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

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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 entry 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; Schaar 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 (Brennan and Maness, 2010; Anney et al., 2010; McAuley et al., 2012; Gilabert-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 neuropeptide somatostatin (SST) has proven a powerful tool for the identification and evaluation of GABAergic interneuron subtypes (Gabbott et al., 1997; Gonchar and Burkhelter, 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 NRGI 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 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−/−/4−/− 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−/−/4−/− 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−/−/4−/−) (Fig. 1). The most prominent effects were observed in the upper and deep layers of 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+ cells (supplementary material Fig. S2C,D) and CR+ cells were unaffected [mean densities (+s.e.m.) of CR+ cells/mm2 mPFC: 59.1±2.5, 49.36±4.4, 43.52±5.6 and 51.9±1.8 for control, St8sia4−/−, St8sia2−/− and St8sia2−/−/4−/− mice, respectively; n=3 each; one-way ANOVA, P>0.1].

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 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>0.1]. 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−/−), St8sia2−/− (2−/−) and St8sia2−/−/4−/− mice compared with a control group (ctrl) 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

Fig. 3. Reduced interneuron densities but no increase in cell death in the mPFC of St8sia4−/− and St8sia2−/− mice compared with wild-type GAD67-GFP mice. (A) GFP+ cells (green) and colocalization of GFP and PV (red) in Cg1 of the mPFC of 3-month-old mice. Ctrl, wild-type GAD67-GFP. Scale bar: 25 μm. (B-E) Densities of GFP+ and PV+ cells in Cg1 (B,C) or mPFC (D,E) at the indicated age. (F,G) Densities of apoptotic cells (TUNEL+) in the mPFC. Per group, mean±s.e.m. from n=3 animals. **P<0.01; *P<0.05; n.s., not significant (P>0.05); one-way ANOVA and Newman–Keuls post-hoc test.
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<0.1].

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 for LGE, CGE and in the pallium of E13.5 embryos. PolySia weights of polysialylated SynCAM1 (also known as CADM1) and neuropilin 2 as other possible carriers of polySia (Curreli 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.

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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 $\text{St8sia4}^{-/-}$ and $\text{St8sia2}^{-/-}$ 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 $\mu$m/h at the expense of cells migrating with an intermediate velocity (30-60 $\mu$m/h) upon removal of polySia with endo (Fig. 6E). Cells migrating faster than 60 $\mu$m/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...
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; Ottmann-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 or its subdivisions in any of the polyST-deficient genotypes studied. Most, if not all, MGE-derived interneurons seem to express CB 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 PV+ interneurons of the rat.
cortex (Alcantara 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 Breannan, et al., 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-densities affect synaptic connectivity and network functions or if they are affected by the reduced numbers of basket cells.

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−/− or St8sia4−/− 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 littermates 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 signal 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, A11036 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: polY-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 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).

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
agarose (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 (PallMRG50, 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 used for analysis and migratory paths were reconstructed by manual tracking assisted by the AxioVision software. In order to summarize the direction of migration, all paths obtained were centered to a common starting point.

**MGE primary culture**

MGEs of E13.5 GAD67-GFP mice were dissected in ice-cold dissection buffer 2 comprising PBS (pH 7.4) containing 0.6% (w/v) 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 DNase (Roche), cells were dissociated by gentle trituration with a 1000 μl pipette. Cells were pelleted at 200 g for 10 min, washed three times with dissection buffer 2 and resuspended in growth medium 2 consisting of Neurobasal medium with 2 mM L-glutamine (Life Technologies), 1× B27 supplement, 1× penicillin/streptomycin, with or without 4 μg/ml endo. For live imaging, 6.6×10⁵ cells/ml were seeded in eight-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, 10⁶ cells/ml were plated on coverslips 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 day in vitro. For live imaging, cells were incubated for 2 h under standard conditions prior to image acquisition with an Axiovert 200 M microscope with live imaging equipment (as above).

**Image acquisition, cell counting, area and length measurements**

For analyses of postnatal stages, three to six sections per brain from at least three mice were analyzed per experimental group. Pairs of consecutive, 50 μm vibratome sections equally spaced between bregma level 1.9 mm and 1.54 mm were selected. PV was labeled together with either CR or CB (three sections each) or with SST or WFA (six sections each). Near confocal images of 5.1 μm optical sections were acquired with structured illumination (ApoTome technology) using an Axiovert 200 M microscope and a 10× Plan-Apochromat objective with 0.45 numerical aperture (Carl Zeiss). Micrographs of entire sections were acquired by the MosaiX image montage module of the AxioVision software. Identical microscope settings were used among different specimens. For evaluation, micrographs were coded and randomized to ensure that the observer was blind to experimental conditions. Both hemispheres were always evaluated. On each image the mPFC was outlined by use of anatomical landmarks, such as the characteristic shape of the forceps minor of the corpus callosum (fmi in Fig. 1A). The mPFC was subdivided into IL, PrL and CgL according to Paxinos and Franklin (2001) and partitioned into upper and deep layers corresponding to layers 1 to 3 and 5 to 6 of the mPFC (lacking a distinct layer 4). The border between upper and deep layers was determined on each section by inspection of nuclear counterstain with DAPI. Cell counting and area measurements were performed as described previously (Schiff et al., 2011). Briefly, areas were measured for each region of interest and the total numbers of labeled cells 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 St8sia2−/−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. and H.H. designed experiments, analyzed the data and wrote the manuscript; T.K., I.R. and H.H. designed experiments, analyzed the data and wrote the manuscript.

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