neurodevelopmental link between genetic variation in polyST genes and interneuron pathology as observed in neuropsychiatric disease.
Introduction

A well known feature of the neural cell adhesion molecule NCAM is the posttranslational addition of polysialic acid (polySia). PolySia is a major regulator of morphogenesis and adult plasticity of the nervous system (reviewed by Bonfanti, 2006; Gascon et al., 2007; Hildebrandt et al., 2007; Maness and Schachner, 2007; Rutishauser, 2008). In the developing mouse brain, polySia is composed of up to 90 α2,8-glycosidically linked N-acetylnervaminic acid residues (Galuska et al., 2008) (Mühlendorff et al., 1998). Being a polyanionic polysaccharide with a high water binding capacity, polySia increases the hydrodynamic radius of the underlying protein causing a steric inhibition of protein interactions and cell-cell apposition (Johnson et al., 2005). Dynamic changes of NCAM isoforms and polySia levels during brain development have been described for rodents (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). In the mouse, polySia appears at embryonic day (E) 9, remains expressed throughout prenatal brain development and peaks during the perinatal phase (Probstmeier et al., 1994; Ong et al., 1998). The majority of polySia-positive cells in the mouse embryonic brain are precursors of the neuronal lineage, but some polySia-positive astrocytes and oligodendrocyte-precursors have also been described (Trotter et al., 1989; Kiss et al., 1993; Blass-Kampmann et al., 1994; Wang et al., 1994). In contrast to the high levels and the widespread occurrence of polySia-NCAM in embryos, expression in adults is restricted to sites with ongoing plasticity and neurogenesis (reviewed by Bonfanti, 2006; Mühlendorff et al., 2009).

Polysialylation of NCAM is carried out by two polysialyltransferases (polySTs), ST8SIA2 and ST8SIA4 (Eckhardt et al., 1995; Nakayama et al., 1995; Kojima et al., 1995; Scheidegger et al., 1995). Both enzymes can polysialylate NCAM independently and show distinct developmental regulation (reviewed by Hildebrandt et al., 2010). The data so far indicate that ST8SIA2 is mainly involved in polySia-synthesis during embryonic development, while ST8SIA4 is the predominant enzyme in the adult brain (Hildebrandt et al., 1998, Ong et al., 1998; Galuska et al., 2006; Oltmann-Norden et al., 2008; Schiff et al., 2009; Nacher et al., 2010). However, during brain development the expression patterns of the two enzymes show a considerable overlap. If one of the polySTs is deleted the remaining enzyme is able to compensate to at least some extent for the function of the other and polysialylation of proteins is still detectable (Galuska et al., 2006; Oltmann-Norden et al., 2008). At peak expression of polySia in the perinatal brain more than 95% of polySia has been found on
NCAM (Galuska et al., 2010). However, a few other polySia carriers have been identified, and some of them may play a yet undefined role in neurodevelopment (for review, see Mühlenhoff et al., in press).

Considering the prominent neurodevelopmental functions assigned to NCAM and polySia-NCAM, *Ncam1*-knockout mice show a fairly mild phenotype (Cremer et al., 1994). Also, mice deficient for *St8sia4* (*St8sia4<sup>−/−</sup>*, Eckhardt et al., 2000) or *St8sia2* (*St8sia2<sup>−/−</sup>*, Angata et al., 2004) have been reported to show no, or just minor defects of brain development. However, strong effects were observed by cross-breeding these deletion strains to generate mice that are deficient for both polySTs and therefore lack any polySia (*St8sia2<sup>−/−</sup>St8sia4<sup>−/−</sup>*) (Weinhold et al., 2005; Hildebrandt et al., 2009). Unique features of the polysialyltransferase-negative but NCAM-positive mice are postnatal growth retardation, precocious death, high incidence of developing a progressive hydrocephalus, and malformation of major brain axon tracts. These defects are reported to establish due to a gain of polySia-free NCAM because they can be reversed by additional deletion of NCAM (Weinhold et al., 2005; Hildebrandt et al., 2009). In contrast, other features are shared by polyST-deficient- and NCAM-knock out mice (reviewed by Hildebrandt et al., 2007). Most prominent in this category is the reduced size of the olfactory bulbs due to compromised migration of olfactory interneuron precursors from the subventricular zone (SVZ).

There are several links of aberrant polySia-NCAM expression to schizophrenia (Vawter, 2000; Brenneman and Maness, 2010). Among other findings, reduced levels of polySia in the hilus region of the hippocampus and in layers 4 and 5 of the dorsolateral PFC have been reported in schizophrenic patients (Barbeau et al., 1995; Gilabert-Juan et al., 2012). In humans, *NCAM1* and both polySTs, *ST8SIA2* and *ST8SIA4*, map to chromosomal regions that harbor susceptibility loci for schizophrenia (11q23.1, 15q26, and 5q21 for *NCAM1, ST8SIA2, and ST8SIA4*) (Lewis et al., 2003; Lindholm et al., 2004; Mazia et al., 2005). Moreover, three independent studies identified polymorphisms of *ST8SIA2* associated with the disease (Arai et al., 2006; Tao et al., 2007; McAuley et al., 2012). Similar associations were found with autism spectrum disorders and bipolar disorder (McAuley et al., 2012; Anney et al., 2010).

Dysfunction in schizophrenia includes imbalance of excitation and inhibition within cortical circuits and alterations of GABAergic neurons (reviewed by Di Cristo, 2007; Lewis et al.,...
This includes the loss of calcium binding proteins (reviewed by Reynolds et al., 2001). In particular, decreased mRNA levels of parvalbumin (PV) and somatostatin (Sst) as well as reduced densities of PV-positive cells were repeatedly observed in the PFC of schizophrenic patients (Beasley et al., 2002; Reynolds et al., 2002; Hashimoto et al., 2003; Morris et al., 2008; Fung et al., 2010). Similarly, reduced densities of specifically the PV- and Sst-positive interneurons were detected in the PFC of polySia-deficient mice (Röckle et al., submitted). These two interneuron subpopulations originate in the medial ganglionic eminence (MGE) and enter the pallium in two migratory streams. Starting at E12, an early stream of interneurons migrates into the marginal zone, while a second more prominent cohort of cells enters the intermediate zone (IZ) at E13 (reviewed by Métin et al., 2006; Gelman and Marín, 2010). The observation of aberrant patterns of tangentially migrating interneuron precursors in the IZ of $St8sia2^{-/-}$ and $St8sia4^{-/-}$ embryonic mice led us to hypothesize that compromised or delayed tangential migration of interneuron precursors might be causing the observed deficits in PV- and Sst-positive interneurons in the PFC of adult animals (Röckle et al., submitted). Direct proof of altered migration, however, is pending and underlying mechanisms are unresolved.

Here we address the effect of polySia deficiency on interneuron precursors and their migration. Using efficient labeling of GABAergic interneurons with the GAD67-GFP transgene (Tamamaki et al., 2003) and an in vitro system of acute, experimentally induced loss of polySia, we detected reduced velocities of interneurons migrating within the cortex, decreased numbers of cells leaving the MGE as well as shorter leading processes of migratory interneurons. Observations in co-cultures of MGE and pallium from $St8sia2^{-/-}$ and wildtype embryos indicate that a partial loss of polySia is sufficient to attenuate interneuron migration from the MGE into the pallium. Evidence for lower interneuron numbers is provided by analyzing polyST-deficient GAD67-GFP transgenic mice. Together, the data reveal a critical role of polySia for the migratory behavior of cortical interneurons and provide a mechanism of how even moderate reductions of polySia can lead to reduced densities of specific interneuron populations in the postnatal brain.
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. Knockout strains for ST8SIA2 (St8sia2<sup>-/-</sup> or 2<sup>-/-</sup>, Angata et al., 2004) and ST8SIA4 (St8sia4<sup>-/-</sup> or 4<sup>-/-</sup>, Eckhardt et al., 2000) have been backcrossed with C57BL/6J mice for at least six generations (Weinhold et al., 2005). GAD67-GFP mice (Tamamaki et al., 2003) were maintained on C57BL/6J background or crossed with St8sia2<sup>-/-</sup> and St8sia4<sup>-/-</sup>. Genotyping of St8sia2<sup>-/-</sup> and St8sia4<sup>-/-</sup> was performed by PCR as previously described (Weinhold et al., 2005). Genotyping of GAD67-GFP mice was performed with primers 5<sup>`</sup>-GGCACAGCTCTCCTCCTTTGTATG-3<sup>`</sup> (TR-1b), 5<sup>`</sup>-GCTCTCCTTTTCGGAGCG-3<sup>`</sup> (TR-3), and 5<sup>`</sup>-CTGCTTGTCGGACCATTGATAGAC-3<sup>`</sup> (TRGFP-8) to detect wildtype (TR-1b and TR-3) or transgene (TR-1b and TRGFP-8), respectively. Expression of GFP in embryos was monitored with a SteReo Discovery.V12 fluorescence stereomicroscope (Carl Zeiss Microimaging, Göttingen, Germany). For staging of embryos, the morning of the vaginal plug was considered as embryonic day (E) 0.5.

Semi-quantitative Western blotting

Forebrains of E13.5 embryos were dissected and lysed in 10 µl/mg of 20 mM Tris-HCl pH 8, containing 2% Triton X-100, 150 mM NaCl, and 5 mM EDTA. After centrifugation for 10 min, supernatants were mixed with reducing electrophoresis buffer. When indicated, endosialidase (endo, Stummeyer et al., 2005) was added to the supernatants at a concentration of 40 µg/ml and samples were incubated for 30 min on ice. Separation of 20 µg or, in the case of polySia detection, 40 µg of total protein per lane was performed on 12.5% SDS polyacrylamide gels. After electroblotting to nitrocellulose membranes, immunodetection was performed using the following polyclonal (pAb) and monoclonal (mAb) antibodies: 0.4 µg/ml Lhx6-specific rabbit pAb (Santa Cruz Biotechnology Inc., Santa Cruz, CA), 3 µg/ml GAD65/67-specific rabbit pAb (Sigma-Aldrich, Taufkirchen, Germany), 1:4000 of calbindin (CB) D-28-k-specific rabbit pAb (Swant, Bellinzona, Switzerland), 0.4 µg/ml NCAM-specific rat mAb H28 (IgG<sub>2a</sub>, Hirn et al., 1983), 1 µg/ml polySia-specific mouse mAb (IgG<sub>2a</sub>,735, Frosch et al. 1985), and 0.4 µg/ml GAPDH-specific mouse mAb (IgG<sub>1</sub>, Life Technologies, Darmstadt, Germany). Primary pAbs were detected with 25 ng/ml IgG-specific IRDye-680 and -800 conjugated antibodies (LI-COR Biosciences, Bad Homburg, Germany),
whereas mAbs were detected with 100 ng/ml IgG-subtype-specific IRDye-680 and -800 conjugated antibodies (Rockland, Gilbertsville, PA). Western blots were analyzed on an Odyssey near-infrared imaging system (LI-COR) and quantified using the Odyssey software (v3.0, LI-COR). Per group, three animals were analysed.

**Sectioning**

Three-month-old male mice and postnatal day one (P1) pups were deeply anesthetized with a mixture of 200mg/kg Ketamin (Gräub AG, Bern, Switzerland) and 8mg/kg Xylazin (Rompun, Bayer Health Care, Leverkusen, Germany) in 0.9% NaCl. Animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After dissection, brains were postfixed over night. Embryonic brains were fixed directly by immersion of the head in 4% paraformaldehyde overnight. 50-µm-thick coronal sections were obtained with a vibrating microtome (Leica Microsystems, Wetzlar, Germany). At least three mice were analyzed for each genotype and stage (E13.5, P1 and three months). To ensure precise staging of embryonic mice, *ST8sia4*/* and *ST8sia2*/* animals were analyzed in comparison to heterozygous littermates.

**Slice cultures, MGE-pallium co-cultures and MGE-explant cultures**

Embryos of the indicated age were dissected in ice-cold dissection buffer 1 composed of 126 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl₂, 1.2 mM NaH₂PO₄, 1.2 mM MgCl₂, 11 mM D-glucose and 25 mM NaHCO₃ in ddH₂O and embedded in 4% low-gelling agarose (AppliChem, Darmstadt, Germany) in PBS, pH 7.4. 225-µm-thick coronal sections were obtained with a vibrating microtome. When indicated, slices were preincubated for 30 min on ice with 4 µg/ml endo (dissolved from stocks of 2.24 mg/ml H₂O) in dissection buffer containing 10 mM HEPES, 1x penicillin/streptomycin (Biochrom, Berlin, Germany), and 50 µg/ml gentamycin (Sigma-Aldrich). When performing slice cultures, slices were transferred to Millicell cell culture inserts (PICM0RG50, Merck-Millipore, Darmstadt, Germany) in six-well-plates and incubated in growth medium 1 consisting of Neurobasal medium (Life Technologies) containing 1x B27 supplement (Life Technologies), 32 mM D-glucose, 2 mM L-glutamine, 1x penicillin/streptomycin and 4 µg/ml endo, where indicated, and cultured for one day at 37°C and 5% CO₂. For MGE-pallial co-cultures, slices were prepared as described above. The MGE was dissected and replaced by a MGE-fragment from a GAD67-GFP embryo of the same age. The polysialyltransferase-genotypes of the MGE and pallium explants are indicated respectively. Co-cultures were maintained for two days *in vitro* (div) as described
above. When performing live imaging, slices of E13.5 embryos were prepared as described, but cultivation was carried out in 8-well imaging slides (µ-slide 8 well, ibidi, Martinsried, Germany). Slices were embedded in rat-tail collagen type I (BD Biosciences, Heidelberg, Germany) at a concentration of 1.3 mg per ml of PBS containing 32 mM D-glucose and 2 mM L-glutamine. After gelling of the collagen matrix, growth medium 1 was added with or without 4 µg/ml endo and slices were incubated for 2 h before starting image acquisition. For MGE-explant cultures, MGEs were dissected from E13.5 GAD67-GFP mice, embedded in collagen as described above, and cultured for three days in 35 mm culture dishes (µ-Dish, ibidi).

MGE primary culture
MGEs of E13.5 GAD67-GFP mice were dissected in ice-cold dissection buffer 2 consisting of PBS, pH 7.4 containing 0.6% D-glucose, centrifuged and digested with 0.25 % trypsin (Biochrom) in dissection buffer 2 for 30 min at 37°C. After addition of 25% horse serum (Biochrom) and 100 µg/ml DNasel (Roche, Mannheim, Germany) cells were dissociated by gentle trituration with a 1000 µl pipette. Cells were pelleted at 200 x g for 10 min, washed three times with dissection buffer 2 and resuspended in growth medium 2, consisting of Neurobasal medium with 2 mM Glutamax (Life Technologies), 1x B27 supplement, 1x penicillin/streptomycin, with or without the addition of 4 µg/ml endo. For live imaging, 6.6 x 10^5 cells per ml were seeded in 8-well imaging slides (ibidi) coated with 6 µg/cm² rat tail collagen type I (BD Biosciences), 25 µg/cm² poly-D-lysine (Sigma-Aldrich) or Matrigel (BD Biosciences, diluted 1:60 in Neurobasal Medium). For immunofluorescence, 10^6 cells per ml were plated on cover slips coated with 25 µg/cm² poly-D-lysine (Sigma-Aldrich) and contained in 12-well-plates. Cells were cultured at 37°C and 5%CO₂. For immunofluorescence, cells were fixed after 1 div. For live imaging, cells were incubated for 2 h under standard conditions, prior to image acquisition in an Axiovert 200 M microscope with live imaging equipment (see below, section “microscopy”)

Immunofluorescence
Cells or brain sections were permeabilized for 15 min with 0.4% Triton X-100 in PBS, pH 7.4 at room temperature (RT) before blocking for 1 h with 2% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS / Triton X-100 at RT. Primary antibodies were dissolved in blocking buffer and incubated overnight at 4°C. CB D-28k-specific rabbit pAb (Swant) and parvalbumin (PV) -specific mouse mAb (IgG1, Swant) were applied according to
the manufacturer’s instructions. PolySia-specific mAb 735 (IgG2a) was used at 100 ng/ml. Rabbit and mouse IgG-specific Cy3- (Chemicon), Alexa488- and Alexa568- (Life Technologies) conjugated secondary antibodies were used as suggested by the suppliers. As first layer controls, samples were incubated in blocking solution lacking primary antibody. For double immunofluorescence staining, cross-reactivity of secondary antibodies was controlled by omitting either of the two primary antibodies. Specimen were mounted on glass object slides (Menzel) using Vectashield mounting medium with DAPI (Vector Laboratories).

**In situ** hybridization

E13.5 embryos were dissected in PBS-DEPC, pH 7.4, their heads were immediately transferred to Tissue-Tek OCT (Sakura, Alphen aan den Rijn, Netherlands) and frozen over dry ice. 20-µm-thick sections were obtained with a CM3050S cryostat (Leica) and mounted on object slides (SuperFrostPlus, Menzel, Braunschweig, Germany). cDNA from E13.5 whole mouse brain was amplified with primers 5´-TGCGCGTCCGGGTGGTTGGC-3´ (TK-5), 5´-GCCGCCCTCTGCTGCCTCTTC-3´ (TK-6), 5´-ACTGGCCGACAGTCAGTTTC-3´ (TK-ISH-17) and 5´-AGCATTTCCAGACTTGTGCC-3´ (TK-ISH-18) to generate templates for the synthesis of riboprobes against *Lhx6* (TK-5 and TK-6) or *St8sia2* (TK-ISH-17 and TK-ISH-18), respectively. Sizes of amplified products were 579 bp (*Lhx6*) and 657 bp (*St8sia2*). DIG-UTP labelled riboprobes were generated by *in vitro* transcription. Primers were elongated with promoters for T7-RNA-polymerase (5´-GCGTAATACGACTCACTATAGGG-3´) in case of *Lhx6*-antisense, *Lhx6*-sense and *St8sia2*-antisense probes, and for SP6-RNA-polymerase (5´-GCGATTTAGGTGACACTATAGAA-3´) in case of *St8sia2*-sense. Riboprobes were diluted to equal concentrations in hybridization buffer composed of 50% formamide, 10% dextran sulfate, 600 mM NaCl, 1mM EDTA, 10 mM Tris-HCl, pH 7.5, 1x Denhardt’s Reagent (Life Technologies), 100 µg/ml salmon sperm DNA (Sigma-Aldrich) in ddH₂O-DEPC. Per slide, 100 µl of riboprobe was applied, covered by a 24 x 50 mm cover slip and hybridized over night at 55°C in a humidified chamber. For stringency washing of sections, SSC buffer consisting of 150 mM NaCl and 50 mM sodium citrate, pH 7.0 (concentrations of salts for 1x SSC buffer) was used. Slides were washed for 20 min each in 2x SSC, 1x SSC, 0.5x SSC and 0.2x SSC at RT, for 60 min in 0.2x SSC at 68°C and for 15 min in 0.2x SSC at RT. After washing for 15 min in 100 mM Tris-HCl, pH 7.5, with 150 mM NaCl (TBS), slices were blocked for 2 h in TBS with 0.5% blocking reagent (Roche) at RT. Alkaline phosphatase (AP) -conjugated anti-DIG antibody (Roche) was diluted 1:5000 and sections were incubated over night at 4°C under cover slips. After washing in TBS for 15 min sections were equilibrated in
AP buffer consisting of 100 mM Tris-HCl, pH 9.5, with 100 mM NaCl and 50 mM MgCl₂ for 10 min at RT before reacting with AP buffer containing 165 µg/ml 5-bromo-4-chloro-indolyl-phosphate (BCIP) and 330 µg/ml nitro blue tetrazolium chloride (NBT).

**Microscopy and data acquisition**

Microscopy was performed using an Axiovert 200 M microscope equipped with an ApoTome device for near confocal imaging, AxioCam MRm digital camera and AxioVision software v4.82 (Carl Zeiss Microimaging, Göttingen, Germany). Fluorescence images were acquired using the MosaiX software module that permits the sample area to be scanned in order to generate one single large image. MGE primary cultures were acquired using a 20x Plan-Apochromat objective (numerical aperture of 0.8), coronal sections from embryos, P1 mice and three-month-old mice, as well as of MGE-pallial co-cultures with a 10x Plan-Apochromat objective (numerical aperture of 0.45), and slice cultures as well as MGE-explant cultures after 3 div with a 5x Plan-Neofluar objective (numerical aperture of 0.15, all Zeiss). Images of single CB-positive interneurons were acquired with a 63x Plan-Apochromat oil-immersion objective (numerical aperture of 1.4, Zeiss). MGE-explant cultures at t=0 were documented by phase contrast microscopy at 5x magnification. *In situ* hybridization was monitored by using brightfield illumination at 10x magnification and images were merged using the transparency and overlay functions in CorelDraw X3 (Corel, Fremont, CA) as indicated.

Area measurements, cell counting and estimation of leading process extents were performed with the assistance of AxioVision software. Densities of GFP- and PV-positive cells in the medial prefrontal cortex (PFC) of P1 mice and three-month-old mice were estimated by counting respective cells in relation to the assessed area. The subdivision of the medial PFC into cingulate cortex area 1 (Cg1) and upper and deep cortical layers (corresponding to layers 1 to 3 and 5 to 6, respectively) was achieved according to Paxinos and Franklin (2001). To analyze the pattern of GAD67-GFP-positive cells in slice cultures, 2-3 sections per animal were obtained from a total of 9 animals and incubated in the presence or absence of 4 µg/ml endo. MGE-pallial co-cultures were analyzed from 4 preparations, each consisting of one GFP-positive MGE-donor and one GFP-negative host animal. MGE-explant cultures were carried out using tissue from 3 animals giving rise to a total of 18 explants that were incubated in the presence or absence of 4 µg/ml endo. With the aid of AxioVision software, the area of the explants was estimated at t=0. Images of cultures after 3 div were exported to imageJ software (Schneider et al., 2012) in TIFF format and the area covered by GFP-
positive cells was analyzed. Areas at t=0 were subtracted from the respective areas at 3 div, resulting in areas covered by migrating cells during cultivation of the explant.

Densitometric quantification of polySia was carried out using imageJ software. With the aid of the ApoTome technology, optical sectioning of the samples was achieved using identical settings among different specimen. Ten consecutive optical sections were merged into one image using AxioVision software, exported to imageJ in TIFF format and “mean grey values” of the intermediate zone migratory stream or the somata of individual CB-positive cells were determined. Two independent sets of experiments were analyzed, each set consisting of 4-6 sections obtained from one animal per genotype.

Live imaging was performed at 37°C and 5% CO₂ using a microscope incubator (Pecon, Erbach, Germany). Time lapse sequences were generated by acquiring images with a 10x objective at an interval of 2 min for up to 14 h. For the quantification of interneuron precursor velocity, a sequence of 180 min (90 images) starting 4 h after the onset of image acquisition was analyzed. Only cells visible throughout the entire sequence were used for analysis and migratory paths were reconstructed using the AxioVision software. In order to summarize the direction of migration, all obtained paths were centred to a common starting point.

Leading processes of GAD67-GFP-positive interneurons were evaluated in slice and MGE primary cultures. Only cells with a clearly discernible leading process were analyzed. Lengths were determined by tracing the process from the tip of the growth cone to its onset defined as the site of somal constriction.
Results

Analyses of polyST-deficient GAD67-GFP mice imply a loss of interneurons

For analyses of interneuron development we used GAD67-GFP transgenic mice, in which GABAergic cells are efficiently labeled (Tamamaki et al., 2003). \( St8sia4^{--} \) and \( St8sia2^{--} \)-GAD67-GFP mice were generated and reductions of interneuron densities as shown previously in the \( 4^{--} \) and \( 2^{--} \) lines (Röckle et al., submitted) were verified in these new lines by analyses of PV-positive interneurons in the cingulate cortex area 1 (Cg1) of three-month-old animals. As expected, all of the PV-positive cells were also positive for GFP (Fig. 1a) and densities of PV-positive cells were significantly reduced in Cg1 of \( 4^{--} \) and \( 2^{--} \)-GAD67-GFP mice (Fig 1a,b). Importantly, densities of the entire population of GAD67-GFP-positive interneurons were also reduced in both polyST-deficient lines (Fig. 1c). The finding that not only protein levels of PV, but also the numbers of the GFP-positive interneurons were diminished strongly suggests a loss of interneurons earlier in development. We therefore also estimated interneuron densities in the medial PFC at postnatal day 1 (P1) and found that in both polyST-deficient lines, densities of GFP-positive interneurons were significantly reduced (Fig. 1d).
Fig. 1 Densities of interneurons expressing GFP and parvalbumin (PV) in the prefrontal cortex (PFC) of St8sia4−/− (4−/−), St8sia2−/− (2−/−) and wildtype (ctrl)-GAD67-GFP mice. (a) Representative images illustrating the colocalization of PV (red) and GFP (green) on cells of the cingulate cortex area 1 (Cg1) of the PFC of 3-month-old mice. Scale bar: 25 µm. (b,c) Densities of GFP-positive (GFP+) and of PV-positive (PV+) interneurons in Cg1 of 3-month-old mice. (d) Densities of GFP+ cells in the PFC of postnatal day one (P1) mice. Per group (genotype), mean values ±SEM from n=3 animals are plotted. Results from one-way ANOVA are indicated and for ANOVA with $P<0.05$, Newman-Keuls post hoc test was applied. *, **, significant difference against control with $P<0.05$ or $P<0.01$, respectively.
No evidence for altered interneuron specification in polyST-deficient mice

The LIM-containing homeodomain transcription factor Lhx6 is induced in the MGE and maintained in PV- and Sst-positive interneurons (Liodis et al., 2007; Du et al., 2008), while calbindin (CB) is an early marker of tangentially migrating interneurons (Anderson et al., 1997; Polleux et al., 2002). To assess the possibility that the observed interneuron deficits of polyST-deficient mice (Röckle et al., submitted) result from abnormal specification of GABAergic neuron progenitors in the MGE, protein levels of Lhx6, CB and the common GABAergic marker glutamic acid decarboxylase (GAD) were analyzed by Western blotting of forebrain lysates from E13.5 ctrl, $4^{-/-}$ and $2^{-/-}$ mice. No significant differences in the levels of Lhx6, CB or the GAD immunoreactive bands at 65 and 67 kDa were detected (Fig. 2). This result argues against major changes of interneuron specification.

Fig. 2 Quantification of Lhx6, glutamic acid decarboxylase 65/67 (GAD65/67) and calbindin (CB) in the forebrain of St8sia4^{-/-} ($4^{-/-}$), St8sia2^{-/-} ($2^{-/-}$) and control (ctrl) mice at E13.5. (a) Representative Western blots illustrating the levels of Lhx6, GAD65/67 and CB. GAPDH was used as loading control. (b-d) Densitometric evaluation of Lhx6, GAD65/67 and CB signals as shown in (a). Mean values ±SEM from n=3 animals per group are plotted. GAPDH was used as internal control. Results from one-way ANOVA are indicated. n.s., not significant ($P>0.05$).
Ablation of ST8SIA4 or ST8SIA2 leads to decreased polySia levels in the area of tangential interneuron migration

Changes of polySia levels in 4\(^{-/-}\) and 2\(^{-/-}\) mice were addressed one day after the onset of interneuron migration from the MGE into the developing cortex, which in the mouse starts at E12.5 (Corbin and Butt, 2011). Lysates obtained from E13.5 forebrains were analyzed by Western blotting. As shown in Fig. 3a, signals obtained with the polySia-specific mAb 735 match the well-known size of polySia-NCAM, but were clearly distinct from bands obtained for alternative polySia acceptors such as polySia-neuropilin2 or polySia-SynCAM1 (Curreli et al., 2007, Galuska et al., 2010). These signals were completely abolished by treatment of the lysate with the enzyme endosialidase (endo) to specifically remove polySia (Fig. 3a, right lane). In contrast, the NCAM-specific mAb H28 yielded only weak signals in the untreated control, but sharp bands, indicative of polySia-free NCAM-140 and -180 were clearly increased after endo treatment (Fig. 3a, lower panels). It may appear puzzling at first sight that no NCAM signals were obtained at the level of the polySia bands in the untreated control. It is, however, a frequent observation that mAb H28 binds preferentially to the polySia-free form of NCAM, most likely because it recognizes an epitope in the extracellular domain, which is shielded in the presence of polySia (see Oltmann-Norden et al., 2008 for an example). Together, these data clearly indicate that NCAM is the primary target of polysialylation in the embryonic forebrain.

Consistent with previous results from early postnatal brain (Galuska et al., 2006, Oltmann-Norden et al., 2008), comparative analysis of E13.5 forebrain lysates revealed reduced levels of polySia in 2\(^{-/-}\) but not in 4\(^{-/-}\) animals (Fig. 3b,c). For a more focused assessment of polySia levels, intensities of polySia immunofluorescence signals were analyzed in the area of tangential interneuron migration. To identify the migratory streams of E13.5 mice, cortical interneurons were labeled by CB immunofluorescence (Anderson et al., 1997; Polleux et al., 2002). As illustrated in Fig. 3d-f, the intensity of polySia immunofluorescence signals in the area of CB-positive cells migrating in the IZ was significantly reduced in 2\(^{-/-}\) and 4\(^{-/-}\) embryos.
Fig. 3 Quantification of polysialic acid (polySia) in the forebrain of St8sia4−/− (4−/−), St8sia2−/− (2−/−) and control (ctrl) mice at E13.5. (a,b) Representative Western blots illustrating the levels of polySia and NCAM in forebrain lysates. In (a) lysates were incubated in the absence (ctrl, left lane) or presence of 40 µg/ml endosialidase (endo, right lane, see text for details). In (b), GAPDH was used as loading control. Note that the same polySia signals are shown as controls in (a) and (b). (c) Densitometric evaluation of polySia signals from Western blots as shown in (b). Mean values ±SEM from n=3 animals per group are plotted. GAPDH served as internal control. (d) Representative images illustrating the expression of calbindin (CB, red) and polySia (green) on coronal forebrain sections. The area of the intermediate zone (IZ) migratory stream is delineated by a dashed line. Scale bar: 100 µm. (e) Detail from (a, ctrl), illustrating colocalization of polySia and CB on interneurons (arrowheads) of the IZ migratory stream. Asterisks mark strongly labeled blood vessels. (f) Quantification of polySia signal intensity in the area of the IZ migratory stream [boxed in (d)]. Mean grey values from matched immunofluorescence images were analyzed and plotted as arbitrary units. Strongly labeled blood vessels were excluded from analysis. Mean values from n=7-8 sections per group (1 animal per group) are plotted. Reproduction of the experiment yielded comparable results (data not shown). (c,f) Results from one-way ANOVA are indicated and Newman-Keuls post hoc test was applied for ANOVA with P<0.05. ***, **, and *, significant difference between indicated groups with P<0.001, P<0.01, and P<0.05 respectively.
Fig. 4 Live imaging of migrating interneurons in E13.5 GAD67-GFP (GFP+) coronal brain slices, cultivated in the absence (ctrl) or presence of endosialidase (endo, 4 µg/ml). (a) Selected frames from representative time-lapse recordings of GFP+ interneurons migrating at different velocities. Images at 0, 60, 120, and 180 min recording time are shown and tracks of the marked cells (arrowheads) are summarized in the last panel of each row. Scale bar 20 µm. (b) Velocities of GFP+ interneurons in the presence or absence of endo, as indicated. Mean values ±SEM from n=4 slices are plotted (a total of 385 cells for ctrl and 504 cells for endo were evaluated). (c) Velocities of cells evaluated in (b) were grouped in bins and depicted as percentage of all cells evaluated for each condition. Mean values ±SEM from n=4 slices are plotted. (d) For each GFP+ cell analyzed, the net direction of migration was determined and results are plotted as percentage of cells, migrated into the indicated quadrant. Mean values ±SEM from n=4 slices are plotted. (e) Detailed representation of the direction of migration for each cell from (d). (b, c) Results from student’s t-test are indicated. ** and *, significant difference with $P<0.01$ and $P<0.05$, respectively.
Loss of polySia inhibits interneuron migration in vitro

Expression of the GAD67-GFP transgene starts in the MGE prior to the onset of interneuron migration (Tamamaki et al., 2003). Thus, coronal slice cultures of E13.5 GAD67-GFP mice could be employed to assess interneuron migration from the MGE into the pallium (Britto et al., 2006). To directly monitor the impact of polySia on the process of tangential interneuron migration endo was used to remove polySia. Efficient degradation of polySia after one day in vitro (div) was confirmed by loss of polySia immunofluorescence (Suppl. Fig. 1). Analyses by time lapsed live cell imaging over a period of 3 h revealed a wide range of different velocities of migrating interneurons (Fig. 4a). In the presence of endo, the mean velocity of precursors was significantly reduced (Fig. 4b). Grouping into categories yielded an increase in the fraction of cells migrating less than 30 µm/h, at the expense of cells with an intermediate velocity between 30 and 60 µm/h (Fig. 4c). The fraction of cells migrating faster than 60 µm/h was not different between controls and endo-treated slices. With or without endo treatment the migrating GAD67 GFP cells in the slice cultures showed no preference for direction (Fig. 4d,e).

Having established that acute loss of polySia attenuates migration of interneurons within the pallium at E13.5, we sought to investigate, if polySia-deficiency affects already the initial transition from the MGE into the pallium. For this purpose, coronal slices were obtained from E12.5 GAD67-GFP brains and cultured in the presence or absence of endo before numbers of GFP-positive cells were evaluated in bins of 100 µm starting at the pallial-subpallial boundary. After one day of culture in the presence of endo, significantly fewer cells were detected within each of the first three bins of the pallium (Fig. 5a,b). Cells intruded for more than 300 µm were not affected. We also analyzed the distribution of interneurons along the ventricular-pial axis of the dorsal telencephalon, but we could not detect any differences between controls and endo-treated slices (Fig. 5c). These in vitro data imply that loss of polySia attenuates interneuron recruitment from the MGE into the pallium, while laminar distribution is unchanged.
Fig. 5 Analysis of the effect of polySia-deficiency on interneuron migration into the pallium in embryonic GAD67-GFP (GFP+) slice cultures and co-cultures of MGE and pallium. (a) Representative images of coronal slice cultures from E12.5 GFP+ brains, cultivated for one day in vitro in the absence (ctrl) or presence of endosialidase (endo, 4 µg/ml). Borders of the tissue are outlined. Scale bar: 100 µm. (b) Migration of GFP+ cells was analyzed by estimating cell numbers in bins with distances of 100 µm, starting at the pallial/subpallial boundary. Mean values ±SEM from n=9 animals per group are plotted. (c) The distribution of GFP+ cells across arbitrary layers of the pallium (I-VI) was analyzed and results are depicted as percent of total cells per condition. Mean values ±SEM from n=9 animals per group are plotted. (d) Representative images of co-cultures from GFP+ MGEs and GFP-negative pallial tissue. As indicated, co-cultures of St8sia2+/+(wt)-MGEs and St8sia2−/−(2−/−)-pallium were analyzed in comparison to co-cultures of 2−/−-MGEs and 2+/+ -pallium. Tissue borders are outlined. Scale bar: 100 µm. (e) Migration of GFP+ cells was analyzed by estimating cell numbers in bins with distances of 100 µm, starting at the co-culture intersection. Mean values ±SEM from n=4 independent co-cultures per group are plotted. Results from student’s t-test are indicated. ** and *, significant difference with \( P<0.01 \) and \( P<0.05 \), respectively.

In a next step, we tried to address the question if the observed deficits in interneuron migration depend on reduced levels of polySia either in the pallium as the target region and cellular environment of the tangentially migrating cells, or on the MGE-derived interneurons themselves, or both. To this end, we performed cultures of MGE explants dissected from E13.5 2−/−-GAD67-GFP mice and placed adjacent to GFP-negative pallial tissue from age-matched 2+/+ animals. Vice versa, co-cultures of 2+/+ -GAD67-GFP MGEs and pallium from
GFP-negative $2^{+/c}$ mice were generated (Fig. 5d). We chose $2^{+/c}$ rather than $4^{+/c}$ animals in this set-up, because only the $2^{+/c}$ mice displayed a robust reduction of polySia on the level of the entire forebrain including pallium and MGE (see Fig. 3, above). After cultivation for 2 days, densities of GFP-positive cells that have entered the respective pallial tissue were evaluated. Highly reminiscent to the results obtained by complete enzymatic removal of polySia in slices comprising MGE and pallium (Fig. 5a,b), the numbers of GFP-positive cells in the pallium close (<300 µm) to the border with the MGE were significantly lower in the co-cultures of $2^{+/c}$ MGEs and wildtype pallium (Fig. 5e). Again, numbers of cells at longer distances (>300 µm) were not altered. These findings support the hypothesis that adequate levels of polySia on the MGE-derived interneurons are required for proper migration.

Fig. 6 Expression of St8sia2 in the E13.5 mouse forebrain. Representative images of in situ hybridization for Lhx6 and St8sia2. An asterisk marks the medial ganglionic eminence. The merged image in the upper panel was produced by overlaying pseudocolored images of St8sia2 (green) and Lhx6 (red) signals obtained from two consecutive sections. In the lower panel, images of control (ctrl) hybridization with the respective sense-probes are shown. hi, hippocampus; lv, lateral ventricle, ctx, cortex, LGE, lateral ganglionic eminence. Scale bar: 200 µm.

In situ hybridization was performed to analyze the expression pattern of St8sia2 in the E13.5 mouse brain. In addition, expression of Lhx6 was addressed, which strongly labels the MGE and therefore was used to identify this structure. As shown in Fig. 6, St8sia2 and Lhx6
signals were clearly colocalized. This documents that St8sia2 is expressed in the MGE. In contrast, no clear signals of St8sia2 expression were obtained in the pallium. The finding of reduced polySia levels around the interneuron stream in the IZ of 2−/− embryos (see Fig. 3) suggested a contribution of ST8SIA2 to polySia synthesis in the pallium, but it remained open, if ST8SIA2 is involved in polySia synthesis of interneurons that have left the MGE. To address this question, intensities of polySia immunofluorescence signals directly associated with CB-positive migratory interneurons of the IZ were evaluated on sections of 2−/− and control embryos at E13.5 (Fig. 7a). Significantly reduced intensities were detected in 2−/− embryos (Fig. 7b) indicating that the loss of ST8SIA2 affects polySia levels of migrating interneurons in the cortex.

![Image](image_url)

**Fig. 7** Quantification of polysialic acid (polySia) on calbindin (CB)-positive interneurons in the pallium of E13.5 control (ctrl) and St8sia2+/− (2+) mice. (a) Representative images, illustrating the colocalization of polySia (green) and CB (red). Scale bar: 10 µm. (b) polySia signal intensity on CB-positive interneurons was quantified. Mean values ±SEM from n=9-12 cells per group are plotted. The result from student’s t-test is indicated. **, significant difference with P<0.01.
PolySia-deficient migratory interneurons have shorter leading processes

Migrating interneurons originating in the MGE show a stereotyped dynamic behavior of leading process protrusion and subsequent saltatory translocation of the nucleus (Bellion et al., 2005). Leading process dynamics is intimately linked to directional guidance of migrating cells, as it is assumed that they sample their environment for guidance cues (reviewed by Ayala et al., 2007; Valiente and Marín, 2010). To study the potential impact of polySia on the morphology of the leading process, coronal slices of E12.5 GAD67-GFP mice were cultured in the presence or absence of endo and the lengths of leading processes were analyzed after 1 div. Compared to controls, leading processes of endo-treated GFP-positive interneurons were significantly shorter (Fig. 8a,b). To analyze, if this is a cell-autonomous effect independent from the cellular environment, primary cultures of dissociated E13.5 MGEs were prepared. Cultures consisting of >80% GFP- and polySia-positive cells were obtained (Fig. 8d). Cultures were run for two days, but efficient migration was not observed on any of the substrates tested (poly-D-lysine, collagen, Matrigel, data not shown). Nevertheless, live cell imaging revealed a highly dynamic pro- and retraction of primary processes (Fig. 8c) and after cultivating primary cells for 1 div in the presence of endo, significantly shorter primary processes were detected (Fig. 8d,e). This points towards a cell-autonomous role of polySia in leading process formation and provides a possible mechanism of how polySia deficiency may affect interneuron migration under in vivo conditions.
Fig. 8 Removal of polysialic acid (polySia) leads to decreased lengths of interneuron leading processes in slice- and medial ganglionic eminence (MGE)-primary cultures of embryonic GAD67-GFP mice. (a) Representative images of embryonic day (E) 12.5 slice cultures, cultured for one day in vitro in the absence (ctrl) or presence of endosialidase (endo, 4 µg/ml). Arrowheads mark the end of a primary process. Scale bar: 20 µm. (b) Lengths of leading processes of GFP-positive cells in slice cultures, as shown in (a). Mean values ±SEM from n=8 animals are plotted. (c) Selected frames of a time-lapse recording of a E13.5 MGE-primary culture illustrating the dynamics of leading processes of non-migrating interneurons. Scale bar: 10 µm. (d) Representative images of GFP-positive cells in E13.5 MGE-primary cultures cultivated for one day in the presence or absence of endo (4 µg/ml) and stained for polySia, as indicated. Nuclei were counterstained with DAPI. The last image in each row shows a higher magnification of the area boxed in the merged image (GAD67-GFP, green; polySia, red; DAPI, blue). Scale bar: 10 µm. (e) Lengths of leading processes of GFP-positive cells in primary cultures, as shown in (d). Mean values ±SEM from n=218 and n=211 cells (ctrl and endo) are plotted. Results from student’s t-test are indicated. *** and **, significant difference with *P*<0.001 and *P*<0.01, respectively.
In order to further investigate a possible cell-autonomous role of polySia for the process of interneuron migration, we conducted MGE-explant cultures from E13.5 GAD67-GFP mice. In these explant cultures collagen was used as a polySia-free matrix. After 3 days of culture in the presence or absence of endo, considerable amounts of cells have migrated into the matrix (Fig. 9a). However, no differences between the area occupied by migrating cells in control and endo-treated cultures were detectable (Fig. 9b). Thus, polySia-deficiency of the MGE does not impair the migratory performance of interneuron precursors into an artificial, cell-free environment. Together with the cell autonomous change of interneuron process lengths even in the absence of migration, these observations support the idea that additional environmental factors are needed for efficient tangential interneuron migration in the pallium.

Fig. 9 Estimation of interneuron precursor migration in medial ganglionic eminence (MGE)-explant cultures from embryonic day 13.5 GAD67-GFP embryos, cultured in collagen in the absence (ctrl) or presence of endosialidase (endo). (a) Representative images of MGE-explants at the onset of the experiment (t=0, upper panel; acquired under brightfield illumination) and after three days in vitro (3 div, lower panel; images of GFP fluorescence). Scale bar: 100 µm. (b) After 3 div, the area occupied by GFP-positive cells was analyzed. Mean values ±SEM from n=3 animals per group are plotted. Student’s t-test revealed no significant difference.
Discussion

We recently described specific reductions of PV- and Sst-positive interneuron populations in the PFC of polySia-deficient mice (Röckle et al., submitted) and proposed that deficits in tangential interneuron migration might cause these alterations. We now tested this hypothesis and found a loss of PFC interneurons in ST8SIA2- and ST8SIA4-deficient GAD67-GFP mice, which indeed establishes during embryonic development. As shown by enzymatic removal of polySia in slice cultures, acute loss of polySia hampered the entry of interneurons from the MGE into the pallium, reduced the speed of a fraction of interneurons migrating at an intermediate velocity, but had no effect on the laminar distribution of interneurons within the pallium. We also present evidence that loss of polySia affects migratory interneurons independent from environmental factors pointing towards a cell autonomous mechanism of polySia-dependent interneuron migration.

Reduced polySia causes slower migration and loss of interneurons

Expression of ST8SIA2 and ST8SIA4 has been demonstrated in the embryonic mouse brain and mRNA levels of both enzymes increase steeply between E12.5 and E14.5, a period of extensive interneuron precursor migration (Ong et al., 1998; Schiff et al., 2009). Nevertheless, at E13.5 polySia levels were virtually unchanged in forebrain extracts of St8sia4−/− and only moderately decreased in St8sia2−/− mice. This is consistent with the enzymatic compensation found during early postnatal development of single polyST knockout mice (Galuska et al., 2006; Oltmann-Norden et al., 2008) but seems to contradict the assumed impact of both polySTs on interneuron migration. Around the migratory stream in the IZ however, reductions of polySia were evident and thus may cause the aberrant patterns of tangential interneuron migration in St8sia2−/− and St8sia4−/− embryos described previously (Röckle et al., submitted).

Cortical integration of interneurons follows a classical “inside-out” pattern, where early-born neurons from the MGE populate preferentially the deep cortical layers before they disperse by changing from tangential to radial migration (reviewed by Marín and Rubenstein, 2003; Miyoshi and Fishell, 2011). The in vitro data obtained in the current study demonstrates that reduced migration upon polySia-deficiency is not associated with altered patterns of laminar distribution. This is consistent with findings of our previous study showing that the reductions of PV- and Sst-positive interneurons in ST8SIA2- and ST8SIA4-deficient mice were found
across all layers (Röckle et al., submitted). Together the data indicate that polySia-deficiency affects tangential migration rather than the process of radial sorting and laminar distribution of cortical interneurons.

The clear requirement of polySia for invasion and subsequent migration of cortical interneurons in the pallium strongly suggests that the loss of interneurons, as detected in the PFC of polyST-deficient mice, is at least in part caused by migration deficits. Other studies support such a causal relationship between disturbed tangential migration of interneurons and their reduction in the cortex. In mice deficient for the brain-derived neurotrophic factor (BDNF)-receptor TrkB, as well as in mice that lack urokinase-type plasminogen activator receptor (u-PAR), an enzyme that activates hepatocyte growth factor (HGF), tangential interneuron migration is compromised and less interneurons are present in either the embryonic or the neonatal frontal and parietal cortex (Polleux et al., 2002; Powell et al., 2001). Similar to the polySia-deficient mouse models, the laminar distribution of interneurons is not disturbed in the TrkB- and u-PAR-mutant mice and the authors of both studies therefore conclude that impairments of interneuron migration into the pallium lead to the observed reductions of these cells in the cortex.

When migrating, interneurons undergo a characteristic sequence of nucleokinesis and somal translocation, which is initiated by movements of the centrosome and the Golgi apparatus into a highly dynamic leading process (Bellion et al., 2005). Tangentially migrating interneurons show a binary leading process architecture that is set up in order to choose the direction of migration (Martini et al., 2009). Thus, leading process motility is a crucial component of the migratory cycle and essential for sampling of guidance cues (reviewed by Ayala et al., 2007; Valiente and Marin, 2010). Upon removal of polySia in slice cultures, we detected a significant reduction of the lengths of interneuron leading processes. This defect also established in primary cultures devoid of the environmental factors that may be relevant for migration in situ and it was observed in the absence of migration, i.e. independent of nucleokinesis. It therefore seems likely that loss of polySia has a cell-autonomous effect on leading process dynamics and this may be a key mechanism by which polySia impacts cortical interneuron migration.

There are prominent examples of cell-autonomous migration deficits of cortical interneurons that are characterized by altered leading process morphology. In mice deficient for the
microtubule-associated protein doublecortin (DCX), migrating cortical interneurons extend shorter leading processes that display increased branching (Friocourt et al., 2007; Kappeler et al., 2006). Interestingly, these mice also exhibit aberrant migration of olfactory bulb interneuron precursors as well as deficits of specifically the CB-positive interneurons in the periglomerular layer of the olfactory bulb (Koizumi et al., 2006). Both features have also been found in polySia-deficient mice (Ono et al., 1994; Hu et al., 1996; Chazal et al., 2000, Röckle et al., submitted) highlighting remarkable similarities between the cell-autonomous function of a protein involved in cytoskeletal organization and polySia as a cell surface factor in the migration of interneurons. Altered leading process morphology leading to cell-autonomous migration deficits has also been described for mice, deficient for the cytoskeleton-associated factor Lissencephaly-1 (LIS1) (Nasrallah et al., 2006), as well as after knockdown of the centrosome-associated protein Disrupted-in-schizophrenia 1 (DISC1) in embryonic mice (Steinecke et al., 2012). In these cases, longer leading processes were observed and this is discussed as an indication of impaired nucleokinesis rather than altered leading process dynamics. Together, these examples highlight that altered leading process morphology, as observed in the current study, is a feature of cell-autonomous migration deficits of cortical interneurons.

Such a cell-autonomous role of polySia is unexpected, because so far, polySia has been reported to regulate interactions of a cell with its environment, either by modulating NCAM-mediated or NCAM-independent cellular interactions, or by acting as a scavenger of soluble factors, to facilitate interactions with the respective cell surface receptors, as suggested for BDNF (for review, see Hildebrandt et al., 2007). However, Conchonaud et al. (2007) observed that removal of polySia with endo decreased the lateral membrane diffusion of NCAM and concluded that this regulation of membrane-associated dynamics provides an intrinsic mechanism which impacts migration of polySia-positive tumor cells. A potential role for polySia-NCAM in the membrane-dependent regulation of cell motility is also implied by a study from Diestel et al. (2007) showing that NCAM is endocytosed and recycled in neurons. The role of polySia in this process has not been addressed, but the authors hypothesize that the NCAM-dependent membrane trafficking may be involved in the regulation of cellular motility, possibly by its effects on integrin membrane turnover, since NCAM has been shown previously to regulate integrin-dependent cell migration in rat neuroblastoma cells (Diestel et al., 2005). In line with this model, polySia may regulate interneuron migration and particularly leading process dynamics by influencing NCAM-dependent membrane trafficking.
Deficits in neuronal migration may be linked to neurodevelopmental and neuropsychiatric disease

Together with previous observations (Röckle et al., submitted), the current data demonstrate that polySia-deficiency leads to defective developmental migration of MGE-derived interneurons and to reduced densities of PV- and Sst-positive interneurons in the PFC. These findings provide a possible link between neurodevelopmental deficits and the emerging evidence that genetic variation in NCAM1 or ST8SIA2 may be associated with schizophrenia. Multiple lines of evidence suggest that a loss of excitatory / inhibitory balance in specific networks may be responsible for some of the symptoms observed in several psychiatric disorders (Di Cristo, 2007) and abnormal inhibitory function in the PFC might cause cognitive disturbances like deficits in attention and working memory observed in schizophrenic patients (Lewis et al., 2005; Uhlhaas and Singer, 2010; Farzan et al., 2010). Alterations of PV and Sst expression are among frequently reproduced findings in schizophrenia and although results are conflicting between studies, reduced numbers of PV-positive interneurons have been found in the Cg1 of schizophrenic patients (Benes et al., 1991; Beasley et al., 2002; Reynolds et al., 2002; Hashimoto et al., 2003; Morris et al., 2008; Fung et al., 2010; Marín, 2012). Similarly, associations between altered migration of interneurons and schizophrenia have been shown for the cell surface receptor ERBB4. Activation of ERBB4 by neuregulin 1 (NRG1) is required for attraction of interneurons to the cortex and decreased ERBB4 signaling is implicated in the susceptibility to schizophrenia (Flames et al., 2004; Gambarotta et al., 2004; Corfas et al., 2004). Another example is DISC1 that is reported as risk factor for schizophrenia (Marín, 2012). Deletion or knockdown of DISC1 leads to accumulation of late-born neurons in the SVZ and the intermediate zone (Kamiya et al., 2005; Duan et al., 2007). Overexpression of a truncated human form of DISC1 in mice leads to reduced PV immunoreactivity in the PFC of three-month-old mice (Hikida et al., 2007). In analogy to these examples, the combined consideration of the current results obtained in mice and evidence from patients indicates a possible link between polySia-dependent interneuron migration and a neurodevelopmental predisposition to schizophrenia.

Altogether our results demonstrate the polySia-levels are critical for the migration of MGE-derived interneurons, strongly suggesting that altered migration is at least one cause for the observed loss of these cells in the perinatal and postnatal PFC of polySia-deficient mice. Despite the abundance of residual polySia in St8sia2−/− mice, migration of ST8SIA2-deficient
interneurons into the pallium is reduced. This reveals that subtle reductions of polySia levels lead to a defective migration of MGE-derived interneurons and points towards a possible link between genetic variation in polyST genes and interneuron pathology as observed in neuropsychiatric disease. Based on the decreased densities of GABAergic interneurons in the PFC, we suggest polyST-deficient mice as an animal model to study the consequences of inhibitory dysbalance as observed in neuropsychiatric disorders like schizophrenia. Future work should focus on the behavioral analyses of \textit{St8sia2}^{+/−} and \textit{St8sia4}^{+/−} mice, in order to investigate, if interneuron deficits are associated with altered schizophrenia-related and PFC-dependent behavior in tests of cognition and sensory functions.
References


Röckle, I, Kröcher, T, Weinhold B, Burkhardt H, Hildebrandt H (submitted) Altered densities and compromised migration of interneurons in the forebrain of mice deficient for polysialic acid or NCAM. submitted to Cerebral Cortex


Supplementary material

Suppl. Fig. 1 Polysialic acid (polySia) in slice cultures can be efficiently removed using endosialidase (endo). Coronal slices of embryonic day 12.5 GAD67-GFP (GFP, green) brains were cultured for one day in vitro in the absence (ctrl) or presence of 4 µg/ml endo. After fixation, sections were stained with polySia-specific antibody (red). Scale bar: 20 µm.
Chapter 3 - Schizophrenia-like phenotype in polysialyltransferase ST8SIA2 deficient mice

This manuscript has been prepared for submission

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Preface - About this manuscript

This study addresses the question, whether polyST deficiency might cause a schizophrenia-like phenotype in mice. Corresponding to clinical parameters in schizophrenia, the volume of the thalamus and the lateral ventricles, as well as the size of the internal capsule was analyzed in $St8sia2^{-/-}$ and $St8sia4^{-/-}$ mice. Furthermore, potential alterations of the thalamocortical connectivity were addressed. In a collaborative effort with the laboratory of A. Zharkovsky (Univ. Tartu, Estonia), mice deficient for ST8SIA2 or ST8SIA4 were subjected to tests of cognition and sensory functions.

My contributions to this manuscript comprised the dissection of mice, histological examination by immunofluorescent staining, microscopical evaluation of fiber tract morphology and Western blot determination of protein levels. Prof. H. Hildebrandt, Prof. A. Zharkovsky and I designed the experiments and wrote the manuscript.
Abstract

Posttranslational modification of the neural cell adhesion molecule (NCAM) by polysialic acid (polySia) is crucial for nervous system development and brain plasticity. PolySia attachment is catalyzed by the polysialyltransferases (polySTs) ST8SIA2 and ST8SIA4, two enzymes with distinct but also common functions during neurodevelopment and in the adult brain. A growing body of evidence links aberrant levels of NCAM and polySia as well as variation in the ST8SIA2 gene to neuropsychiatric disorders, including schizophrenia. To investigate whether polyST deficiency might cause a schizophrenia-like phenotype, St8sia2−/− mice, St8sia4−/− mice and their wildtype littermates were assessed neuroanatomically and subjected to tests of cognition and sensory functions. St8sia2−/− but not St8sia4−/− mice displayed enlarged lateral ventricles and a size reduction of the thalamus accompanied by a smaller internal capsule and a highly disorganized pattern of fibers connecting thalamus and cortex. Reduced levels of the vesicular glutamate transporter VGLUT2 pointed towards compromised glutamatergic thalamocortical input into the frontal cortex of St8sia2−/− mice. Both polyST-deficient lines were impaired in short- and long-term recognition memory, but only St8sia2−/− mice displayed impaired working memory and a deficit in prepulse inhibition, which could be attenuated by clozapine treatment. Furthermore, St8sia2−/− mice exhibited anhedonic behavior and increased sensitivity to amphetamine-induced hyperlocomotion. Together, these data reveal that reduced polysialylation in St8sia2−/− mice leads to pathological brain development and schizophrenia-like behavior. We therefore propose that genetic variation in ST8SIA2 has the potential to confer a neurodevelopmental predisposition to schizophrenia.
Introduction

Schizophrenia is a complex mental disorder that is clinically characterized by delusions, conceptual disorganization, hallucinations, cognitive dysfunction, and psychosocial impairments, which emerge during adolescence or early adulthood. Increasing evidence points towards a neurodevelopmental mechanism for schizophrenia, and dysconnectivity resulting from impairments during development may be an important factor in the etiology of the disease (Friston, 1998; Stephan et al., 2009; Insel, 2010; Rapoport, 2012).

The neural cell adhesion molecule NCAM has been shown to be involved in the regulation of neurodevelopment and synaptic plasticity, (Edelman, 1986; Amoureux et al., 2000; Kiss and Muller, 2001). Posttranslational addition of α-2,8-polysialic acid (polySia) attenuates NCAM-mediated binding and reduces overall cell-cell apposition (Hildebrandt et al., 2007; Rutishauser, 2008). PolySia is essential for brain development and involved in neuroblast migration, neurite outgrowth, axonal pathfinding and axon tract formation as well as synaptogenesis (Hildebrandt et al., 2007; Rutishauser, 2008). In the adult brain, the expression of polySia is reduced to areas of persistent neurogenesis and plasticity (Bonfanti, 2006). Addition of polySia to NCAM occurs through two Golgi-associated polysialyltransferases (polySTs), ST8SIA2 and ST8SIA4 (for review, see Hildebrandt et al., 2010). Expression of the two enzymes shows considerable overlap and both are able to compensate for each other to at least some extent. Mice with genetic ablation of either ST8SIA2 or ST8SIA4 have been generated to study common and distinct functions of the two polySTs (Eckhardt et al., 2000; Angata et al., 2004; Stoenica et al., 2006; Nacher et al., 2010). Taken together, these studies imply that loss of ST8SIA2 affects mainly brain development, while ST8SIA4 is the dominant form of the postnatal brain (Hildebrandt et al., 1998; Ong et al., 1998; Oltmann-Norden et al., 2008, Nacher et al, 2010). Since each of the two polyST-deficient lines retained polySia expression, simultaneous deletion of both genes was needed to completely ablate polySia (Weinhold et al., 2005). Phenotypic analyses of these St8sia2 and St8sia4 double knockout mice highlighted the importance of balanced polySia and NCAM levels for brain development (Weinhold et al., 2005; Angata et al., 2007). Notably, reduced polysialylation of NCAM impairs the formation of major brain axon tracts like anterior commissure, internal capsule and corpus callosum (Hildebrandt et al., 2009). The complete loss of polySia in the St8sia2 and St8sia4 double knockout mice causes severe pathfinding errors of thalamocortical axons (Schiff et al., 2011).
Thalamic abnormalities and thalamocortical dysconnectivity are frequently observed in schizophrenic patients (Douaud et al., 2007; Mitelman et al., 2007; Begré and Koenig, 2008; Schmitt et al., 2011). Deficits of thalamocortical networks are thought to have a neurodevelopmental origin and may account for many of the clinical and cognitive symptoms observed in schizophrenia (Woodward et al., 2012 and references therein). Thus, it would be of interest to assess if the thalamocortical deficits in polyST-negative mice are linked to schizophrenia-like behavioral impairments. However, the severe and postnatally lethal phenotype of these mice prevents any behavioral analyses. So far, it is not known if thalamocortical connectivity is affected in St8sia2- or St8sia4-deficient mice. Concerning behavior, the assessment of the two single knockout lines performed so far indicates altered fear behavior in St8sia2 knockout mice (Angata et al., 2004), impaired contextual and spatial learning in the absence of St8sia4 (Senkov et al., 2006, Markram et al., 2007) as well as a divergent impact of St8sia2- and St8sia4-deficiency on social interaction and aggression (Calandreau et al., 2010).

Altered levels of polySia and NCAM have been repeatedly associated with neuropsychiatric disorders (for review, see Vawter, 2000; Brennaman and Maness, 2010). The first evidence for aberrant polySia expression in schizophrenia came from post-mortem studies showing reduced numbers of polySia-positive cells in the dentate gyrus of schizophrenic brains (Barbeau et al., 1995). ST8SIA2 maps to chromosome 15q26, a common susceptibility region for both schizophrenia and bipolar disorder (Maziade et al., 2005). Additionally, ST8SIA4 is located at 5q21, another susceptibility region for schizophrenia (Lindholm et al., 2004). Two independent studies conducted on Japanese and Chinese Han populations revealed significant associations of polymorphisms in the promoter region of ST8SIA2 but not ST8SIA4 with schizophrenia (Arai et al., 2006; Tao et al., 2007). Very recently, McAuley et al. (2012) reported on a risk haplotype within ST8SIA2 that is also associated with this disorder.

Based on the role of polySia-NCAM in developmentally regulated connectivity and its possible involvement in the pathogenesis of schizophrenia, we performed a comparative neuroanatomical and behavioral assessment to explore whether mice deficient for St8sia2 or St8sia4 exhibit a schizophrenia-like phenotype.
Methods

Animals

All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Experiments were undertaken by individuals who hold an appropriate license, and all efforts were made to minimize the number of animals used and pain and discomfort caused to the animals. The generation of ST8SIA2 and ST8SIA4 knockout mice (St8sia2⁻/⁻, Angata et al., 2004; St8sia4⁻/⁻, Eckhardt et al., 2000) has been described previously. St8sia2⁻/⁻ and St8sia4⁻/⁻ mice were back-crossed to C57BL/6J mice for at least six generations. Male and female F1 and F2 generation knockout mice and their wildtype littermates (St8sia2+/+ and St8sia4+/+) at the age of 2-4 months and with an average weight of 24.0 g were used. Animals were housed in groups (five or six mice per cage) under a 12-h light-dark cycle in a temperature and humidity controlled room. All mice had free access to food and water. In behavioral tests, three different experimental groups were used: the first group was subjected to object recognition test, T-maze test, sucrose preference test and taste aversion test. The second group was subjected to prepulse inhibition test (first without and then with drug treatment). The third group was used to measure locomotor activity (first without and then with drug treatment). The time interval between any behavioral tests was at least one week.

Morphology of the lateral ventricles, thalamus and internal capsule

For the quantification of the volume of the lateral ventricles and thalamus every sixth section, and for the volume of total brain every twelfth section throughout the structure was collected (n=7-8). Sections were incubated in a 0.05% trypsin solution, containing 0.1 M Tris-HCl, 0.1% CaCl and 0.9% NaCl for 10 min followed by an incubation in 0.25% Triton X-100 for 1 hour and in 70% ethanol containing 1% HCl for 10 sec. The sections were stained using hematoxylin-eosin, washed with phosphate-buffered saline (PBS; 0.1 M, pH 7.4) and cover-slipped with a water-based mounting medium (Vector Laboratories, UK). The volume was estimated according to Cavalieri’s principle by summing up the points falling on the cross-sectional area of all sections and by multiplying the distance between two sections by the thickness of the sections and by the area associated with each point. The size of the internal capsule was assessed on serial coronal vibratome sections from three-month-old mice, as described previously (Weinhold et al., 2005). Images of unstained free-floating sections were acquired with a SteReo Discovery.V12 stereomicroscope (Zeiss, Germany) under a modified dark field illumination, and the rostrocaudal extent of the internal capsule was calculated as
the product of the number of coronal sections on which the internal capsule was present, multiplied by the thickness of the sections (50 μm).

**Western blot analysis**

Frontal cortices were dissected and lysed in 10 μl/mg of following buffer: 2% Triton X-100, 150 mM NaCl, 5 mM EDTA, 20 mM Tris-HCl, pH 8. After centrifugation for 10 min, extracts were subjected to SDS-PAGE (30 μg total protein / lane) and electroblotted on nitrocellulose membranes. Immunostaining was performed using rabbit polyclonal antibodies against VGLUT1 and VGLUT2 (Synaptic Systems, Goettingen, Germany, #135303 and #135402, 1:5000, each). Loading controls were performed with either actin- or GAPDH-specific mouse monoclonal antibodies (Chemicon, Temecula, CA, #mAB1501, 1:10.000 and Ambion Life Technologies, Carlsbad CA, #AM4300, 1:40.000, respectively) Primary antibodies were detected with 25 ng/ml of respective IgG-specific IRDye-680 and -800 conjugated antibodies (LI-COR Biosciences, Bad Homburg, Germany). Each sample was reacted with VGLUT2- and, after stripping, with VGLUT1-specific antibody. Western blots were analyzed on an Odyssey near-infrared imaging system (LI-COR) and quantified using the Odyssey software (v3.0, LI-COR). The ratio VGLUT2/VGLUT1 was calculated from 6 technical replicates per sample. Per group, six animals were analyzed.

**Immunofluorescence**

Fluorescence labeling of parvalbumin (PV), neurofilament (NF) and perineuronal nets (PNN) was performed on free-floating 50-μm-thick vibrating microtome sections, permeabilized with 0.4% Triton X-100 and blocked with 2% normal goat serum (Vector Laboratories) for 1 h. PV and NF were detected using specific mouse monoclonal IgG, antibodies (Swant, Bellinzona, Switzerland, 1:5000; and 2H3, Developmental Studies Hybridoma Bank, Iowa City, IA; 1:500) and mouse IgG-specific Cy3- or Alexa568-conjugated secondary antibodies (Jackson, West Grove, PA, and Molecular Probes, Karlsruhe, Germany; 1:500, each). PNNs were labeled with biotinylated *Wisteria floribunda* agglutinin (WFA, 1:500, Sigma, Tauфикirchen, Germany) and detected with Cy3-conjugated streptavidin (1:1000, Rockland Immunochemicals, Gilbertsville, PA). Cross-reactivity of secondary antibodies was controlled by omitting either the first antibody or WFA. Stained sections were washed with ddH2O and mounted on glass object slides (SuperFrostPlus, Menzel, Braunschweig, Germany) using Vectashield mounting medium with DAPI (Vector Laboratories). Fluorescence microscopy was performed with an Axiovert 200 M microscope equipped with MosaiX and ApoTome module, AxioCam MRm.
digital camera and AxioVision software (Zeiss, Germany). Where indicated, optical sections with a thickness of 5.1 or 1.2 μm were obtained by ApoTome technology using 10x or 20x Plan-Apochromat objectives with numerical apertures of 0.45 or 0.8, respectively (Zeiss, Germany).

Object recognition test

Eight mice from all genotypes were individually habituated to an open-field box (50 x 50 cm; height, 30 cm) for one day. During training sessions, two identical solid impermeable objects were placed into the open field and the animal was allowed to explore the area for 5 min. The time spent exploring each object was recorded. During retention tests (2 and 24 hours after the training), the animals were placed back into the same box, in which one of the familiar objects used during training was replaced by a novel object (different material, shape and color), and mice were allowed to explore freely for 5 min. Recognition memory was quantified as the proportion of time spent exploring the new object.

Spatial working memory test on T-maze

The T-maze delayed alternation task was performed as described by Reisel et al. (2002). The wooden T-maze consisted of a start arm (50 cm) and two identical goal arms (39 cm) surrounded by a 30 cm high wall. A plastic food well was located 3 cm from the end of each goal arm. Both transgenic mice and wildtype littermates were maintained on a restricted feeding schedule at 85% of their free-feeding weight and habituated to the maze, and to drinking sweetened, condensed milk (diluted 50/50 with water), over several days before the trials. Each individual trial consisted of a sample run and a choice run. On the sample run, the mice were forced either left or right by closing one of the goal arms, in a pseudorandom sequence (with an equal number of left and right turns per session, and no more than two consecutive turns in the same direction). A 0.1 ml reward was available in the food well at the end of the arm. On the choice run, the mouse was placed, facing the experimenter, at the beginning of the start arm and allowed a free choice of either goal arm. The time interval between the sample and choice run was 15 sec. The animal was rewarded for choosing the previously unvisited arm (i.e., for alternating). In order to prevent the sensory cues to interfere (help the mice to choose the correct arm just by the smell), the upper sides of the walls in both choice arms were lubricated with the same reward - sweetened condensed milk (diluted 50:50 with water). So, the background of the smell was even within both choice arms and the test could not have been confounded just by sensory cues. Entry into the arm was
defined when a mouse placed all four paws into the arm. Mice were run one trial at a time with an intertrial interval (ITI) of approximately 10 min. Each block consisted of a total of eight trials, conducted on two consecutive days with four trials per day. Mice received 40 trials in total over 10 days of testing.

**Prepulse inhibition of acoustic startle and clozapine treatment**

The prepulse inhibition (PPI) was assessed using three automated SR-Lab System chambers (San Diego Instruments, San Diego, CA, USA). A speaker located in the ceiling of the chamber provided background noise (65 dB) and the acoustic stimuli. A piezoelectric sensor attached to the base transduced the startle response. Ten mice from each genotype were subjected to this test. After a 5 min acclimation period, four successive trials of 40 msec noise bursts at 120 dB were presented (not included in the analysis). Subjects were then exposed to five different types of acoustic stimuli in a randomized order: pulse alone (120 dB noise for 40 msec), no stimulus (only background noise), and three separate prepulse + pulse combinations, with prepulse set at three sound levels of 70, 75 and 80 dB for 20 msec followed by a 40 msec pulse at 120 dB. There were 100 msec between the prepulse and the pulse. A total number of 12 trials under each acoustic stimulus condition were presented with an average 20-sec variable intertrial intervals randomly ranging from 5-25 sec. The startle amplitude for each trial was measured for 65 msec starting from the onset of the startle stimulus. Percent prepulse inhibition of startle response was calculated as: \[1- \frac{\text{startle response to prepulse + pulse}}{\text{startle response to pulse alone}}\] x 100. The antipsychotic clozapine (C6305, Sigma-Aldrich, USA) was dissolved in 0.1 M HCl in saline and neutralized to pH 6–7 with 0.1 M NaOH. Clozapine was injected intraperitoneally (i.p.) 30 min before the PPI test and the acute effect of the drug was monitored on a prepulse level of 75 dB. The dose of clozapine (3 mg/kg) was chosen on the basis of previous studies of doses required to obtain behavioral effects in mice (Brody et al., 2004; Duncan et al., 2006). The effect of clozapine treatment was evaluated using a random crossover experimental design (n=8). In a first session, half of the wildtype and \(St8sia2^{-/-}\) mice were pretreated with clozapine, while the other half received vehicle. In a second session pretreatment for each mouse was reversed. In order to limit crossover effects, the second session was conducted after a 7-day washout period.
Sucrose preference test
In order to determine anhedonic behavior, mice from all genotypes (n=8) were subjected to a sucrose preference test that was conducted on 14 consecutive days. Each mouse was placed in a separate cage where it was given a free choice to drink from two graduated bottles – one with 0.8% sucrose solution and another with tap water. To prevent habituation to place preference in drinking, the position of bottles was changed every 24 hours. No previous food or water deprivation was applied prior to testing. The consumption of both liquids was estimated every day at the same time by measuring the level of liquids in the bottle. Sucrose preference was calculated as the sucrose solution intake as a percentage of the total amount of liquid drunk.

Taste aversion test
Mice from all genotypes (n=4) were given a free choice between two graduated bottles, one with a taste solution (0.1 M HCl) and another with tap water. To prevent habituation to side preference in drinking, the position of the bottles was changed every 24 hours. The consumption of both liquids was estimated every day at the same time by measuring the level of liquid, and the liquid preference was calculated as the percentage of taste solution intake of the total amount of liquid drunk.

Locomotor activity and amphetamine treatment
Horizontal locomotor activity was assessed in standard polypropylene cages (15 x 25 cm) that were uniformly illuminated with dim lighting. A light-sensitive video camera, connected to the computer, was mounted about 1.5 m above the observation cage and the locomotor activity of eight animals at the same time was monitored and analyzed using VideoMot2 software (TSE Systems, Germany). 16-18 mice from all genotypes were used. At first, baseline locomotion was measured as the total distance covered in centimeters in 60 min. After that, mice received an injection either with vehicle (0.9% saline) or D-amphetamine hemisulfate (1 mg/kg, A5580, Sigma-Aldrich, USA) i.p. and were subjected again for the assessment of locomotor activity for a 60-minute testing period.

Statistical analysis
All data are given as mean ± SEM. Statistical analysis was performed by unpaired Student’s t-test and ANOVA, where appropriate. Post hoc comparisons were made using Bonferroni’s or Dunnett’s test.
Results

Neuroanatomical assessment

Structural neuroimaging studies have revealed consistent evidence for brain abnormalities in schizophrenic patients such as an enlargement of the lateral ventricles which may be linked to shrinkage of the thalamus (Shenton et al., 2001; Gaser et al., 2004). In addition, decreased size or other signs of structural anomalies of the internal capsule, particularly of its frontal thalamocortical aspects, were reported frequently (Hulshoff Pol et al., 2004; Douaud et al., 2007; Mitelman et al., 2007; Begré and Koenig, 2008; Wobrock et al., 2008; Wobrock et al., 2009; Sussmann et al., 2009; Woodward et al., 2012; Lee et al., 2013). To determine if polyST deficiency leads to schizophrenia-like alterations in neuroanatomy, we histologically examined brain regions, which might be relevant for a schizophrenia-like phenotype. Inspection of histological sections revealed a significant enlargement of the lateral ventricles in \textit{St8sia2}⁻/⁻ mice relative to wildtype littermates (Fig. 1A,B). Less pronounced, but similar to the \textit{St8sia2} and \textit{St8sia4} double knockout mice (Weinhold et al., 2005), the extent of ventricular dilatation varied greatly between individuals and some of the \textit{St8sia2}⁻/⁻ mice displayed normal-sized ventricles (3 out of 8 animals analyzed). Dilated ventricles were never observed in \textit{St8sia4}⁻/⁻ mice (8 animals analyzed). Moreover, the \textit{St8sia2}⁻/⁻ mice exhibited a marked decrease in the volume of the thalamus (Fig. 1A,C) and a significant reduction in the size of the internal capsule (Fig. 1A,D). There was, however, no correlation between these defects and the volume of the lateral ventricles. No differences were found in total brain volume (mean volume ± SEM: 189.7 ± 2.941 mm³, 185.2 ± 10.23 mm³ and 175.8 ± 3.088 mm³ for wildtype, \textit{St8sia2}⁻/⁻, and \textit{St8sia4}⁻/⁻, respectively).
Fig. 1 Morphometric evaluation of the size of lateral ventricles, thalamus and internal capsule in wildtype, \textit{St8sia2}^- (2^-) and \textit{St8sia4}^- (4^-) mice. (A) Representative microphotographs of unstained coronal vibratome sections acquired under a modified dark field illumination. lv, lateral ventricle; Th, thalamus; ic - internal capsule. Scale bar: 1 mm. (B) Volume of the lateral ventricles. (C) Volume of the thalamus. (D) Rostrocaudal extent of the internal capsule. Values in (B-D) represent mean ± SEM (one-way ANOVA followed by Dunnett's multiple comparison test, *$P<0.05$, **$P<0.001$, n=8, for (B) and (C); n=4, for (D)).

Together with the overall size reduction of the thalamus in the \textit{St8sia2}^- brain, staining of the parvalbumin-immunoreactive neurons of the reticular thalamic nucleus (Rt) revealed a marked change in the shape of this structure, which arises from the ventral thalamus and forms a shell-like envelope of GABAergic neurons at the interface between thalamus and internal capsule (Fig. 2A-C). Since thalamocortical and corticothalamic fibers are major constituents of the internal capsule, we next sought to analyze possible changes of these fibers in the \textit{St8sia2}^- mice, in which the internal capsule was affected. Taking advantage of the fact, that all fibers running between the thalamus and the internal capsule traverse the Rt, we analyzed these fibers at the level of the Rt using immunofluorescence staining with a neurofilament-specific antibody. Due to same species primary antibodies, the neurofilament
staining could not be combined with parvalbumin immunofluorescence. Instead, *Wisteria floribunda* agglutinin (WFA), which specifically labels the perineuronal nets around Rt neurons (Schiff et al., 2011), was used for identification of the Rt (Fig. 2D-F). Double-labeling of Rt neurons and traversing fibers revealed highly ordered trajectories of parallel fibers in wildtype and *St8sia4*<sup>-/-</sup> mice (three out of three, each). In contrast, a completely disorganized pattern was observed in all three *St8sia2*<sup>-/-</sup> mice that were analyzed by neurofilament staining (Fig. 2G-I).

Fig. 2 Smaller thalamus, altered shape of the reticular thalamic nucleus and aberrant trajectories of traversing thalamocortical and corticothalamic fibers in *St8sia2*<sup>-/-</sup> mice. (A-C) Representative brain sections of wild type, *St8sia2*<sup>-/-</sup> and *St8sia4*<sup>-/-</sup> mice at the level of the thalamus (Th). The reticular thalamic nucleus (Rt) is labeled by parvalbumin (PV, red). (D-F) Identification of the Rt by staining of perineuronal nets (PNN) with WFA lectin (red). ApoTome images approximately corresponding to the sites indicated by the boxed areas in (A-C). (G-I) Patterns of neurofilament-positive fibers running between internal capsule and thalamus (NF, green). ApoTome images obtained from sites within the reticular thalamic nucleus as indicated by the boxed areas in (D-F). WFA staining of PNN, red. Scale bars: (A-C), 1 mm; (D-F), 100 µm, (G-I), 20 µm. Nuclei in (A-I) were counterstained with DAPI (blue).
Glutamatergic afferent neurons connecting cortex and thalamus are known to exhibit mostly discrete expression of the vesicular glutamate transporters 1 and 2 (VGLUT1, VGLUT2) (Graziano et al., 2008 and references therein). Expression of VGLUT2 is characteristic for thalamocortical axon terminals (Kaneko and Fujiyama, 2002) (Fig. 3A). In order to further analyze the disorganized fibers at the level of the Rt in St8sia2\textsuperscript{-/-} mice, we quantified VGLUT1 and VGLUT2 levels in frontal cortex lysates of St8sia2\textsuperscript{+/+}, St8sia4\textsuperscript{+/+} and wildtype mice at the age of three months. Protein levels of VGLUT1 were comparable in all genotypes (Fig. 3B,D), and expression of VGLUT2 was similar in wildtype and St8sia4\textsuperscript{+/+} mice (Fig. 3B,C). Only St8sia2\textsuperscript{-/-} mice displayed a significant reduction of VGLUT2 that, expressed in relation to VGLUT1, leads to a decrease of the ratio VGLUT2 / VGLUT1 (Fig. 3E). These results argue for a reduction of thalamocortical projection to the frontal cortex in St8sia2\textsuperscript{-/-} mice.

**Fig. 3 Reduced levels of vesicular glutamate transporter 2 in the frontal cortex of St8sia2\textsuperscript{-/-} mice.** (A) Diagram of corticothalamic and thalamocortical axons passing through the reticular thalamic nucleus (Rt), distinguished by complementary distribution of the vesicular glutamate transporters VGLUT1 and VGLUT2. Th, thalamus; Cx, cortex. (B) Representative Western blots illustrating the protein levels of VGLUT1 and VGLUT2 in wildtype (ctrl), St8sia2\textsuperscript{+/+} (2\textsuperscript{+/+}) and St8sia4\textsuperscript{+/+} (4\textsuperscript{+/+}) mice. (C-E) Densitometric evaluation of VGLUT2 (C), VGLUT1 (D) and VGLUT2 relative to VGLUT1 signals (E). Values represent mean ± SEM (one-way ANOVA with Tukey's multiple comparison post hoc test, ** P<0.01, * P<0.05, n=6, each).
Object recognition test

In a first test, cognitive function of polyST-deficient mice was assessed in a novel object recognition task. During training, mice were presented with two different objects for 5 minutes. Wildtype and polyST-deficient mice showed no preference for any of the objects. After a delay of 2 or 24 hours, mice were presented with one of the two objects replaced by a novel one. Unlike the wildtype controls, St8sia2<sup>−/−</sup> and St8sia4<sup>−/−</sup> mice displayed no preference for the novel object after 2 hours (Fig. 4A and 4B; t=3.574, df=13, P=0.0034 and t=2.479, df=16, P=0.0247, respectively) and after 24 hours delay (Fig.4C and 4D; t=2.411, df=13, P=0.0315 and t=3.356, df=16, P=0.0040, respectively). These results indicate impaired short and long term recognition memory in both polyST-deficient lines.

Fig. 4 Performance of St8sia2<sup>−/−</sup> and St8sia4<sup>−/−</sup> mice, as well as of respective wildtype littermates in object recognition test after 2 (A,B) and 24 hours delay (C,D). Data are expressed as the proportion (％) of time spent exploring the new object. Values represent mean ± SEM (Student’s t-test, * P<0.05, ** P<0.01, n=8).
**Spatial working memory on T-maze**

To examine changes in spatial working memory, wildtype, St8sia2\(^{-/-}\) and St8sia4\(^{-/-}\) mice were subjected to a T-maze delayed alternation task. Mice of all genotypes performed above the chance level. Of all the choices made during 10 days, the percentages of total correct choices made were (mean ± SEM) 82.03% ± 2.044, 69.38% ± 6.422, 76.88% ± 3.917 for wildtype, St8sia2\(^{-/-}\) and St8sia4\(^{-/-}\) mice, respectively. The comparison of the percentage of correct choices (i.e. alternations) revealed that the performance of St8sia2\(^{-/-}\) mice was overall worse than that of wildtype littermates during the whole period of testing (Fig. 5A). Two-way ANOVA demonstrated a highly significant main effect of genotype (F1,70=7.36, P=0.0084), but no main effect of block (F4,70=0.83, P=0.5102) or group-by-block interaction (F4,70=0.13, P=0.9701). In contrast, the performance of St8sia4\(^{-/-}\) mice was not significantly different from wildtype littermates (Fig. 5B). No statistically significant differences between any genotypes were found in locomotor activity (data not shown).

![Fig. 5 Performance of St8sia2\(^{-/-}\) (2\(^{-/-}\), A) and St8sia4\(^{-/-}\) mice (4\(^{-/-}\), B), compared to respective wildtype littermates in spatial working memory task on the T-maze in 5 consecutive blocks. Data are expressed as a percentage of correct choices made in each test block. Values represent mean ± SEM (two-way ANOVA followed by Bonferroni’s multiple comparison test, * P<0.05, n= 8).](image)

**Prepulse inhibition of acoustic startle response**

A possible role of polySTs in sensorimotor gating was addressed by testing for prepulse inhibition (PPI) of the acoustic startle response. Differences between St8sia2\(^{-/-}\), St8sia4\(^{-/-}\) and their wildtype littermate controls were analyzed with a two-factor ANOVA for repeated measurements with genotype and prepulse intensity level as factors. PPI was significantly decreased in the group of St8sia2\(^{-/-}\) mice compared to wildtype littermates (Fig. 6A, genotype: F1,34=4.98, P=0.0394). ANOVA also revealed a significant effect of prepulse intensity (F2,34=116.91, P=0.0001) and an interaction between genotype and prepulse intensity (F2,34=5.85, P=0.0066). Bonferroni’s *post hoc* comparison showed a significant
disruption of PPI at the prepulse level of 75 ($P<0.05$) and 80 dB ($P<0.05$). In contrast, $St8sia4^−$ mice showed no significant reduction in PPI. Startle amplitudes were unaltered in $St8sia2^−$ and $St8sia4^−$ mice as compared with wildtype mice (Fig. 6B). Trying to normalize PPI by antipsychotic drug treatment, the acute effect of the atypical antipsychotic clozapine was evaluated in $St8sia2^−$ mice and their wildtype littermates. Clozapine (3 mg/kg, i.p.) had no significant effect on PPI in the wildtype, but restored PPI in $St8sia2^−$ mice to the level of clozapine-treated wildtype animals (Fig. 6C, two-way ANOVA; genotype: $F_{1,14}=2.60$, $P=0.1290$; treatment: $F_{1,14}=0.04$, $P=0.8480$; treatment-by-genotype interaction: $F_{1,14}=2.30$, $P=0.1519$). However, consistent with previous findings in C57BL/6J mice (Dirks et al., 2003), clozapine significantly reduced mean startle amplitudes (Fig. 6D, $F_{1,14}=54.41$, $P<0.001$). The genotype-by-treatment interaction was not significant ($F_{1,14}=0.86$, $P>0.05$).

![Fig. 6](image)

**Fig. 6** Prepulse inhibition (PPI) of the acoustic startle response is altered in $St8sia2^−$ (2−) but not in $St8sia4^−$ (4−) mice. (A) PPI of the acoustic startle response at three prepulse levels: 70, 75 and 80 dB. Values represent means % PPI ± SEM. * $P<0.05$ as compared with wildtype mice (ctrl) (two-way ANOVA for repeated measurements followed by Bonferroni’s multiple comparison test, $n=10$). (B) Average startle response. Data are expressed as the startle amplitude produced by pulse alone (120 dB). Values represent mean ± SEM; $n=10$. (C) PPI of the acoustic startle response after a single clozapine (3mg/kg) treatment on prepulse level of 75 dB in wildtype (ctrl) and $St8sia2^−$ mice. Values represent mean % PPI ± SEM (two-way ANOVA followed by Bonferroni’s multiple comparison test, ** $P<0.01$, n=8). (D) Average startle response after clozapine treatment. Data are expressed as the startle amplitude produced by pulse alone (120 dB). Values represent mean ± SEM (two-way ANOVA followed by Bonferroni’s multiple comparison test, *** $P<0.001$, n=8).
Sucrose preference test

During the entire testing time, wildtype and *St8sia4*−/− mice demonstrated a clear preference for sucrose solution, consuming around 70-75% of sucrose solution of the whole fluid intake (Fig. 7A). *St8sia2*−/− mice had no preference for the sucrose solution and consumed around 50% of sucrose solution (ANOVA for repeated measurements followed by Bonferroni’s post hoc test, F1,273=6.93, \( P=0.0156 \)). The amount of total fluid consumed on the basis of body weight did not differ between any genotype (Fig. 7B). In order to explore the possibility that the reduced preference for sucrose consumption in *St8sia2*−/− mice might result from the impairment in the development of taste cells, mice were also subjected to taste aversion test. When animals were given free choices between tap water and 0.1 M HCl solution, mice from all genotypes showed clear preference for tap water. Although altered perception of sweet taste can not be excluded, these data indicate that taste processing in general is not impaired in *St8sia2*−/− mice.

![Figure 7](image-url)

**Fig. 7** Sucrose consumption by wildtype (ctrl), *St8sia2*−/− and *St8sia4*−/− mice in the sucrose preference test. (A) Percentage of sucrose solution of the total fluid volume consumed on 14 consecutive days. (B) Total fluid consumption (ANOVA for repeated measurements followed by Bonferroni’s post hoc test, \( P<0.02 \), (A), not significant, (B), n=8, each).
Locomotor activity after amphetamine treatment

St8sia2<sup>-/-</sup> and St8sia4<sup>-/-</sup> mice showed no differences in baseline locomotor activity compared to wildtype littermates (Fig. 8A; mean distance ± SEM: 5458 ±473, 5813 ± 468 and 6082 ± 411 cm for St8sia2<sup>-/-</sup>, St8sia4<sup>-/-</sup> and wildtype, respectively). Similar results were seen after saline injection. A relatively low dose of amphetamine (1 mg/kg) was applied causing a small, transient increase of locomotor activity in wildtype and St8sia4<sup>-/-</sup> mice, which is in good accordance with published data for C57BL/6 mice (van den Buuse, 2010). In contrast, St8sia2<sup>-/-</sup> mice responded with a huge increase in locomotor activity (Fig. 8B; genotype: F1,29=6.88, P=0.0137) indicating increased sensitivity to amphetamine treatment.

![Figure 8](image.png)

**Fig. 8** Baseline activity (A) and saline- and amphetamine-induced locomotor activity (B) in wildtype (ctrl), St8sia2<sup>2-/-</sup> and St8sia4<sup>4-/-</sup> mice. Data are expressed as the distance travelled in centimeters over a period of 60 minutes (baseline) or 15 minutes (saline / amphetamine treated). Values represent mean ± SEM (ANOVA for repeated measurements followed by Bonferroni’s post hoc test, **P<0.01, n=16-18).**
**Discussion**

The results of the present study demonstrate that mice deficient for either the polyST ST8SIA2 or ST8SIA4 exhibit impaired short- and long-term recognition memory. In contrast, only the loss of ST8SIA2 results in additional cognitive and sensory deficits such as impairment in a working memory task and alterations in prepulse inhibition. Moreover, St8sia2−/− mice displayed anhedonic behavior in the sucrose preference test and increased amphetamine sensitivity. Notably, ablation of ST8SIA2, which leads to a partial loss of polySia in particularly the embryonic and early postnatal period, also causes deficits of thalamocortical connectivity, which are not detected in St8sia4−/− mice. Thus, deficiency in polySia-NCAM during neurodevelopment may lead to the cognitive and sensory deficits detected in adult St8sia2−/− mice.

Our previous morphometric analysis of young polySia- and NCAM-deficient mice indicated that among the fiber tracts affected in the St8sia2 and St8sia4 double knockout mice only the size of the internal capsule was clearly reduced in the St8sia2−/− line, whereas anterior commissure and corpus callosum were not significantly smaller than in the wildtype (Hildebrandt et al., 2009). The specific deficits of thalamocortical connections in St8sia2−/− mice as described in the current study add significantly to previous results demonstrating that size reductions of the internal capsule in four-week-old mice strictly correlate with the level of polySia-free NCAM during development (Hildebrandt et al., 2009) and that thalamocortical pathfinding defects during embryonic development lead to the internal capsule deficits in St8sia2 and St8sia4 double knockout mice (Schiff et al., 2011). Projection errors of thalamocortical axons have been described in NCAM knockout mice (Enriquez-Barreto et al., 2012), but they occur exclusively in the dorsal telencephalon, and therefore are clearly distinct from the defect in St8sia2−/− mice. In contrast to the variable size of the lateral ventricles in ST8SIA2-deficient (this study) or St8sia2 and St8sia4 double knockout mice (Weinhold et al., 2005), the lack of NCAM causes a highly consistent ventricular dilatation (Wood et al., 1998; Weinhold et al., 2005) indicating different causes and, possibly, different consequences of this morphological phenotype. Possibly, a yet unknown mechanism can compensate for ventricular dilatation caused by the constitutive lack of polysialylation, and the compensation levels might differ between individual mice. The reductions of thalamus and internal capsule in ST8SIA2-deficient mice were consistent and not correlated with the variable size of the lateral ventricles, indicating that they were not caused by the ventricular...
dilatation. Conversely, however, a smaller thalamus and internal capsule could lead to structural instability and an expansion of the ventricles in some of the $St8sia2^{-/-}$ mice.

The neuroanatomical changes in ST8SIA2-deficient mice are consistent with the neuropathology of schizophrenia. Examinations on first-episode and unmedicated patients have shown selective regional deficits in brain volume and ventricular enlargement (Shenton et al., 2001). The latter was accompanied by the shrinkage of gray and white matter structures near the ventricles including the total thalamus or its subnuclear regions (Sim et al., 2006 and references therein). Decreased volume of particularly the thalamocortical aspects of the internal capsule, reduced size and shape deformations of the thalamus as well as glutamatergic abnormalities are characteristic features of schizophrenic patients (Clinton and Meador-Woodruff, 2004; Mitelman et al., 2007; Watis et al., 2008; Wobrock et al., 2009; Adriano et al., 2010; Cronenwett and Csernansky, 2010). Additionally, recent studies revealed structural and volume deficits of the anterior limb of the internal capsule (ALIC) connecting thalamus and frontal cortex (Oh et al., 2009; Mamah et al., 2010; Levitt et al., 2012; Hazlett et al., 2012). Indications for a disorganized and decreased connection between thalamus and frontal cortex in $St8sia2^{-/-}$ mice arise from the dramatic disorganization of fibers between internal capsule and thalamic nuclei at the level of the Rt as well as from reduced protein levels of the thalamocortical axon-specific marker VGLUT2 in the frontal cortex.

Cognitive impairments including poor performance in tasks requiring working memory are a hallmark of schizophrenia (Green et al., 2000) and impaired PPI of the startle response as an operational measure of sensorimotor gating has been consistently demonstrated in patients (Braff et al., 2001). Thus, the deficits of working memory and PPI as observed in $St8sia2^{-/-}$ mice are reminiscent to abnormal behavior of schizophrenic patients. Increased amphetamine sensitivity, a rodent correlate of schizophrenia-like positive symptoms (Arguello and Gogos, 2010; van den Buuse, 2010) supports a schizophrenia-like behavioral phenotype of ST8SIA2 deficient mice. Furthermore, $St8sia2^{-/-}$ mice lack sucrose preference indicating anhedonia (Ellenbroek and Cools, 2000), which is one of the core symptoms of depression-related disorders, but also a common negative symptom in schizophrenia (Barch and Dowd, 2010; Anticevic and Corlett, 2012). None of these behavioral deficits was observed in $St8sia4^{-/-}$ mice. Therefore, as discussed below, it is conceivable that disturbed brain development in $St8sia2^{-/-}$ mice and in particular the pronounced ST8SIA2-specific
impact on the thalamocortical system is a major cause for at least some of the schizophrenia-like traits.

Impaired short- and long-term recognition memory was observed in both polyST-deficient lines. It is not clear, whether these common deficits result from common or distinct alterations of polySia-synthesis in the absence of ST8SIA2 or ST8SIA4. Evidently, ST8SIA2 plays a more prominent role in brain development (Hildebrandt et al., 1998; Ong et al., 1998; Oltmann-Norden et al., 2008) and the completely different polySia patterns in the adult brain of St8sia2−/− and St8sia4−/− mice indicate non-overlapping functions of the two polySTs (Nacher et al., 2010). However, biochemical analyses and morphometric comparisons between mice with differently combined wildtype and knockout alleles of St8sia2 and St8sia4 also document the cooperative activity of the enzymes in polySia synthesis during the developmental period (Galuska et al., 2006; Oltmann-Norden et al., 2008; Hildebrandt et al., 2009). Thus, yet undetected common reductions of polySia synthesis in St8sia2−/− and St8sia4−/− mice may cause the shared deficits in novel object recognition memory. These may, for instance, affect the perirhinal cortex, which is implicated in object recognition (for a recent summary, see Barker and Warburton, 2011) and shows high susceptibility for learning and memory-related changes of polySia (Conboy et al., 2010). Overlapping but in detail different deficits of polySia synthesis in, for example, hippocampal development and plasticity may also be responsible for impaired fear conditioning of St8sia2−/− and St8sia4−/− mice (Angata et al., 2004; Senkov et al., 2006) and impaired spatial and reversal learning of St8sia4−/− mice in the Morris water maze (Markram et al., 2007). As another common feature of St8sia2−/− and St8sia4−/− mice, decreased motivation of social interaction has been observed (Calandreau et al., 2010). The same study reported on increased aggression of specifically the St8sia2−/− mice, which may be linked to reduced anxiety-like behavior and enhanced locomotion in the open field as reported in the initial description of these mice (Angata et al., 2004).

A number of observations in schizophrenic patients and in animal models suggest that the alterations in brain morphology as detected in the St8sia2−/− mice may be linked to the cognitive and behavioral impairments of these animals. The neural models of schizophrenia implicate different aspects of thalamic function (for review, see Sim et al., 2006). First, psychotic features of schizophrenia have been linked to a breakdown of the sensory filter or gating role of the thalamus. Second, the pathology of the prefrontal cortex in schizophrenia
points to defects of primarily middle zone layers that are receiving projections from thalamic nuclei, and thalamic pathology often emerges together with prefrontal cortex pathology, a combination that could result in clinical symptoms (Andreasen et al., 1994, Lewis et al., 2001). Third, it has been proposed that disruptions of the cortical-cerebellar-thalamic-cortical circuitry lead to cognitive impairments (Andreasen et al., 1996). In rodents, lesions or inhibition of the mediodorsal and intralaminar thalamic nuclei projecting to the prefrontal cortex are known to cause reductions of working memory and prepulse inhibition (Romanides et al., 1999; Swerdlow et al., 2001; Bailey and Mair, 2005; Mair and Hembrook, 2008). Similarly, in humans, there is a close link between thalamic infarction around the mediodorsal and intralaminar nuclei and deficits of executive functions ascribed to the prefrontal cortex (Van der Werf et al., 2000). Notably, reduced neuron numbers and altered mRNA expression of VGLUT2 were reported in the mediodorsal thalamic nucleus (Pakkenberg, 1990; Smith et al., 2001) and reduced glutamatergic gene expression patterns in specifically the thalamocortical projection neurons of the mediodorsal thalamic nucleus suggest that the circuitry required for communication between the thalamus and the frontal cortex is affected in schizophrenia (Sodhi et al., 2011). Thus, the schizophrenia-related symptoms of \textit{St8sia2\textasciitilde} mice, like deficits of spatial working memory and impaired sensorimotor gating, might be linked to disturbed thalamic input into the prefrontal cortex.

Taken together, our data demonstrate that neurodevelopmental deficits of polySia synthesis in the \textit{St8sia2\textasciitilde} mice result in changes of thalamocortical connectivity and a behavioral phenotype, which together are highly reminiscent to pathology and symptoms seen in schizophrenic patients. Therefore, \textit{St8sia2\textasciitilde} mice may provide an attractive animal model to explore effective antipsychotic drug treatment of at least some aspects of this disorder.
References


3 - General Discussion

Modification of NCAM with polySia is tightly associated with nervous system development and plasticity (Hildebrandt et al., 2007, Rutishauser, 2008) and several lines of evidence link aberrant polySia-NCAM expression to schizophrenia (Barbeau et al., 1995; Poltorak et al., 1995; van Kammen et al., 1998; Vawter, 2000; Vawter et al., 2001). The studies of this thesis addressed (i) polySia-dependent changes of interneurons in brain regions relevant to the pathophysiology of schizophrenia, (ii) the role of polySia for migration of cortical interneurons, and (iii) if loss of ST8SIA2- or ST8SIA4-dependent polySia synthesis is linked to a schizophrenia-like phenotype in mice.

PolySia-deficiency leads to compromised migration of MGE-derived interneurons and to reduced densities of parvalbumin- and somatostatin-positive cells in the PFC

In the first study of this thesis, we observed that densities of interneurons expressing parvalbumin or somatostatin were reduced in the PFC of adult NCAM- or polySia-deficient mice. Analyses of calbindin and perineuronal nets as additional characteristic markers of interneurons disclosed alterations of specifically basket cells and, most likely, other subtypes, such as chandelier cells or somatostatin-positive Martinotti cells. Seeking for mechanisms that lead to reduced interneuron densities, we demonstrated altered patterns of tangentially migrating interneuron precursors in the pallium of embryonic St8sia2−/− and St8sia4−/− mice. Parvalbumin and somatostatin are expressed in non-overlapping patterns, and both subtypes of interneurons originate in the MGE (Marín and Rubenstein, 2003; Danglot et al., 2006; Gelman and Marín, 2010; Corbin and Butt, 2011). In the second study we therefore investigated the effect of polySia-deficiency on migrating MGE-derived interneurons. In vitro assays revealed that migration is compromised upon enzymatic removal of polySia and that attenuation of polySia levels on the interneurons is critical for these cells to enter the pallium.

Seeking for the mechanisms that lead to disturbed migration of interneurons, we observed shorter leading processes upon removal of polySia not only in slice cultures but also in primary cultures, i.e. in the absence of environmental factors that are present in vivo. Because leading process motility is a crucial component of the interneuron migratory cycle (Ward et al., 2005), we propose that altered leading process morphology is connected to the defective migration of interneurons upon polySia-deficiency and that the underlying mechanisms of polySia-dependent migration are independent from environmental factors.
Such a cell-autonomous role of polySia is unexpected, because so far, polySia has been reported to regulate interactions of a cell with its environment, either by modulating NCAM-mediated or NCAM-independent cellular interactions, or by acting as a scavenger of soluble factors, to facilitate interactions with the respective cell surface receptors, as suggested for BDNF (for review, see Hildebrandt et al., 2007). However, evidence for a cell-autonomous mechanism of how polySia may impact migration arises from a study demonstrating a role of polySia in the regulation of NCAM-dependent membrane dynamics that affect the migration of tumor cells (Conchonaud et al., 2007). Other studies point towards a role of NCAM-dependent membrane trafficking for integrin-mediated cell migration (Diestel et al., 2005, 2007). Corresponding to these examples, polySia on NCAM may modulate membrane dynamics of interneuron leading processes and this could be the key-mechanism by which polySia controls interneuron migration cell-autonomously. Direct evidence demonstrating a contribution of NCAM in the polySia-dependent migration of interneurons is missing, but, as described in the first study of this thesis, Ncam1-/- and polyST-deficient mice display comparable reductions of interneurons in the adult PFC. Thus, as suggested by this model of polySia-dependent leading process dynamics, the loss of NCAM may have a similar effect on interneuron migration. To test this hypothesis, further studies on the mechanisms of interneuron migration should include analyses in NCAM-deficient mice.

Assuming that polySia affects membrane dynamics in the leading process, it remains unclear how this translates into altered migration. Cell-autonomous migration deficits of cortical interneurons that are characterized by altered leading process morphology involve intrinsic regulation of the cytoskeleton. This has been demonstrated to result in shorter leading processes as shown for doublecortin (DCX) knockout mice, but also in longer processes as shown for lissencephaly 1 (LIS1) knockout mice and after knockdown of Disrupted-in-schizophrenia 1 (DISC1) (Kappeler et al., 2006; Nasrallah et al., 2006; Friocourt et al., 2007; Steinecke et al., 2012). DCX and LIS1 bind to microtubules to promote their stability (reviewed by Manent et al., 2011). Moreover, LIS1 binds to the centrosome in migrating neurons and LIS1, DCX and DISC1 function with dynein or with dynein motor-related proteins during neuronal migration (Tanaka et al., 2004; Kamiya et al., 2005, 2008). Loss of these cytoskeleton-associated factors, therefore, is likely to have direct consequences on the movements of the centrosome and the Golgi apparatus into the leading process, and hence could impair nucleokinesis and somal translocation, which are characteristic for migrating interneurons (Bellion et al., 2005). Accordingly, changes in the lengths of leading processes
may indicate altered rates of nuclear translocation and the shorter leading processes observed in response to removal of polySia could be a result of altered nucleokinesis rather than a direct effect of polySia-deficiency on the structure and integrity of the leading process. It remains to be resolved whether polySia-regulated migration of interneurons directly depends on membrane-dynamics, nucleokinesis, or both. These possibilities should be explored in future studies.

In addition to impaired developmental migration of interneurons, other factors might contribute to a decrease of interneuron densities in the PFC of polySia-deficient mice. Loss of polySia has been demonstrated to cause premature differentiation of neuronal precursors in vitro and in vivo (Petridis et al., 2004; Burgess et al., 2008; Röckle et al., 2008), as well as reduced proliferation, enhanced survival and improved differentiation of neuroblastoma cells (Seidenfaden et al., 2003, 2006). Similarly, altered differentiation may contribute to the observed interneuron deficits. These effects, however, are induced in a cell-contact dependent manner and depend on the altered interactions of polySia-free NCAM (Seidenfaden et al., 2003; Röckle et al., 2008). Incompatible with this mechanism, we observed altered interneuron densities in polySia-negative mice in the presence (St8sia2−/− St8sia4−/−) as well as in the absence of NCAM (St8sia2−/− St8sia4−/− Ncam1−/−). It therefore seems highly unlikely that altered differentiation due to a gain of polySia-free NCAM interactions contributes to the reduced interneuron densities in polySia-deficient mice.

Another potential factor that may account for a decrease of interneurons is apoptosis. In the normal mouse brain, about 40% of developing cortical interneurons are eliminated through apoptosis during postnatal life. Cell death in the neocortex is highest at P7 and gradually declines to nearly undetectable levels at P20 (Southwell et al., 2012). This demonstrates that cortical interneurons are prone to apoptosis at specific stages of their development. Since we did not observe increased cell death in ST8SIA2- or ST8SIA4-deficient mice at P10, a time point within the phase of highest apoptosis, it seems that polySia-deficiency does not lead to increased cell death of interneurons during this sensitive period of postnatal development. However, we observed reduced interneuron densities in the PFC of P1 polyST-deficient mice. Thus, increased apoptosis may eliminate cortical interneurons in polyST-deficient mice at earlier time points and further investigations should clarify if altered migration leads to enhanced interneuron death during embryonic development.
The finding that polySia levels were reduced *in vivo* is an important link between impaired interneuron migration caused by polySia-deficiency *in vitro* and the shorter migratory streams at E13.5 as well as the reduced interneuron densities in the postnatal PFC. As shown by analyses of whole brain lysates at P1, a loss of ST8SIA4 is completely compensated by ST8SIA2 and more than 50% of NCAM remain polysialylated in *St8sia2*−/− mice (Galuska et al., 2006; Oltmann-Norden et al., 2008). Correspondingly, our data demonstrate only partial reductions of polySia during interneuron migration. Thus, a partial reduction of polysialylation appears to be sufficient to hamper the migration of interneurons. Evidently, polySia synthesis based on the expression of ST8SIA2 in interneurons is critical for the cell-autonomous regulation of migration at least during the phase when migrating cells enter the pallium. In contrast, the impact of ST8SIA4 on interneuron migration remains to be resolved. Unlike the robust reduction of polySia observed in Western blots from extracts of the entire forebrain of ST8SIA2-deficient embryos, ST8SIA4-deficiency caused only a subtle decrease of polySia levels in the area of the intermediate zone migratory stream. Thus, ST8SIA4 is involved in polysialylation during the migration of interneurons in the embryonic brain and its loss has consequences on polySia synthesis. With regard to the high level of compensation, this was not necessarily expected and broadens the view on the role of ST8SIA4, which so far has been assigned predominantly to polySia-synthesis of the adult brain (Hildebrandt et al., 1998; Ong et al., 1998; Galuska et al., 2006; Oltmann-Norden et al., 2008; Schiff et al., 2009; Nacher et al., 2010). Although speculative, it is possible that interneuron migration depends not only on the cell-autonomous effects of ST8SIA2 but also on polySia in the environment, which may be provided by the activity of ST8SIA4. Further studies on the polySia-dependent migration should therefore focus on this potential ST8SIA4-mediated mechanism.

**Various cortical interneuron populations are affected in polySia-deficient mice**

Contrary to a decrease of parvalbumin- and somatostatin-positive cells in the PFC of polySia- or NCAM-deficient mice, the first study of this thesis shows that calbindin- and calretinin-positive interneurons were unaffected by a partial or a complete loss of polySia. At this point, it remains unclear why polySia-deficiency differently affects distinct subpopulations of interneurons. Concerning the calretinin-positive interneurons, a possible explanation could arise from the fact that most of these cells originate in the CGE, whereas the MGE is the source for cortical parvalbumin- and somatostatin-positive interneurons (reviewed by Gelman and Marin, 2010; Corbin and Butt, 2011). Possibly, polySia is not expressed by migratory interneurons from the CGE. Alternatively, polySia may be dispensable for migration of CGE-
derived interneurons to at least the PFC, or the loss of polySia has no effect on the calretinin-positive subtype, while other CGE-derived subpopulations may be affected. Besides the calretinin-positive, mainly bipolar cells, the CGE gives also rise to interneurons expressing combinations of reelin and vasointestinal peptide (Fogarty et al., 2007). Thus, possible changes of these cells should be addressed in further studies together with the question, if the loss of interneurons is confined to the PFC or detected in other brain regions.

Assuming a developmental origin of altered parvalbumin- and somatostatin-positive interneuron densities in the PFC, it is puzzling, why calbindin-positive interneuron populations were not affected although they also derive from precursors of the MGE. One reason may be that most, if not all MGE-derived interneurons seem to express calbindin in the embryonic and early postnatal phase (Anderson et al., 1997; Polleux et al., 2002) before undergoing a phenotypic shift as shown for the postnatal development of parvalbumin-positive interneurons of the rat cortex (Alcantara et al., 1996). It therefore seems possible that mainly the maturation process of this transition is impaired by the reduced numbers or the inappropriate timing of interneurons arriving in the PFC. Such a mechanism could lead to reduced production of just the somatostatin–positive and the parvalbumin-positive, calbindin-negative interneuron populations.

Together with a decrease of parvalbumin-positive cells in the PFC of adult polyST-deficient mice, an unexpected increase of parvalbumin-positive cells in the CA field of the hippocampus was observed, while the dentate gyrus was unaffected. The reasons for this inverse relationship remain unclear. The MGE is known to produce hippocampal interneurons that migrate preferably to CA1/2 and avoid the dentate gyrus (reviewed by Danglot et al., 2006). Therefore, polySia-deficiency not only affects MGE-derived interneurons in the PFC but also in the hippocampus. Besides the finding that the velocities of interneurons are reduced upon removal of polySia in slice cultures, we also demonstrated that a subpopulation of fast cells was unaffected. Since the migratory route of interneurons into the hippocampus is by far the longest (Danglot et al., 2006), the fraction of fast cells, which migrate independent of polySia, may be destined to populate the hippocampus. Direct evidence is missing, but this model could explain why densities of parvalbumin-positive cells in the hippocampus are not reduced in polySia-deficient mice. The increase of parvalbumin-positive cells specifically within the CA field could arise from altered distribution within the hippocampus. This may differ between the analyzed polySia- and NCAM-deficient genotypes.
since the distribution of cells in the CA1/2 subregions is differently affected in these lines. For example, the fraction of parvalbumin-positive cells in the stratum radiatum and lacunosum-moleculare is increased in all but the ST8SIA4-deficient line, which, in contrast, displayed the highest densities in the stratum pyramidale. Another possible explanation for increased parvalbumin-positive cells in the hippocampus could be that this establishes after the period of polySia-dependent migration. So far, we analyzed only interneuron migration at E13.5 and further experiments should address the impact of polySia at different time points of developmental interneuron migration as well as the time course of the interneuron increase in the hippocampus.

All polySia- and NCAM-deficient lines, including the St8sia2\(^{-}\) and the St8sia4\(^{-}\) mice, displayed significant reductions of the calbindin-positive interneuron subpopulation in the glomerular cell layer of the olfactory bulb, while densities of calretinin- and tyrosine hydroxylase-positive cells were unaffected. Considering the prominent role of polySia for the migration of postnatally generated olfactory interneurons from the SVZ toward the olfactory bulb (Rutishauser, 2008), altered densities of interneurons in the olfactory bulb of single polyST knockout mice were unexpected, since unchanged polySia expression of migrating cells in the postnatal rostral migratory stream and a normal morphology of the rostral migratory stream and the olfactory bulb have been reported for St8sia2\(^{-}\) and St8sia4\(^{-}\) mice (Eckhardt et al., 2000; Angata et al., 2004). Contrary to polySia-negative mice (Weinhold et al., 2005), evidence for postnatal migration deficits in single polyST knockout mice is therefore missing. As for the MGE-derived interneurons, subtle, yet undetected reductions of polySia levels could be sufficient to affect the migration of postnatally born olfactory bulb interneurons. Another possible explanation would be that reduction or loss of polySia during embryonic but not postnatal migration of olfactory interneurons affects specifically the calbindin-positive interneuron population in the glomerular layer, whereas only the complete absence of polySia leads to the disruptions of the rostral migratory stream leading to the loss of granule cells and the smaller olfactory bulbs as seen in the Ncam1\(^{-}\) and the St8sia2\(^{-}\), St8sia4\(^{-}\) double knockout mice (Chazal et al., 2000; Weinhold et al., 2005). Two lines of evidence support this assumption. First, the mode of interneuron migration from the embryonic MGE closely resembles the migration of olfactory bulb interneurons generated in the embryonic LGE, but differs from the persistent migration of olfactory bulb interneurons in the postnatal rostral migratory stream. MGE- and LGE-derived interneurons disperse and migrate rather individually, whereas the postnatally born olfactory interneurons migrate in
chains of cells that move in close contact with each other (Marín and Rubenstein, 2003). Second, different interneuron populations of the glomerular layer of the olfactory bulb are preferentially born during different time windows (De Marchis et al., 2007, Batista-Brito et al., 2008). For example, the calbindin-positive interneurons of the olfactory bulb are mainly of embryonic and perinatal origin, while calretinin-positive cells are born later. Therefore, reduced densities of calbindin-positive cells in the glomerular layer of polyST-deficient mice could indeed result from migration deficits during embryonic development and the underlying mechanisms may correspond to those that cause defective migration of MGE-derived cortical interneurons. However, it remains to be studied if polySia levels during postnatal migration of olfactory bulb interneurons are affected in \textit{St8sia2}^{-/-} and \textit{St8sia4}^{-/-} mice and / or if polySia-deficiency affects the migration of interneurons from the embryonic LGE.

\textbf{ST8SIA2-deficient mice exhibit a schizophrenia-like phenotype}

In the third study of this thesis we investigated whether mice deficient for ST8SIA2 or ST8SIA4 exhibit a schizophrenia-like phenotype. We found that both polyST-deficient lines displayed impaired short- and long-term recognition memory but only \textit{St8sia2}^{-/-} mice demonstrated additional cognitive and sensory deficits such as impairment in a working memory task and alterations in prepulse inhibition as well as anhedonic behavior and increased amphetamine sensitivity. Because all of these defects that establish exclusively in mice deficient for ST8SIA2 are highly reminiscent to behavioral parameters in schizophrenia, we proposed that deficiency in ST8SIA2-dependent polySia synthesis during brain development may lead to a schizophrenia-like phenotype in these mice. Notably, altered thalamocortical projections and disturbed thalamic input into the frontal cortex were observed only in the \textit{St8sia2}^{-/-} mice. As discussed in detail in the third study of this thesis, these neuroanatomical changes may at least in part cause the deficits of spatial working memory and sensorimotor gating. The first part of this thesis demonstrates that \textit{St8sia2}^{-/-} mice also display alterations of interneurons in the adult PFC, another schizophrenia-like trait, which however is shared with the \textit{St8sia4}^{-/-} mice. It can be assumed that the decreased numbers of interneurons in the PFC cause a dysbalance in inhibitory / excitatory circuits, which may contribute to the observed symptoms and similar mechanisms are discussed in schizophrenia (Di Cristo, 2007). This raises the intriguing question, whether the thalamocortical defects alone, or in combination with the interneuron deficits in the PFC lead to the schizophrenia-like behavioral phenotype of specifically the \textit{St8sia2}^{-/-} mice.
In contrast, the common deficits of novel object recognition memory of $St8sia2^{-/-}$ and $St8sia4^{-/-}$ mice may result from the deficits of PFC interneurons or other, yet unknown consequences of altered polySia synthesis shared by the two lines. Besides the PFC, the perirhinal cortex is implicated in object recognition (Barker and Warburton, 2011) and shows high susceptibility for learning and memory-related changes of polySia (Conboy et al., 2010). It therefore will be of interest to analyze interneuron deficits or alterations in polySia-dependent plasticity in, for example, the perirhinal cortex of polyST-deficient mice, which may underlie this common behavioral trait.
Conclusions and perspectives

The studies of this thesis demonstrate that polySia is critical for the migration of cortical interneurons indicating that reduced developmental polySia levels cause the observed alterations of specific interneuron-populations in the PFC, hippocampus and olfactory bulbs. A schizophrenia-like phenotype was observed in St8sia2\textsuperscript{-/-}, but not St8sia4\textsuperscript{-/-} mice, potentially induced by reduced glutamatergic projections to the frontal cortex. Since St8sia2\textsuperscript{-/-} mice combine two striking defects, interneuron-deficits in the PFC and defective glutamatergic thalamocortical projections into the frontal cortex, one could speculate that this combination of defects is required for the development of a schizophrenia-like behavior. We therefore propose that St8sia2\textsuperscript{-/-} mice are an attractive tool to study schizophrenia-related alterations in GABAergic inhibition and thalamocortical connectivity in the PFC. Most interesting would be to explore the behavioral phenotype of mice, in which the two neuroanatomical traits are dissected by conditional ablation of St8sia2 in, for example, either the MGE or the thalamus.

Besides alterations of specific interneuron populations and a defective thalamocortical projection, also neuroanatomical defects like reduced dimensions of the thalamus and internal capsule, increased volumes of lateral ventricles and anomalies of brain fiber tracts in polySia-deficient mice are reminiscent to observations in schizophrenia. While one study reported that polySia levels in the PFC of schizophrenic patients were reduced (Gilabert-Juan et al., 2012), another study demonstrated that a schizophrenia-associated polymorphism in ST8SIA2 leads to increased promoter activity (Arai et al., 2006). Future work should therefore address the variations in ST8SIA2 in schizophrenic patients and assess the functional consequences for polySia-NCAM expression. Brain imaging studies should be performed to analyse a correlation of these factors with disturbed thalamocortical connectivity and other neuroanatomical parameters in healthy subjects and schizophrenic patients.
4 - Methods

Standard laboratory chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), AppliChem (Darmstadt, Germany) and Merck (Darmstadt, Germany). When performing cell or slice culture experiments, chemicals with the grade “cell culture tested” were used. Unless otherwise stated, incubation steps were performed at room temperature.

4.1 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. Knockout strains for ST8SIA2 ($St8sia2^{-/-}$ or $2^{-/-}$, Angata et al., 2004), ST8SIA4 ($St8sia4^{-/-}$ or $4^{-/-}$ Eckhardt et al., 2000) and NCAM ($Ncam1^{-/-}$ or $N^{-/-}$, Cremer et al., 1994) have been backcrossed with C57BL/6J mice for at least six generations. Single knockout strains were intercrossed to obtain double knockout ($St8sia2^{-/-} St8sia4^{-/-}$, $2^{-/-}4^{-/-}$) or triple knockout ($St8sia2^{-/-} St8sia4^{-/-} Ncam1^{+/-}$; $2^{-/-}4^{-/-}N^{+/-}$) animals (Weinhold et al. 2005).

GAD67-GFP mice (Tamamaki et al., 2003) were maintained on C57BL/6J background or crossed with $St8sia2^{-/-}$ and $St8sia4^{-/-}$ mice. Genotyping of $St8sia2^{-/-}$; $St8sia4^{-/-}$ and $Ncam1^{-/-}$ was performed by PCR as previously described (Weinhold et al. 2005). Genotyping of GAD67-GFP mice was performed with primers 5´-GGCACAGCTCTCCCTTCTGTTTGC-3´ (TR-1b), 5´-GCTCTCCTTTCGCGTTCCGACAG-3´ (TR-3), and 5´-CTGCTTGTCGGCCATGATAGACG-3´ (TRGFP-8) to detect wildtype (TR-1b and TR-3) or transgene (TR-1b and TRGFP-8), respectively. Expression of GFP in embryos was checked with a SteReo Discovery.V12 fluorescence stereomicroscope (Carl Zeiss Microscopy, Göttingen, Germany). To ensure precise staging of embryonic mice, $St8sia2^{+/-}$ and $St8sia4^{+/-}$ embryos were analyzed in comparison to heterozygous littermates ($St8sia2^{-/-}$ and $St8sia4^{-/-}$).

In experiments, where $St8sia2^{-/-}$- and $St8sia4^{-/-}$-GAD67-GFP mice were analyzed, respective wild type animals ($St8sia2^{+/-}$- and $St8sia4^{+/-}$-GAD67-GFP) were used as controls. For staging of embryos, the morning of the vaginal plug was considered as embryonic day (E) 0.5.
4.2 Western blotting and quantification of protein levels

Time pregnant mice were euthanized by cervical dislocation and embryos were transferred to ice cold PBS, pH 7.4. Forebrains were dissected under a stereomicroscope (Leica Microsystems, Wetzlar, Germany), transferred to microcentrifuge tubes and after estimation of their weight, snap-frozen in liquid nitrogen. Tissue was stored at -80°C until further processing. Forebrains were homogenized in 10 µl/mg ice cold lysis buffer (2% (v/v) Triton X-100; 150 mM NaCl; 5 mM EDTA; 20 mM TrisHCl, pH 8; 1x Complete Mini EDTA-free protease inhibitor (Roche, Mannheim, Germany)) using a microcentrifuge tube tissue grinder and samples were kept on ice for 10 min. After centrifugation for 10 min at 13000 x g and 4°C, protein concentrations were estimated using a BCA Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA). Where indicated, endosialidase (endo, Stummeyer et al., 2005) was added to the lysates at a concentration of 40 ng/µl and samples were incubated for 30 min on ice. Samples were mixed with 5x sample buffer (20% (v/v) glycerol; 5% (v/v) SDS; 5% (v/v) β-mercaptoethanol; 50 µg/ml bromphenol blue; 0.5 M Tris-HCl, pH 6.8) and subjected to SDS-PAGE (40 µg total protein per lane for quantification of polySia, otherwise 20 µg per lane) using a Minigel Twin electrophoresis device (Biometra, Göttingen, Germany). After electrophoresis, proteins were electroblotted on Protran nitrocellulose transfer membranes (Whatman, Kent, UK) using a Fastblot semidry blotting device (Biometra) with a current of 15 mA/cm² for 60 min.

Membranes were blocked for 60 min in blocking buffer (PBS, pH 7.4 containing 20% (v/v) Licor blocking buffer (LI-COR Biosciences, Bad Homburg, Germany)). Immunostaining was performed for 8-12 hours at 4°C on an orbital shaker (Heidolph, Schwabach, Germany) using following polyclonal (pAb) and monoclonal (mAb) antibodies: 0.4 µg/ml Lhx6-specific rabbit pAb (Santa Cruz Biotechnology Inc., Santa Cruz, CA), 3 µg/ml GAD65/67-specific rabbit pAb (Sigma-Aldrich), CB D-28-k-specific rabbit pAb (Swant, Bellinzona, Switzerland; 1:4000), 0.4 µg/ml NCAM-specific rat mAb H28 (IgG2α, Hirn et al., 1983), 1 µg/ml polySia-specific mouse mAb 735 (IgG2α, Frosch et al. 1985), 0.4 µg/ml GAPDH-specific mouse mAb (IgG1, Life Technologies, Darmstadt, Germany), as well as VGLUT1- and VGLUT2- specific rabbit pAbs (both Synaptic Systems, Goettingen, Germany, 1:5000 each). After incubation with primary antibodies, membranes were washed three times for 15 min with wash buffer (PBS, pH 7.4 containing 0.1% (v/v) Tween-20) and incubated with secondary antibodies in blocking buffer for 60 min. Primary pAbs were detected with 25 ng/ml IgG-specific IRDye-680 and -800 conjugated antibodies (LI-COR Biosciences), whereas mAbs were detected with 100 ng/ml
IgG-subtype-specific IRDye-680 and -800 conjugated antibodies (Rockland, Gilbertsville, PA). Membranes were washed three times for 15 min with wash buffer and analyzed on an Odyssey near-infrared imaging system (LI-COR Biosciences).

Protein levels were quantified using the Odyssey application software v3.0 (LI-COR Biosciences), yielding signal intensities for the respective proteins of interest.

4.3 Vibratome sectioning

Three-month-old male mice and postnatal day 1 pups were deeply anesthetized with an intraperitoneal injection of a mixture of 200mg/kg Ketamin (Gräub AG, Bern, Switzerland) and 8mg/kg Xylazin (Rompun, Bayer Health Care, Leverkusen, Germany) in 0.9% (w/v) NaCl. Animals were perfused transcardially with 30 ml of fixation buffer (0.1 M phosphate buffer, pH 7.4 containing 4% (w/v) paraformaldehyde) using a P-1 peristaltic pump (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Brains were dissected and postfixed in fixation buffer for 8-12 hours at 4°C. Embryos were dissected after cervical dislocation of time pregnant mothers. Brains were removed and fixed by immersion in fixation buffer for 8-12 hours at 4°C. After fixation, brains were washed in 0.1 M phosphate buffer, pH 7.4 and stored at 4°C until further processing. For sectioning, brains were embedded in liquid and prechilled agar (0.1 M phosphate buffer, pH 7.4 containing 4% (w/v) agar). 50-µm-thick coronal sections were obtained with a VT1000S vibrating microtome (Leica Microsystems). Sections were collected in 0.1 M phosphate buffer, pH 7.4 in 24-well-plates (Greiner Bio-One, Frickenhausen, Germany) and stored at 4°C.
4.4 Tissue culture

4.4.1 Slice cultures

Time pregnant mice were euthanized by cervical dislocation and embryos were transferred to ice cold dissection buffer (126 mM NaCl; 2.5 mM KCl; 2.5 mM CaCl₂; 1.2 mM NaH₂PO₄; 1.2 mM MgCl₂; 11 mM D-glucose; 25 mM NaHCO₃). Brains were dissected and embedded in low-gelling-temperature agarose (PBS, pH 7.4 containing 4% (w/v) low gelling and melting agarose (AppliChem)). 225-µm-thick coronal sections were obtained with a vibrating microtome and transferred to postholding buffer (dissection buffer containing 10 mM HEPES; 1x penicillin / streptomycin (Biochrom, Berlin, Germany); 50 µg/ml gentamycin (Sigma-Aldrich)). For enzymatic removal of polySia, slices were preincubated for 30 min on ice with 4 µg/ml endo in postholding buffer. Slices were transferred to Millicell cell culture inserts (PICM0RG50, Merck) in six-well-plates (Greiner Bio-One) and incubated in growth medium 1 (Neurobasal medium (Life Technologies) containing 1x B27 supplement (Life Technologies); 32 mM D-glucose; 2 mM L-glutamine (Biochrom); 1x penicillin / streptomycin) with or without 4 µg/ml endo. Slices were cultured for one day at 37°C and 5% CO₂ in a humidified incubator. After cultivation, slices were transferred to object slides (SuperFrostPlus, Menzel, Braunschweig, Germany) and fixed for 30 min in fixation buffer.

For live imaging studies, sections were acquired as described above, transferred to 8-well imaging slides (µ-slide 8 well, ibidi, Martinsried, Germany) and embedded in a three dimensional collagen matrix (PBS, pH 7.4 containing 1.3 mg/ml collagen (rat-tail collagen type I, BD Biosciences, Heidelberg, Germany); 32 mM D-glucose; 1x L-glutamine; 8 mM NaOH). To allow gelling of the collagen, slices were incubated for 30 min at 37°C. Growth medium 1 with or without 4 µg/ml endo was added and slices were preincubated for 2 hours at 37°C and 5% CO₂ in a humidified incubator before the onset of live imaging.

4.4.2 Co-cultures of MGE and pallium

Age-matched St8sia2⁻/⁻ and St8sia2⁺/+ embryos from independent matings were acquired. Time pregnant mice were euthanized by cervical dislocation and embryos were transferred to ice cold dissection buffer. Brains were dissected under a stereomicroscope and host brains were embedded in low-gelling-temperature agarose. 225-µm-thick coronal sections were obtained with a vibrating microtome and transferred to postholding buffer. Host tissue MGEs were dissected and discarded. MGEs from GFP+ brains were dissected under a stereomicroscope and cut into four pieces of equal size. Host slices were transferred to
Millicell cell culture inserts in six-well-plates and GFP+ MGE tissue was placed adjacent to the host slice, so that it replaces the host MGE. Co-cultures were incubated in growth medium 1 and cultures were maintained for two days at 37°C and 5% CO₂ in a humidified incubator. After cultivation, slices were transferred to object slides and fixed for 30 min in fixation buffer.

4.4.3 MGE explant cultures

Time pregnant mice were euthanized by cervical dislocation and embryos were transferred to ice cold dissection buffer. MGEs were dissected under a stereomicroscope and cut into four pieces of equal size. For removal of polySia, slices were preincubated with 4 µg/ml endo in postholding buffer for 30 min on ice. MGE tissue was embedded in a three dimensional collagen matrix in 35 mm culture dishes (µ-Dish, ibidi), as described above. After gelling of the collagen, growth medium 1 with or without 4 µg/ml endo was added. Explant cultures were maintained for three days at 37°C and 5% CO₂ in a humidified incubator. Without further processing, cultures were immediately imaged at the end of the experiment.

4.5 MGE primary cultures

Time pregnant mice were euthanized by cervical dislocation and embryos were transferred to ice cold PBS / Glc (PBS, pH 7.4 containing 38 mM D-glucose). MGEs were dissected under a stereomicroscope and transferred to fresh PBS / Glc. After centrifugation for 3 min at 200 x g, MGEs were incubated for 30 min at 37°C in digestion buffer (PBS / Glc containing 0.25 % (v/v) trypsin w/o EDTA (Biochrom)). Final concentrations of 25% (v/v) horse serum (Biochrom) and 100 µg/ml DNasel (Roche) were added and cells were dissociated by gently pipetting up and down 25 times with a 1000 µl pipette (Eppendorf, Hamburg, Germany). After centrifugation for 10 min at 200 x g and 4°C, cells were washed twice with PBS / Glc and resuspended in growth medium 2 (Neurobasal medium containing 1x B27 supplement; 2 mM Glutammax (Life Technologies); 1x penicillin / streptomycin) with or without 4 µg/ml endo. Cells were seeded at a density of 1x10⁶ per ml on poly-D-lysine coated (25 µg/cm²; Sigma-Aldrich) cover slips (20 mm, Menzel) in 12-well-plates (Greiner Bio-One). Cultures were maintained for one day and fixed for 30 min in fixation buffer.

For live imaging, 6.6 x 10⁵ cells per ml were seeded in 8-well imaging slides, coated with 6 µg/cm² rat tail collagen type I, 25 µg/cm² poly-D-lysine or Matrigel (BD Biosciences, diluted
1:60 in Neurobasal Medium). Cultures were preincubated for 2 hours at 37°C and 5% CO₂ in a humidified incubator before the onset of image acquisition.

4.6 Immunofluorescence

Cells grown on cover slips, tissue attached to object slides and free-floating coronal sections were subjected to the same protocol for immunofluorescence staining. Samples were permeabilized for 15 min in PBS / Triton (PBS, pH 7.4 containing 0.4% (v/v) Triton X-100) before they were blocked for 60 min with blocking buffer (PBS / Triton containing 2% (v/v) normal goat serum (Vector Laboratories, Burlingame, CA, USA)). Primary antibodies were incubated in blocking buffer for 8-12 hours at 4°C. The following monoclonal (mAb) or polyclonal antibodies (pAb) were applied: CB D-28k-specific rabbit pAb (Swant, 1:5000), PV-specific mouse mAb (IgG1, Swant, 1:5000), Sst-specific rat mAb (IgG2b; Merck, 1:200), 100 ng/ml polySia-specific mouse mAb 735 (IgG2a), and NF-specific mouse mAb 2H3 (IgG1, Developmental Studies Hybridoma Bank; Iowa, IA, USA; 1:500). After incubation with primary antibodies, samples were washed three times for 15 min with PBS / Triton and incubated for 60 min with secondary antibodies in blocking buffer. Rabbit, mouse and rat IgG-specific Cy3- (Merck), Alexa488- and Alexa568- (Life Technologies) conjugated secondary antibodies were used as suggested by the suppliers. As first layer controls, samples were incubated in blocking buffer lacking primary antibody. In double stained immunofluorescence samples, cross-reactivity of secondary antibodies was controlled by omitting either of the two primary antibodies. Staining with the biotinylated lectin WFA (Sigma-Aldrich, 1:500) was performed as described for antibodies. WFA was detected with Cy3-conjugated streptavidin (Rockland, 1:1000). After incubation with secondary antibodies or streptavidin, samples were washed three times for 15 min with PBS / Triton and mounted on cover slips / object slides using Vectashield mounting medium with DAPI (Vector Laboratories).
4.7 \textit{In situ} hybridization

If possible, buffers were DEPC-treated to inactivate RNases. DEPC was added to a final concentration of 0.1% (v/v) and incubated for up to 4 hours at room temperature on a magnetic stirrer (Heidolph). Residual DEPC was inactivated by autoclaving.

4.7.1 Generation of DIG-labeled riboprobes

Riboprobes were generated by amplifying cDNA with gene specific primers. First, RNA was isolated from E13.5 mice. Time pregnant mothers were euthanized by cervical dislocation and embryos were transferred to PBS. Brains were dissected under a stereomicroscope and total RNA was isolated using a NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. After addition of 1x Ribolock RNase Inhibitor (Thermo-Scientific), residual DNA was digested using RQ1 DNase (Promega, Madison, WI, USA) as suggested by the supplier. Random hexamer primers (Life technologies) were added to a final concentration of 25 ng/µl and samples were incubated for 10 min at 70°C. Synthesis of cDNA was carried out using RevertAid Premium Reverse Transcriptase (Thermo-Scientific) as suggested by the supplier, using a total of 2.5 µg RNA per preparation. DIG-labeled riboprobes were synthesized using a two step protocol. First, cDNA was amplified using primers 5´-TGCGCTCCGGTGTGTGTC-3´ (TK-5), 5´-GCCGCCCTCTGCTGCCTCTTC-3´ (TK-6), 5´-ACTGGCCGACAGTCAGTTTC-3´ (TK-ISH-17) and 5´-AGCATTTCCAGACTTTTGCC-3´ (TK-ISH-18) to generate gene specific cDNA for LHX6 (primers TK-5 and TK6) and ST8SIA2 (primers TK-ISH-17 and TK-ISH-18), respectively. PCR was carried out using Phusion Hot Start High Fidelity Polymerase (Thermo-Scientific) according to manufacturer’s instructions, with an annealing temperature of 64°C for both targets. Sizes of PCR products were 579 bp (LHX6) and 657 bp (ST8SIA2).

In a second step, these products were again PCR amplified, using combinations of the above mentioned primers, as well as additional primers 5´-GCCGTAATACGACTCAGGTTGCGCGTGGTTTGGTC-3´ (TK-ISH-5), 5´-GCCGTAATACGACTCAGGTTGCGCGTGGTTTGGTC-3´ (TK-ISH-6), 5´-GCCGTAATACGACTCAGGTTGCGCGTGGTTTGGTC-3´ (TK-ISH-11) and 5´-GCCGATTTAGGGATGAGGAGGTC-3´ (TK-ISH-23) that contained promoter sequences for RNA-Polymerases T7 and SP6 (5´-GCCGATTTAGGGATGAGGAGGTC-3´ and 5´-GCCGATTTAGGGATGAGGAGGTC-3´). To generate templates for RNA-specific antisense probes, primers TK-5 and ISH-6 (LHX6) and
ISH-17 and ISH-11 (ST8SIA2) were used. Templates for control sense probes were generated using primers TK-6 and ISH-5 (LHX6) and ISH-18 and ISH-23 (ST8SIA2). With these templates, riboprobes were synthesized. RNA-Polymerases T7 and SP6 (Roche) were used according to manufacturer's instructions, with DIG-11-UTP (Roche) added to a final concentration of 0.35 mM. After Synthesis, DIG-labeled riboprobes were precipitated by addition of final concentrations of 100 mM LiCl and 75% (v/v) prechilled ethanol. After incubation for 6-8 hours at -20°C, samples were centrifuged for 15 min at 13000 x g and 4°C. Riboprobes were washed twice with ice-cold 70% ethanol and air-dried. Probes were resolubilized in H2O and stored in aliquots at -80°C.

4.7.2 Sectioning and hybridization

Time pregnant mice were euthanized by cervical dislocation and embryos were transferred to PBS. Heads were dissected, embedded in Tissue-Tek OCT in Biopsy Cryomolds (both Sakura, Alphen aan den Rijn, Netherlands) and immediately frozen over dry ice. Samples were stored at -80°C until sectioning. 20-µm-thick coronal sections were obtained with a CM3050S cryostat (Leica Microsystems) and mounted on SuperFrostPlus object slides (Menzel). Sections were fixed in PBS / PFA (PBS, pH 7.4 containing 4% (w/v) paraformaldehyde) for 30 min. RNases were inactivated by carbethoxylation, twice for 15 min in PBS with 0.1% (v/v) fresh DEPC. Sections were incubated with prehybridization buffer (50% (v/v) formamide; 10% (w/v) dextran sulfate; 600 mM NaCl; 1mM EDTA; 10 mM Tris-HCl, pH 7.5; 1x Denhardt’s Reagent (Life Technologies); 100 µg/ml salmon sperm DNA (Simga-Aldrich)) for 2 hours at 58°C. Riboprobes were diluted to equal concentrations in prehybridization buffer and added to the sections on 24 x 50 mm cover slips for 8-12 hours in a humidified chamber at 58°C in a hybridization oven (GE Healthcare Bio-Sciences). After hybridization, sections were washed in SSC buffer (1x SSC: 150 mM NaCl; 50 mM sodium citrate, pH 7.0) as followed: 20 min each in 2x SSC, 1x SSC, 0.5x SSC and 0.2x SSC at room temperature, 60 min in 0.2x SSC at 68°C and 15 min in 0.2x SSC at room temperature. Sections were washed with Tris / NaCl (150 mM NaCl; 100 mM Tris-HCl, pH 7.5) for 15 min. Blocking was achieved by incubation in blocking buffer (Tris / NaCl containing 0.5% (w/v) Blocking Reagent (Roche)) for 2 hours. Alkaline phosphatase(AP)-conjugated anti-DIG antibody (Roche) was diluted 1:5000 in blocking buffer and added to the samples on cover slips for 8-12 hours at 4°C. Sections were washed with Tris / NaCl for 15 min and pH was equilibrated by incubation in AP-buffer (100 mM NaCl; 50 mM MgCl2; 100 mM Tris-HCl, pH 9.5) for 10 min. Staining was performed by incubation with 330 µg/ml NBT and 165 µg/ml
BCIP in AP-buffer for 4-20 hours in a dark box and checked occasionally using an Eclipse TS100 microscope (Nikon, Düsseldorf, Germany). Reaction was stopped by washing sections with tap water for 5 min and with ddH₂O for 2 min. Air-dried sections were embedded in Euparal (Waldeck, Münster, Germany) and coverslipped.

4.8 Microscopy and data acquisition

Microscopy was performed using an Axiovert 200 M microscope equipped with an ApoTome device for near confocal imaging, AxioCam MRm digital camera and AxioVision software v4.8.2 (all from Carl Zeiss Microscopy). The latter includes the MosaiX module that permits the sample area to be scanned in order to generate one single large image.

4.8.1 Analyzing interneurons on coronal sections

MosaiX-images of coronal sections were acquired using the ApoTome technology with a 10x Plan-Apochromat objective with a numerical aperture of 0.45. All samples were matched for exposure times. In adult mice, the subdivision of the medial prefrontal cortex into Cg1, PrL and IL, as well as into upper and deep cortical layers (corresponding to layers 1 to 3 and 5 + 6) was achieved according to Paxinos and Franklin (2001). Cells were counted by visual inspection with the aid of the interactive event counting tool of AxioVision software. The estimated cell numbers were related to the area of the respective region. Cell densities were estimated per section and respective cell densities per animal were calculated by estimating the mean of up to six sections. Extents of migratory streams on embryonic coronal sections were analyzed by estimating the distance of the very last interneuron in a migratory stream to the pallial / subpallial boundary.

4.8.2 Densitometric quantification of polySia on sections

PolySia levels in the area of migrating interneurons on coronal sections were quantified by analyzing images acquired using the ApoTome technology with a 10x Plan-Apochromat objective. Exposure times were identical among all samples. Ten optical sections with a distance of 0.55 µm along the z axis and each with a thickness of 5.1 µm were acquired and merged into one image using AxioVision software. These images were exported as TIFF files in 16 bit grayscale to imageJ software v1.44p (Schneider et al., 2012). Background was subtracted using the rolling ball method, the radius set to 10 pixels. The area of the intermediate zone migratory stream was outlined and analyzed, yielding mean grey values. Strongly polySia-positive blood vessels were excluded from analysis.
PolySia levels on somata of calbindin-positive cells were quantified as described above. Images were acquired using a 63x Plan-Apochromat oil-immersion objective with a numerical aperture of 1.4. Optical sections had a distance of 0.25 µm and a thickness of 0.81 µm.

### 4.8.3 Counting of GFP-positive cells in slice- and co-cultures

MosaiX-images of slice cultures were acquired by ApoTome technology using a 5x Plan-Neofluar objective with a numerical aperture of 0.15. All samples were matched for exposure times. With the aid of AxioVision software, curves with ascending distances of 100 µm starting from the intersection of strongly GFP-positive ganglionic eminence and pallium were drawn and overlaid into the image. The curves hereby resembled the shape of the intersection. This way, bins of ascending distances to the subpallial / pallial boundary were created and numbers of GFP-positive cells within these bins were estimated.

MosaiX-images of slice cultures were acquired by ApoTome technology using a 10x Plan-Apochromat objective. Numbers of GFP-positive cells were estimated as described before. The intersection of GFP-positive MGE and GFP-negative host tissue served as starting point for the creation of bins.

### 4.8.4 Estimation of migration in MGE explant cultures

Mosaix-images of MGE-explant cultures were acquired using a 5x Plan-Neofluar objective. At the beginning of the experiment (t=0), micrographs were acquired by phase contract microscopy. After 3 days in vitro, Mosaix-images of GFP-fluorescence were acquired with using ApoTome technology. With the aid of AxioVision software, the area of the explant was estimated at t=0. Images of cultures after 3 days in vitro were exported in TIFF format in 8 bit grayscale and the area covered by GFP-positive cells was analyzed using imageJ software. The required threshold-settings were adjusted identically in all analyzed samples and scaling of the images was set according to the original images from AxioVision software. Areas at t=0 were subtracted from the respective areas at 3 days in vitro, resulting in areas covered by migrating cells during cultivation of the explant.
4.8.5 Analysis of interneuron leading processes

Images of MGE primary cultures were acquired using the ApoTome technology with a 20x Plan-Apochromat objective with a numerical aperture of 0.8. With the aid of AxioVision software, extents of leading processes of GFP-positive cells were estimated. Lengths were determined by tracing the process from the tip of the growth cone to its onset defined as the site of somal constriction. Only cells with a clearly discernible leading process were analyzed.

4.8.6 Live imaging

Live imaging was performed at 37°C and 5%CO₂ using a humidified incubator (Pecon, Erbach, Germany) mounted to the microscope. Using multiwell imaging slides, up to 6 independently cultured slices were analyzed in one experiment. Time lapse sequences were generated by acquiring images with a 10x Plan-Apochromat objective at an interval of 2 min for up to 14 hours. For the quantification of interneuron precursor velocity, a sequence of 180 min starting 4 hours after the onset of image acquisition was analyzed. Only cells visible throughout the entire sequence were used for analysis and migratory paths were reconstructed using the AxioVision software. The resulting distances were used to estimate the velocity of migrating cells. Binning of velocities was achieved by using the frequency distribution function of GraphPad Prism software v4 (GraphPad Software Inc., La Jolla, CA, USA). In order to summarize the direction of migration for one experimental condition, all obtained paths were centered to a common starting point. The end points of the migratory paths were marked in AxioVision software and TIFF images were exported to ImageJ in two color mode. Within ImageJ software the coordinates of the marks were estimated and exported to GraphPad Prism software to create a graph illustrating the final position of every cell in relation to the starting point.

4.8.7 Documentation of in situ hybridization

MosaiX-images were acquired using brightfield-microscopy with a 10x Plan-Apochromat objective. Merged images of micrographs were created using the transparency and overlay functions in CorelDraw software X3 (Corel, Fremont, CA, USA).
4.9 Morphological analysis of the internal capsule

Images of unstained free-floating coronal sections were acquired with a SteReo Discovery.V12 stereomicroscope under a modified dark field illumination. The rostrocaudal extent of the internal capsule was calculated as the product of the number of coronal sections on which the internal capsule was present, multiplied by the thickness of the sections (50 μm).

4.10 Statistical analysis

All statistical analyses were performed using GraphPad Prism software and applying unpaired Student’s t-test or ANOVA with indicated post hoc tests, where appropriate.
5 - References


Bélanger M-C, Di Cristo G (2011) Sensory experience differentially modulates the mRNA expression of the polysialyltransferases ST8SiaII and ST8SiaIV in postnatal mouse visual cortex. PloS One 6(9):e24874


Edelman G (1987) CAMs and Igs: cell adhesion and the evolutionary origins of immunity. Immunological Reviews 100:11–45


Foley D, Swartzentruber K, Colley K (2009) Identification of sequences in the polysialyltransferases ST8Sia II and ST8Sia IV that are required for the protein-specific polysialylation of the neural cell adhesion molecule, NCAM. The Journal of Biological Chemistry 284(23):15505–16


Young K, Fogarty M, Kessaris N, Richardson W (2007) Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb. The Journal of Neuroscience 27(31):8286–96


Affidavit

I herewith declare that I autonomously carried out the PhD-thesis entitled “Role of polysialic acid in the genesis of GABAergic neurons of the cerebral cortex and for thalamocortical connectivity”.

No third party assistance has been used.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institution: Institute of Cellular Chemistry, Hannover Medical School

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

_______________________________
January 28, 2013
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