A crucial role for polysialic acid in developmental interneuron migration and the establishment of interneuron densities in the mouse prefrontal cortex

Tim Kröcher1,2,*†, Iris Röckle1‡, Ute Diederichs1, Birgit Weinhold1, Hannelore Burkhardt1, Yuchio Yanagawa3, Rita Gerardy-Schahn1,2 and Herbert Hildebrandt1,2,§

ABSTRACT

Polysialic acid (polySia) is a unique glycan modification of the neural cell adhesion molecule NCAM and a major determinant of brain development. Polysialylation of NCAM is implemented by the two polysialyltransferases (polySTs) ST8SIA2 and ST8SIA4. Dysregulation of the polySia-NCAM system and variation in ST8SIA2 has been linked to schizophrenia and other psychiatric disorders. Here, we show reduced interneuron densities in the medial prefrontal cortex (mPFC) of all polyST-deficient lines, whereas calretinin-positive cells and the parvalbumin-negative fraction of calbindin-positive cells were unaffected. Reduced interneuron numbers were corroborated by analyzing polyST-deficient GAD67-GFP knock-in mice. The accumulation of precursors in the ganglionic eminences and reduced numbers of tangentially migrating interneurons in the pallium were observed in polyST-deficient embryos. Removal of polySia by endosialidase treatment of organotypic slice cultures led to decreased expression of GAD67-GFP-positive interneurons from the ganglionic eminences into the pallium. Moreover, the acute loss of polySia caused significant reductions in interneuron velocity and leading process length. Thus, attenuation of polySia interferes with the developmental migration of cortical interneurons and causes pathological changes in specific interneuron subtypes. This provides a possible link between genetic variation in polyST genes, neurodevelopmental alterations and interneuron dysfunction in neuropsychiatric disease.

KEY WORDS: Neural cell adhesion molecule, NCAM, Protein glycosylation, Cortical interneuron migration, Mouse prefrontal cortex, Parvalbumin, Somatostatin

INTRODUCTION

The sugar polymer polysialic acid (polySia) is a major regulator of cellular plasticity in brain development (Rutishauser, 2008; Schnaar et al., 2014). PolySia modulates cell interactions by multiple mechanisms including the attenuation of homo- and heterophilic cell-cell and cell-matrix adhesion, adjustment of receptor functions and, possibly, membrane dynamics. Two independently regulated polysialyltransferases (polySTs), ST8SIA2 and ST8SIA4, are able to produce polySia and, as shown in the developing mouse brain, the vast majority of polySia is attached to the neural cell adhesion molecule NCAM (NCAM1) (Mühlenhoff et al., 2013). In humans, several studies suggest a link between dysregulation of the polySia-NCAM system and variation in ST8SIA2 with schizophrenia and other psychiatric disorders (Brennaman and Maness, 2010; Anney et al., 2010; McCauley et al., 2012; Gilbert-Juan et al., 2012). In rodents, expression of the two polySTs shows considerable overlap, but ST8SIA2 is predominantly involved in polySia biosynthesis during brain development, whereas ST8SIA4 seems to be the major polyST of the adult brain (Hildebrandt et al., 2010). Accordingly, ST8SIA2-deficient mice were originally described to have neurodevelopmental defects manifesting in the aberrant topology of hippocampal mossy fiber projections, which might be linked to altered fear behavior (Angata et al., 2004). By contrast, and consistent with the prevalent expression of St8sia4 in the adult, mice lacking ST8SIA4 exhibit no detectable morphological defects but impaired synaptic plasticity in the CA1 subregion of the hippocampus (Eckhardt et al., 2000). Simultaneous ablation of St8sia2 and St8sia4 (St8sia2−/−4−/−) yielded entirely polySia-negative mice. These animals show a number of additional, severe defects, including postnatal growth retardation and premature death, a high incidence of hydrocephalus, as well as malformation of major brain axon tracts (Weinhold et al., 2005; Hildebrandt et al., 2009). In addition, St8sia2−/−4−/− mice show a reduction in the size of the olfactory bulb caused by a migration deficit of subventricular zone-derived interneurons (Weinhold et al., 2005), and the altered migration of neural precursors during the cortical development of St8sia2−/−4−/− mice has been suggested (Angata et al., 2007). Immunohistochemical detection of the calcium-binding proteins parvalbumin (PV; or PVALB), calbindin [CB; also known as calbindin 1 (CALB1)] and calretinin [CR; also known as calbindin 2 (CALB2)] as well as the neureptide somatostatin (SST) has proven a powerful tool for the identification and evaluation of GABAergic interneuron subtypes (Gabbott et al., 1997; Gonchar and Burkhalter, 1997; Kawaguchi and Kubota, 1997; Gelman and Marin, 2010; Anastasiades and Butt, 2011). Reminiscent of clinical studies reporting interneuron alterations in the prefrontal cortex (PFC) of schizophrenic patients (Lewis et al., 2012; Marin, 2012), loss-of-function mouse models of major schizophrenia risk genes, such as NRG1 and DISC1, display defects in cortical interneuron development or altered interneuron numbers in the medial prefrontal cortex (mPFC) (Flames et al., 2004; Hikida et al., 2007; Shen et al., 2008; Steinecke et al., 2012). Motivated by these findings, and...
based on the well-known role of polySia in the migration of olfactory bulb interneurons (Ono et al., 1994; Chazal et al., 2000; Weinhold et al., 2005), we examined whether cortical interneurons, particularly those of the mPFC, would be affected by polySia deficiency. The results indicate that even moderate interference with NCAM-based polySia during brain development leads to reduced densities of PV+ and SST+ interneuron populations. Aberrant allocation of migratory interneurons between the medial and lateral ganglionic eminences (MGE and LGE) and the pallium in St8sia2−/− and in St8sia4−/− embryos, as well as the slower migration of GAD67-GFP-labeled interneurons after acute enzymatic removal of polySia in slice cultures, suggest that disturbed tangential migration accounts at least in part for the observed interneuron phenotype in the mPFC of polyST-deficient mice.

RESULTS

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

Owing to the high mortality of St8sia2−/− mice after 4 weeks of age (Weinhold et al., 2005), all comparative analyses involving mice of this genotype were restricted to 1-month-old animals. For analyses of St8sia2−/− mice, which have a high incidence of hydrocephalus (Weinhold et al., 2005), only those with moderate ventricular dilatation and no cortical thinning were used. Compared with the control group, the densities of PV+ cells in the upper and deep layers of the mPFC were significantly lower in both polyST single-knockout lines (St8sia2−/− and St8sia4−/−) as well as in the double knockout (St8sia2−/−, St8sia4−/−) (Fig. 1). The most prominent effects were observed in the upper and deep layers of the cingulate cortex area 1 (Cg1), which harbor the highest densities of PV+ cells in the wild type (supplementary material Fig. S1A,B). Significant reductions, or at least a trend, were detected in the upper and deep layers of the infralimbic (IL) as well as in the deep layers of the prelimbic (PrL) cortex (supplementary material Fig. S1A,B). Evaluation of PV and CB double immunofluorescence revealed reduced densities of PV+ CB+ cells in the mPFC of all polyST-deficient lines (supplementary material Fig. S1C,D) and of PV+ CB+ cells in the upper but not in the deep layers (supplementary material Fig. S2A,B). Densities of PV+ CB+ (supplementary material Fig. S2C,D) and CR+ cells were unaffected [mean densities (±s.e.m.) of CR+ cells/mm2 mPFC: 59.1±2.5, 49.3±4.4, 43.5±5.6 and 51.9±1.8 for control, St8sia4−/−, St8sia2−/− and St8sia2−/− mice, respectively; n=3 each; one-way ANOVA, P>0.01].

Expression of PV starts at about postnatal day (P) 7 and throughout the next weeks these neurons slowly mature (Powell et al., 2012). Therefore, a developmental delay could have caused the reductions of PV+ cells in 4-week-old mice. However, in 3-month-old St8sia4−/− and St8sia2−/− mice, PV+ cells were still significantly reduced in the upper and deep layers of the mPFC (Fig. 2), as well as in all subdivisions, except for the IL cortex (supplementary material Fig. S3A,B). This excludes a developmental delay or transient reduction but leaves the possibility of a permanent downregulation of PV expression or either loss or agenesis of the respective interneuron populations during development.

To assess if the observed effects were confined to the expression of PV as a marker we analyzed the abundance of perineuronal nets (PNNs). PNNs are formed around mature basket cells and can be labeled with Wisteria floribunda agglutinin (WFA) (Härtig et al., 1992). In the mPFC of 3-month-old mice, WFA marked a subpopulation of PV+ interneurons (Fig. 2C). In the upper and deep layers, densities of PV+ cells with PNNs were significantly reduced in St8sia4−/− and St8sia2−/− mice (Fig. 2D,E) and, except for the deep layers of the IL cortex, all subdivisions were affected (supplementary material Fig. S3C,D). By contrast, numbers of PV+ cells with PNNs were unaffected [mean densities (±s.e.m.) of PV− PNN+ cells/mm2 mPFC: 16.4±1.6, 20.7±0.3 and 20.6±4.3 for control, St8sia4−/− and St8sia2−/− mice, respectively; n=3 each; one-way ANOVA, P>0.01]. These results argue against a specific loss of PV expression in polyST-deficient mice. Moreover, significantly reduced densities of SST+ interneurons were found in the upper layers of the mPFC of 3-month-old St8sia4−/− and St8sia2−/− mice and in all of its subdivisions (Fig. 2F, supplementary material Fig. S3E), and a trend towards lower numbers of SST+ cells was observed for the deep layers, significantly affecting PrL and IL cortex (Fig. 2G; supplementary material Fig. S3F).

**Fig. 1.** Parvalbumin immunoreactivity in the medial prefrontal cortex at P30. (A) Illustration of the mouse medial prefrontal cortex (mPFC), consisting of cingulate cortex area 1 (Cg1), prelimbic (PrL) and infralimbic (IL) cortex, and of the division into upper and deep layers (see also Paxinos and Franklin, 2001; see Materials and Methods for details). The square marks the position of the micrographs in B, fmi, forceps minor of the corpus callosum. (B) Parvalbumin (PV)+ cells in the Cg1 in different genotypes. Scale bar: 50 μm. (C,D) Densities of PV+ cells in the upper layers (C, cortical layers 1-3) and deep layers (D, cortical layers 5, 6) of the mPFC of St8sia4−/− (4/4+), St8sia2−/− (2/−) and St8sia2−/−, St8sia4−/− (2/−, 4/−) mice compared with a control group (ctl) consisting of one St8sia2−/−, St8sia4−/− and two St8sia2−/−, St8sia4−/− mice. Per group, mean±s.e.m. from n=3 animals. ***P<0.001; **P<0.01; one-way ANOVA and Newman–Keuls post-hoc test.
Interneuron deficits of polyST-deficient mice are established during development

GAD67-GFP knock-in mice were used to address the entire population of GABAergic interneurons (Tamamaki et al., 2003). Together with reductions of PV+ cells, lower densities of GFP+ interneurons were detected in the Cg1 of 3-month-old St8sia4−/− and St8sia2−/− GAD67-GFP mice (Fig. 3A-C). Densities of GFP+ interneurons in the mPFC of both polyST-deficient lines were already reduced at P1, but not at embryonic day (E) 16.5 (Fig. 3D,E). Analyses of TUNEL staining at P1 or E16.5 yielded no signs of increased apoptotic cell death in the mPFC (Fig. 3F,G). Together, these data suggest that the interneuron deficit of polyST-deficient mice develops between E16.5 and P1 but is not caused by lower survival rates in the mPFC.

Fewer migratory interneurons in the pallium of polyST-deficient embryos

The large majority of the SST+ and PV+ interneurons originates in the MGE (Gelman and Marin, 2010). Thus, compromised migration from the MGE into the developing neocortex might account for the observed deficits in polyST-deficient mice. In 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 zones (Anderson et al., 1997; Polleux et al., 2002). CB-immunoreactive cells with typical morphologies of migrating interneurons were detected in sections of control, St8sia4−/− and St8sia2−/− brains at E13.5 (Fig. 4A). Double labeling with polySia-specific antibody revealed that polySia is present on the migratory neurons as well as on structures in close contact with them (Fig. 4B). Densitometric analysis in the area of the intermediate zone indicated...
a clear reduction of polySia signal intensity in St8sia4+/− and, slightly more prominently, in St8sia2+/− embryos (Fig. 4C).

Concomitantly, migratory streams formed by CB+ cells in the intermediate zone were significantly shorter in St8sia4+/− and St8sia2+/− mice than in controls and numbers of CB+ cells were reduced in the pallium at E13.5 (Fig. 4D,E). As a distinct population of interneurons, we sought to analyze tangentially migrating CR+ precursors in the pallium, which are born between E14.5 and E16.5 and derive mainly from the caudal ganglionic eminence (CGE) (Xu et al., 2004). Thus, E16.5 was chosen for analysis, and because counting was hampered by the abundance of CR+ fibers entering the cortex at this stage (Fonseca et al., 1995), GAD67-GFP embryos were used to reliably identify interneurons. As shown in Fig. 4E (lower right), the numbers of CR and GFP double-positive cells were unaffected in the pallium of St8sia4+/− and St8sia2+/− embryos.

**CB+ cells accumulate in the dorsal MGE and LGE of polyST-deficient embryos**

Interneurons born in the MGE migrate towards the LGE, which they pass to reach the cortex. In the subventricular zone of the MGE and the LGE of E13.5 St8sia4+/− and St8sia2+/− embryos, polySia immunoreactivity was reduced and CB+ cells accumulated mainly in the dorsal MGE (Fig. 5A–C). Accordingly, CB immunoreactivity was significantly increased in the MGE of both knockout lines (Fig. 5B) and a slight increase was also observed in the LGE (Fig. 5C). In addition, the numbers of TUNEL+ apoptotic cells were increased in the MGE of St8sia4+/− and St8sia2+/− embryos at E13.5 (Fig. 5D,E). However, the TUNEL+ cells were not perceivably enriched in the dorsal MGE, where the accumulation of CB+ cells was detected. Apoptosis was not significantly altered in LGE, CGE and in the pallium of E13.5 embryos [mean densities (±s.e.m.) of TUNEL+ cells/mm2: 44.1±3.7, 61.4±11.6 and 59.8±8.8 for LGE; 69.9±6.1, 82.6±3.6 and 84.6±5.3 for CGE; and 70.9±8.3, 77.1±4.7 and 73.1±3.7 for pallium; of control (n=6), St8sia4+/− (n=4) and St8sia2+/− (n=6) mice, respectively; one-way ANOVA, P<0.01].

To assess overall changes in MGE interneurons, protein levels of GAD65/67 (also known as GAD2/1), CB and LHX6, a characteristic marker of MGE-derived interneuron populations (Lavdas et al., 1999; Liodis et al., 2007), were analyzed in E13.5 forebrain lysates by western blot. No changes in any of these proteins could be detected (supplementary material Fig. S4). These findings argue against drastic changes in MGE-derived interneuron numbers and are consistent with the immunohistological results demonstrating an accumulation of migratory interneurons in the ganglionic eminences and a corresponding reduction in the pallium of polyST-deficient mice.

**Acute loss of polySia leads to reduced velocities of migrating interneurons in slice cultures**

Interneuron migration was further studied in coronal slice cultures obtained from E12.5 or E13.5 GAD67-GFP embryos treated with endosialidase (endo) to remove polySia (supplementary material Fig. S5). Western blot analysis of E13.5 forebrain lysates demonstrating an accumulation of migratory interneurons in the ganglionic eminences and a corresponding reduction in the pallium of polyST-deficient mice.
endo for 1 day the number of GFP+ cells was significantly reduced in the pallium close to the pallial-subpallial boundary, whereas more distant cells were unaffected (Fig. 6A,B). Consistent with the lower numbers of CB+ cells in the pallium and their accumulation in the GE of St8sia4−/− and St8sia2−/− embryos at E13.5, these results argue for a decreased entry of interneurons into the pallium. Thus, lower interneuron numbers in the PFC of polyST-deficient mice might arise from a reduced ability to cross the subpallial-pallial boundary or from deficits in the migratory process itself. To test the latter, we sought to directly determine the impact of polySia on interneuron migration by live imaging of GAD67-GFP+ cells in slice cultures, which were treated with endo for 2 h to acutely remove polySia prior to a 3 h observation period. Owing to the high density of GAD67-GFP+ cells, analysis of migration within the ganglionic eminences was not feasible. Live imaging of interneurons in the intermediate zone migratory stream of the pallium in E13.5 slices revealed migration at vastly different velocities (Fig. 6C). In the presence of endo, the mean velocity of all GFP+ cells was significantly reduced (Fig. 6D; supplementary material Movies 1 and 2). Grouping into different velocity bins highlights a significant increase in the fraction of cells moving slower than 30 μm/h at the expense of cells migrating with an intermediate velocity (30-60 μm/h) upon removal of polySia with endo (Fig. 6E). Cells migrating faster than 60 μm/h were unaffected. Relative to their starting position, interneurons migrated in all directions with a slight preference for dorsomedial orientation. This is consistent with previous reports (Britto et al., 2006; Tanaka et al., 2006) and was not altered by endo treatment (Fig. 6F,G).

**Acute loss of polySia leads to the decreased length of interneuron leading processes**

Cortical interneurons migrate by extending a dynamic leading process followed by saltatory translocation of the nucleus (Bellion et al., 2005; for a review see Marin et al., 2010). Alterations of leading process morphology have been repeatedly linked to migration deficits (Nasrallah et al., 2006; Friocourt et al., 2007; Wang et al., 2011; Steinecke et al., 2012; Luccardini et al., 2013). 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 evaluated after 1 day in vitro. Leading processes of GFP+ interneurons were significantly shorter after endo treatment (Fig. 7A,B).

To analyze whether this effect depends on cues provided by the cortical environment, primary cultures of dissociated E13.5 MGE were prepared, which consisted of >80% GFP and polySia
double-positive cells (Fig. 7D). Cultures were run for 2 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 protraction and retraction of primary processes (Fig. 7C; supplementary material Movie 3) and significantly shorter primary processes were detected after cultivation for 1 day in the presence of endo (Fig. 7D,E). These data indicate that loss of polySia perturbs the migration of MGE-derived interneurons by interfering with leading process formation and/or stability.

DISCUSSION

Based on the findings that PV+ and SST+ interneuron populations are persistently reduced in the mPFC of polyST-deficient mice, we tested the hypothesis that deficits in cortical interneuron migration might cause these alterations. Evaluation of genetically labeled interneurons in ST8SIA2- and ST8SIA4-deficient GAD67-GFP mice pointed towards cell loss during embryonic development. Accumulation of precursors in the ganglionic eminences and reduced numbers of tangentially migrating interneurons in the pallium were observed in polyST-deficient embryos. Enzymatic removal of polySia in embryonic slice cultures revealed that acute loss of polySia hampered the entry of interneurons from the MGE into the pallium, reduced the speed of a fraction of cortical interneurons migrating at an intermediate velocity and affected the lengths of their leading processes. Thus, polySia is essential for the migration of interneurons from the MGE into the cortex, suggesting that impaired tangential migration is a primary cause for the lack of mPFC interneurons in mice with compromised polySia synthesis.

PolySia is exclusively synthesized by the two polySTs ST8SIA2 and ST8SIA4 (Weinhold et al., 2005). ST8SIA2 is almost completely downregulated during postnatal development of the rodent and human cortex (Hildebrandt et al., 1998; Ottemann-Norden et al., 2008; McAuley et al., 2012), and ST8SIA4 is solely responsible for polySia expression in mature interneurons of the adult mouse cortex (Nacher et al., 2010). By contrast, and supportive of a common developmental origin of interneuron deficits in both polyST knockout lines, ST8SIA2 and ST8SIA4 are abundantly detected in the embryonic mouse brain and the 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).

As shown in the current study, acute removal of polySia in slice cultures leads to slower migration of cortical interneurons associated with shorter leading processes. Owing to the high density of GAD67-GFP interneurons in the ganglionic eminences these observations were restricted to the pallium. However, the accumulation of CB+ interneuron precursors in the MGE as well as the reduced numbers of interneurons invading the pallium at early stages of migration in polyST-deficient mice or after acute polySia removal in slice cultures suggest that polySia depletion impairs the migration of MGE interneurons from the beginning. Furthermore, cultured MGE interneurons generated shorter processes after polySia removal, indicating that the impact of polySia on leading process morphology is independent of factors provided by the cortical environment.

Previously, the slower migration of interneurons has been linked with either longer or shorter leading processes. For example, longer leading processes have been observed in mice deficient for LIS1 and DISC1, in which impaired migration seems to be linked to altered nucleokinesis (Nasrallah et al., 2006; Steinecke et al., 2012). By contrast, ablation of either CXCR4 or CXCR7 chemokine receptor caused increased or reduced interneuron motility with longer or
shorter leading processes, respectively (Wang et al., 2011). Of particular interest, perturbation of N-cadherin-mediated cell adhesion produced slower MGE-derived interneurons with shorter and less stable leading processes (Luccardini et al., 2013). Similarly, altered cell adhesion might be the primary mechanism by which polySia affects leading process morphology and interneuron migration. However, other possibilities, such as altered interactions of cell surface receptors or different membrane dynamics in the presence or absence of polySia should be considered and explored in future studies.

Reminiscent of the findings in polyST-deficient mice, altered precursor migration from the MGE and reduced numbers or loss of mainly PV interneurons in the postnatal cortex were observed in mouse models with deficiencies of NRG1/ERBB4 (Flames et al., 2004; Fisahn et al., 2009) or DISC1 (Hikida et al., 2007; Shen et al., 2008; Steinecke et al., 2012), which are among the most compelling schizophrenia risk genes in human (Ross et al., 2006), and in mice lacking the brain-derived neurotrophic factor receptor TRKB (also known as NTRK2) (Polleux et al., 2002) or the urokinase-type plasminogen activator receptor uPAR (also known as PLAUR) (Powell et al., 2001, 2003). Collectively, these results indicate that the joint reduction of PV+ and SST+ cells in the mPFC of polyST-deficient mice originates from deficits in the tangential migration of central and ventral MGE-derived cortical interneuron precursors.

Assuming that defects in migration from the central and ventral MGE are the primary cause of altered PV+ and SST+ densities it is surprising that PV− CB+ interneurons were not affected in the mPFC derived cortical interneuron precursors.

The loss or a reduction of NCAM-bound polySia affects PV+ interneurons, which mainly comprise basket and chandelier cells, as well as SST− interneurons, which are a heterogeneous population with many being PV− Martinotti cells (Kawaguchi and Kubota, 1997; Gonchar and Burkhalter, 1997; Markram et al., 2004; Gelman and Marin, 2010). Fate-mapping studies indicate that interneurons of the adult cortex expressing PV, SST and CB are preferentially generated from precursors of the central and ventral MGE as characterized by expression of the NKX2.1 and LHX6 transcription factors, whereas a CR and SST double-positive fraction of Martinotti cells and the bipolar CR-expressing interneurons are generated from the dorsal MGE and from outside the MGE, respectively (Xu et al., 2004; Fogarty et al., 2007; Anastasiades and Butt, 2011). Nkx2.1 knockout mice, which die at birth, lack SST+ cells at E19.0 (Anderson et al., 2001), and in the cortex of 2-week-old Lhx6 null mice dramatic reductions of PV+ and SST+ 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 were associated with the delayed tangential migration and defective differentiation of MGE-derived progenitors into PV+ and SST+ interneurons. Together, these earlier studies support the assumption that the joint reduction of PV+ and SST+ cells in the mPFC of polyST-deficient mice originates from deficits in the tangential migration of central and ventral MGE-derived cortical interneuron precursors.

Fig. 7. Removal of polySia leads to decreases in the length of interneuron leading processes in slice cultures and in MGE-derived primary cultures from embryonic GAD67-GFP mice. (A) E12.5 slice cultures after 1 day in vitro in the absence (ctrl) or presence of endosialidase (endo). Arrowheads mark the end of a primary process. (B) Lengths of leading processes of GFP+ cells. Mean±s.e.m. from n=8 animals for each condition. A total of 186 cells for ctrl and 273 cells for endo were evaluated. (C) Selected frames of a time-lapse recording of an E13.5 MGE primary culture illustrating the dynamics of leading processes of non-migrating interneurons. The arrowhead marks the end of a primary process. (D) GFP+ cells in E13.5 MGE primary cultures cultivated for 1 day in the absence (ctrl) or presence of endo and immunostained as indicated. Nuclei were counterstained with DAPI. The boxed region of the merge is magnified to the right. Residual polySia immunoreactivity after endo treatment was confined to an intracellular compartment close to the nucleus, most likely representing newly synthesized polySia in the Golgi. (E) Lengths of leading processes of GFP+ cells. Mean±s.e.m. from n=16 wells each. A total of 321 cells for ctrl and 335 cells for endo were evaluated. (B,E) **P<0.01; ***P<0.001; Student’s t-test. Scale bars: 20 µm in A and D (left); 10 µm in C and D (right).
corpus (Aldantara et al., 1996). Together with recent evidence for a common clonal origin of at least some of the PV+ and SST+ interneurons (Brown et al., 2011), the available data seem compatible with the possibility that such a transition, i.e. the differentiation of CB+ MGE-derived progenitors into PV+ or SST+ interneurons, is impaired by the inappropriate timing of the slower migrating interneurons and/or affected by the reduced numbers of cells arriving in the mPFC of polyST-deficient mice. Such a scenario would also be consistent with earlier data indicating that the definite expression profiles of PV and CB, as well as other parameters such as the laminar position, can be influenced by environmental cues within the cortex (Mione et al., 1994; Valcanis and Tan, 2003).

The persistent reduction of PV+ interneurons includes, but was not confined to, cells with WFA+ PNNs. This special type of extracellular matrix is formed at the end of the critical period of cortical plasticity (Rhodes and Fawcett, 2004) and is characteristic for a subpopulation of basket cells (Ojima, 1993; Wegner et al., 2003). Notably, changes in basket cells have been found in other mouse models with altered polySia or NCAM levels. Transgenic mice overexpressing the extracellular domain of NCAM show reduced perisomatic innervation from basket cells and impaired synaptic plasticity in the PFC (Pillai-Nair et al., 2005; Brennan and Maness, 2008; Brennan and Maness, 2011). Premature removal of polySia induced untimely maturation of perisomatic innervation and the onset of critical period plasticity in the visual cortex (Di Cristo et al., 2007). The balanced regulation of polySia and NCAM therefore has sequential functions during the development of PV+ basket cells.

It remains unknown whether and how the reduced interneuron densities affect synaptic connectivity and network functions or if they lead to compensatory mechanisms in one or both of the polyST-deficient lines. However, altered mPFC interneuron functions might lead to compensatory mechanisms in one or both of the polyST-deficient lines. This special type of extracellular matrix is formed at the end of the critical period of cortical plasticity (Rhodes and Fawcett, 2004) and is characteristic for a subpopulation of basket cells (Ojima, 1993; Wegner et al., 2003). Notably, changes in basket cells have been found in other mouse models with altered polySia or NCAM levels. Transgenic mice overexpressing the extracellular domain of NCAM show reduced perisomatic innervation from basket cells and impaired synaptic plasticity in the PFC (Pillai-Nair et al., 2005; Brennan and Maness, 2008; Brennan and Maness, 2011). Premature removal of polySia induced untimely maturation of perisomatic innervation and the onset of critical period plasticity in the visual cortex (Di Cristo et al., 2007). The balanced regulation of polySia and NCAM therefore has sequential functions during the development of PV+ basket cells.

In summary, our study demonstrates that polySia is essential for cortical interneuron development and provides a possible link between neurodevelopmental deficits and the emerging evidence that genetic variation of the polySia-NCAM system may be associated with schizophrenia (Arai et al., 2006; Tao et al., 2007; Brennan and Maness, 2010; McAuley et al., 2012). Reporter assays with a risk haplotype for ST8SIA2 suggested higher expression (Arai et al., 2006), whereas another risk variant is associated with lower ST8SIA2 mRNA levels in post-mortem PFC samples (McAuley et al., 2012). Accordingly, the phenotype of ST8SIA2−/− mice can provide clues as to how reduced ST8SIA2 expression might actually lead to an increased risk for schizophrenia. Future work should focus on analyzing common or distinct alterations of synaptic and network activities in the mPFC of ST8SIA2−/− and ST8SIA4−/− mice and explore whether the interneuron deficits of polyST-deficient mice are necessary or sufficient to explain altered behavior.

**MATERIALS AND METHODS**

**Mice**

C57BL/6J and mutant mice were bred at the central animal facility at Hannover Medical School. All protocols for animal use were in accordance with the guidelines for animal experiments established by the European Union and approved by the local authorities. ST8SIA2 and ST8SIA4 knockout strains, backcrossed with C57BL/6J mice for six generations, were intercrossed to obtain ST8SIA2+/− −/− double-knockout animals (Weinhold et al., 2005) or cross-bred with GAD67-GFP knock-in mice (Tamamaki et al., 2003) to obtain ST8SIA2−/− or ST8SIA4−/− mice heterozygous for the transgene (ST8SIA2−/− GAD67-GFP and ST8SIA4−/− GAD67-GFP). Genotyping was performed by PCR as previously described (Tamamaki et al., 2003; Weinhold et al., 2005). For staging of embryos, the morning of the vaginal plug was considered as E0.5. To ensure that embryos were matched in developmental stage, heterozygous and homozygous litters were used and staging was controlled by external features such as the separation of digits (Kaufman, 1992).

**Immunofluorescence and TUNEL labeling**

Sectioning, immunofluorescence staining, detection of PNNs and terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick end labeling (TUNEL) were performed as described previously (Schiff et al., 2011). Specificity of TUNEL signals was controlled by omitting either the digoxigenin-conjugated dUTP or the terminal deoxynucleotidyl transferase; neither control gave any signal. The following monoclonal (mAb) or polyclonal (pAb) antibodies were applied according to the manufacturers’ instructions: CR- and CB D-28k-specific rabbit pAb (7699/4 and CB38a, Swant; 1:5000), PV-specific mouse mAb (IgG1, 235, Swant; 1:5000), SST-specific rat mAb (IgG2b, MAB 354, Chemicon; 1:200), and respective Cy3-conjugated (AD124P, Chemicon; 1:500) and Alexa Fluor 488-, 568- or 647-conjugated (A11029, A11006, A21206, A11306 or A21236, Invitrogen/Molecular Probes; 1:500) secondary antibodies. PolySia-specific mouse mAb 735 (IgG2a) (Frosch et al., 1985) was used at 5 µg/ml. All immunostaining procedures were controlled by omission of primary antibody. In double-stained samples, cross-reactivity of secondary antibodies was controlled by omitting either of the two primary antibodies. For nuclear counterstain, sections were mounted in Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

**Western blotting**

Embryonic forebrains were dissected, lysed and extracts were subjected to SDS-PAGE and western blotting with 20 or 40 µg total protein per lane as described previously (Hildebrandt et al., 2009). Detection and densitometric evaluation were performed with the Odyssey Infrared Imaging System (LI-Cor Biosystems). The following antibodies were used: polySia-specific mAb 735 (1 µg/ml), NCAM-specific rat mAb H28 (0.4 µg/ml) (Hirn et al., 1983), GAD65/67-specific rabbit pAb (3 µg/ml; Sigma-Aldrich, G 5163), LHX6-specific rabbit pAb (0.4 µg/ml; Santa Cruz Biotechnology, sc-98607), and CB D-28k-specific rabbit pAb (1:4000; Swant). As loading control, membranes were stripped and reacted with GAPDH-specific mouse mAb (0.4 µg/ml; Life Technologies, AM 4300). Primary pAbs were detected with 25 ng/ml IgG-specific IRDye 680- and 800-conjugated antibodies (LI-COR Biosciences, 926-32221, 926-32211 or 131002); mAbs were detected with 100 ng/ml IgG subtype-specific IRDye 700- and 800-conjugated antibodies (Rockland, 610-430-041 and 610-432-040).

**Cortical slice preparation and culture**

E12.5 or E13.5 brains obtained from GAD67-GFP embryos were dissected in ice-cold dissection buffer 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 double-distilled H₂O and embedded in 4% low-gelling
agarse (AppliChem) in PBS (pH 7.4). Per embryo, two or three 225 μm coronal slices were obtained with a vibrating microtome. Slices were preincubated for 30 min on ice in dissection buffer supplemented with 10 mM HEPES, 1× penicillin/streptomycin (Biochrom) and 50 μg/ml gentamycin (Sigma-Aldrich). Where indicated, slices were incubated with 4 μg/ml endosialidase (endo) (Stummeyer et al., 2005), which reliably and with high specificity removes polySia from the surface of living cells in culture and in tissues (Jakobsson et al., 2012). After transfer to Millicell cell culture inserts (PCLM0RG50, Merck-Millipore) in six-well plates, slices were cultured for 1 day at 37°C and 5% CO2 in growth medium 1 comprising Neurobasal medium (Life Technologies) containing 1× B27 supplement (Life Technologies), 32 mM D-glucose, 2 mM L-glutamine, 1× penicillin/streptomycin, and 4 μg/ml endo, where indicated. For live imaging, slices of E13.5 embryos were prepared as described above, but cultivation was carried out in eight-well imaging slides (μ-slide 8 well, ibidi) and slices were embedded in rat-tail collagen type I (BD Biosciences) at 1.3 mg/ml in 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.

**Time-lapse imaging**

Live imaging was performed at 37°C and 5% CO2 using a microscope incubator (Pecon). Time-lapse sequences were generated by acquiring images with a 10× objective at an interval of 2 min for up to 14 h using an Axiovert 200 M microscope equipped with an AxioCam MRm digital camera and AxioVision software (Carl Zeiss). Pairs of endo-treated and control slices were recorded simultaneously using the automated stage of the microscope controlled by the ‘Mark and Find’ feature of the AxioVision software. For 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 counted by visual inspection assisted by the interactive event counting tool of the AxioVision software. To avoid bias by the slight reduction in overall cortex size in Shtsia2−/− 4−/− mice (Hildebrandt et al., 2009), cell counts for each evaluated region were normalized to the respective area.

For analyses of embryonic stages, two to four coronal sections at the level of the MGE/LGE, the CGe or the mpFC from at least three E13.5 or E16.5 embryos per genotype were double stained for polySia and CB or CR, or subjected to TUNEL, respectively. Acquired micrographs were blinded and evaluated either by cell counting as described above or by densitometry (see below). Leading processes of GAD67-GFP+ interneurons were tracked on images of slice or MGE primary cultures and track lengths were evaluated with AxioVision software.

**Densitometric quantification and assessment of migratory stream lengths**

Densitometric quantification of polySia immunoreactivity in the pallium was carried out using ImageJ software (Schneider et al., 2012). Ten consecutive optical sections in a z-stack were merged into one image using AxioVision software, exported to ImageJ in TIFF format and mean gray values of the intermediate zone migratory stream were determined. Two independent sets of experiments were analyzed, each set consisting of four to six sections obtained from one animal per genotype. Tangential interneuron 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. Per animal, four consecutive sections were evaluated. Densitometric quantification of polySia and CB immunoreactivity in the MGE or LGE was performed at E13.5 on at least four coronal sections per structure and embryo. Structures were outlined on double-stained sections and mean intensities of polySia and CB signals were determined from the same region of interest using ZEN 2012 software (Carl Zeiss).

**Statistical analysis**

All data are given as mean±s.e.m. Statistical analyses were performed with Prism 4 software (GraphPad) using unpaired Student’s t-test and ANOVA with Newman–Keuls multiple comparison post-hoc test, as indicated.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

T.K., I.R., U.D. and H.B. performed all experiments; B.W. organized breeding of polyST-deficient mice; Y.Y. provided GAD67-GFP mice; R.G.-S. critically revised the manuscript; T.K., I.R. and H.H. designed experiments, analyzed the data and wrote the manuscript.

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Deficiency of neural cell adhesion molecule or its polysialylation modulates functions in regulating interneuron migration. Development 133, 2167-2176.


