In humans, variations in the polysialic acid-producing enzyme ST8SIA2 and disturbances in the cortical inhibitory system are associated with neurodevelopmental psychiatric disorders such as schizophrenia and autism. In mice, the ST8SIA2-dependent formation of polysialic acid during embryonic development is crucial for the establishment of interneuron populations of the medial prefrontal cortex. However, the spatial pattern and the neurodevelopmental mechanisms of interneuron changes caused by loss of ST8SIA2 function have not been fully characterized. Here, we use immunohistochemical analysis to demonstrate that densities of parvalbumin-positive interneurons are not only reduced in the medial prefrontal cortex, but also in the adjacent motor and somatosensory cortices of St8sia2-deficient male mice. These reductions, however, were confined to the rostral parts of the analyzed region. Mice with conditional knockout of St8sia2 under the interneuron-specific Lhx6 promoter, but not mice with a deletion under the Emx1 promoter that targets cortical excitatory neurons and glia, largely recapitulated the area-specific changes of parvalbumin-positive interneurons in the anterior cortex of St8sia2−/− mice. Live imaging of interneuron migration in slice cultures of the developing cortex revealed a comparable reduction of directional persistence accompanied by increased branching of leading processes in slice cultures obtained from St8sia2−/− embryos or from embryos with interneuron-specific ablation of St8sia2. Together, the data demonstrate a cell-autonomous impact of ST8SIA2 on cortical interneuron migration and the distribution of parvalbumin-positive interneurons in the anterior cortex. This provides a neurodevelopmental mechanism for how dysregulation of ST8SIA2 may lead to disturbed inhibitory balance as observed in schizophrenia and autism.

**Keywords:** autism, parvalbumin-positive interneurons, polysialic acid, prefrontal cortex, PSA-NCAM, schizophrenia.

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**Abbreviations used:** ANOVA, analysis of variance; Cat. #, catalog number; Cg1, cingulate cortex area 1; DAPI, 4,6-diamidino-2-phenylindole; M1, primary motor cortex; M2, secondary motor cortex; mAb, monoclonal antibody; MGE, medial ganglionic eminence; mPFC, medial prefrontal cortex; pAb, polyclonal antibody; polySia, polysialic acid; PV“CB”, parvalbumin-positive, calbindin-negative; ROI, region of interest; RRID, Research Resource Identifier (see scicrunch.org); S1, primary somatosensory cortex; SEM, standard error of the mean.
loss of parvalbumin-positive interneurons in the prefrontal cortex is a frequently reported neuropathological finding (Benes and Berretta 2001; Marin 2012; Hashemi et al. 2017). Earlier, we demonstrated reduced densities of parvalbin- and somatostatin-expressing interneurons as well as reduced densities of parvalbumin-positive perisomatic synapses formed by basket cells on pyramidal neurons in the medial prefrontal cortex (mPFC) of St8sia2-deficient mice (Kröcher et al. 2014; Curto et al. 2019). Accumulation of precursors in the embryonic medial ganglionic eminence (MGE) and reduced number of tangentially migrating interneurons in the pallium of St8sia2−/− embryos indicated that the changes in these major interneuron populations are caused by altered migration of their precursors from the MGE toward and into the cortical anlage (Krocher et al. 2014). This received support by the observation that a depletion of polySia by enzymatic treatment of embryonic brain slice cultures led to a decreased entry of interneurons from the ganglionic eminences into the pallium and to a slower migration of interneurons that already had entered the pallium. However, in contrast to these consequences of an acute and complete removal of polySia by interneuron migration in St8sia2−/− embryos may be affected in a different way, mainly because they show normal expression of ST8SIA4 and therefore retain significant amounts of polySia in the ganglionic eminences and the pallium (Krocher et al. 2014). Furthermore, polySia is present on the migrating interneurons as well as in their cortical environment, but so far it is not known, whether polySia impacts interneuron migration cell autonomously or as an environmental cue.

Here we address major open questions concerning the spatial pattern and the neurodevelopmental mechanisms of interneuron changes caused by loss of ST8SIA2 function. Focusing on parvalbin-positive cells of the deep cortical layers, we demonstrate that cortical interneurons are not just reduced in the mPFC of St8sia2-deficient mice, but also in some but not all of the adjacent cortical areas. Conditional deletion of St8sia2 under the MGE-specific Lhx6 promoter (Fogarty et al. 2007) mimics these alterations. In contrast, no reduction of cortical interneurons was observed in mice with a deletion of St8sia2 under the Enx1 promoter that targets St8sia2 in cortical excitatory neurons and glia, but not in interneurons (Gorski et al. 2002). These findings match results obtained by live cell imaging of interneuron migration in the embryonic pallium demonstrating a robust cell-autonomous impact of ST8SIA2 on directional persistence and leading process branching of migrating interneurons.

Methods

Animals

All mice used in this study were bred at the central animal facility of Hannover Medical School. Protocols for animal use were in compliance with the German Animal Welfare Act and approved by the local authorities (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, permissions no. 33.9-42502-04-10-0169 and –15/1902). Mice heterozygous for a loxp sequence-insertion flanking exon 4 of St8sia2 (St8sia2fl/+ ) were generated by a commercial supplier (C57BL/6-St8sia2tm2Cre/+). Taconic Biosciences GmbH and two breeding pairs were transferred to the animal facility of Hannover Medical School. A targeting vector comprising loxp sequences flanking exon 4 and a neomycin resistance flanker by FRT sites inserted into intron 3 were transfected into C57BL/6N Tac embryonic stem cells and positive clones were implanted in a surrogate mother. By FLP-mediated removal of the neomycin resistance, mice with the conditional allele (St8sia2fl/fl) were obtained. In these mice, recombination by CRE-recombinase will result in the deletion of exon 4, encoding a significant portion of the sialyl motif L, which is essential for sialyltransferase activity (Datta and Paulson 1995). The same strategy has been applied for the generation of St8sia2−/− mice (Angata et al. 2004). Mice with the conditional St8sia2-allele were then crossbred with Zp3-Cre mice (de Vries et al. 2000; RRID:IMSR_JAX:002655; obtained from Jackson Laboratories) to obtain St8sia2−/− mice, or with mice expressing Cre-recombinase either under a pallium-specific promoter targeting Cajal–Retzius cells, glutamatergic neurons, astrocytes and oligodendrocytes, but not GABAergic neurons [Emx1-Cre, (Gorski et al. 2002); RRID:IMSR_JAX:005628, obtained from Jackson Laboratories], or under an MGE-specific promoter. For the latter, we decided to use mice that carry a bacterial artificial chromosome to express Cre recombinase under the transcriptional control of Lhx6 (Lhx6-Cre mice; RRID:IMSR_JAX:026555; created by Nicoletta Kessaris, University College London and generously provided by Oscar Marin, King’s College London), because a comparative analysis of Lhx6–Cre and Nkx2.1–Cre reporter mice indicates that nearly 100% of the parvalbin-positive interneurons are generated from Lhx6-expressing precursors, whereas only about 80% are derived from Nkx2.1-expressing precursors (Fogarty et al. 2007). Lhx6-Cre mice are commonly used for post-mitotic ablation of target genes in studies of MGE-derived interneuron development and show no abnormalities of cortical interneuron migration or distribution (Nobrega-Pereira et al. 2008; Luccardini et al. 2013). All mouse strains were backcrossed with C57BL/6N mice for at least six generations. Mice lacking all isoforms of NCAM [Ncam1fl/+], RRID:IMSR_JAX:020405 were obtained from Harold Cremer (Developmental Biology Institute of Marseille) and three 30-day-old Ncam1fl/+ mice that have been processed in parallel with the St8sia2fl/+ and control mice analyzed by Kröcher et al. (2014), were used to comparatively evaluate mPFC interneurons. For live imaging experiments, the initially described St8sia2fl/+ line was used, holding the same deletion exon 4 (Angata et al. 2004), and St8sia2fl/fl;GAD67GFP mice, St8sia2−/−;GAD67GFP, St8sia2fl/+;GAD67GFP and Lhx6-Cre;St8sia2fl/+ mice were generated by crossing back with GAD67-GFP knock-in mice to label the vast majority of cortical interneurons, including the MGE-derived population of parvalbin- and somatostatin-positive cells (Tamamaki et al. 2003). St8sia4−/− mice (Eckhardt et al. 2000) were crossed with Lhx6-Cre;St8sia2fl/+ to obtain Lhx6-Cre;St8sia2−/−,St8sia4−/− mice. St8sia2−/− and St8sia4−/− mice were genotyped by PCR as previously described (Tamamaki et al. 2003; Weinhold et al. 2005). Genotyping of the conditional St8sia2 allele, Lhx6- and Emx1-Cre was performed utilizing the following primers:
cond-1b (5’-GAGACGACACTAGAGGAAATAACA-3’) with cond-2 (5’-CTCTAGAGTTGTTGTTGC-3’) for the floxed St8sia2α allele, and cond-1b with cond-4 (5’-ACAGTTGAGAACACAC-CCCTTC-3’) for the recombined St8sia2α allele; iCreF (5’-GAGGGCTACCTCTGTACC-3’), iCreR (5’-TGCCCAAGGT-CATCCCTGGC-3’), ctrl-F (5’-ACTGGGAATCTCGAACTC TTTGG-3’) and ctrl-R (5’-GATGGTGGGCCACTGCTCATCA-3’) for the Lhx6-Cre allele; Emx-s (5’-CCGGTGCTGGAC-TAAAACATCT-3’), Emx-as (5’-GTGAACACGCATTGCTG-TACCT-3’), EmxWT-s (5’-AAGGTGTGTTCCAGAAATCGC-3’) and EmxWT-as (5’-CTCTCCACCCAGAAGCTGAG-3’) for the Emx1-Cre allele. For staging of embryos, the morning of the vaginal plug was considered as E0.5. For the phenotypic analyses, 3-month-old Lhx6-Cre;St8sia2f/f, Emx1-Cre;St8sia2f/f and Lhx6-Cre;Emx1-Cre;St8sia2f/f mice and, whenever possible, St8sia2f/f littersmates were obtained.

Mice were housed in individually ventilated cages with free access to food and water. Litters consisted of 3–12 pups. After weaning, a maximum of five littersmates of the same sex were housed together. No single-housed mice were included in this study. A total of 62 embryos and 36 three-month-old male mice were used and because no inclusion or exclusion criteria were pre-determined in this exploratory study, no animals were excluded from analyses. However, for phenotypical analyses of 3-month-old mice, some of the immunostained areas could not be evaluated because of damage in the tissue of interest. Therefore, no randomization was performed to allocate subjects in an immunostained area could not be evaluated. For the evaluation of the floxed allele; 5’-CTCTCGAGACATTATT-CATTA-3’ and 5’-GGAGGGGTTCATAGGTTG-3’ for detection of the recombined allele; 5’-TCTCATGCTACGTTGCT-3’ and 5’-GGACTGCCAGACTGATG-3’ and 5’-CCACAGTC-GGAATGGTGAT-3’ for the housekeeping gene PPIA), 0.2 mM dNTPs, 2.25 mM MgCl2, 1.6 µL SYBR Green (diluted 1:1000; Thermo Fisher Scientific, Cat. #4367808), 0.2 µL ROX reference dye (Thermo Fisher Scientific, Cat. #12223012) and 4 µL cDNA (1:10) in Maxima Hot Start reaction buffer (Thermo Fisher Scientific, Cat. #EP0601). Mixtures were pre-heated to 50°C for 20 s, and then maintained at 95°C for 10 min. qPCR was performed in 40 cycles at 95°C for 15 s and 60°C for 1 min (7500 Fast Real-time PCR system, Applied Biosystems) and evaluated using the comparative threshold method (ΔΔCt).

Antibodies

The following monoclonal (mAb) or polyclonal (pAb) antibodies were used: Calbindin–specific rabbit pAb D28-k (Swant Cat. #CB38, RRID:AB_2721225, 1 : 5000), parvalbumin-specific goat pAb (Swant Cat. #PVG-214, RRID:AB_2313848, 1 : 5000), beta III-tubulin-specific mouse mAb (IgG2c; Sigma-Aldrich, St Louis, MO, USA Cat. #T8660, RRID:AB_477590, 1:200), polySiA-specific mouse mAb 735 (Frosch et al. 1985) (RRID:AB_2619682, IgG2a, 2 µg/mL), produced in-house as described by Werneburg et al. (2015), LHX6-specific rabbit pAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA Cat. #sc-98607, RRID:AB_2135838, 1 : 400) and respective Alexa Fluor 488-conjugated goat anti-mouse IgG (Thermo Fisher Scientific, Cat. #A-21141, RRID:AB_2535778), Alexa Fluor 568 conjugated goat anti-mouse IgG (Thermo Fisher Scientific, Cat. #A-21134, RRID:AB_2535773), Alexa Fluor 647-conjugated goat anti-rabbit IgG H + L (Thermo Fisher Scientific, Cat. #A-21141, RRID:AB_2535778), Alexa Fluor 488-conjugated donkey anti-mouse IgG (H + L) (Thermo Fisher Scientific, Cat. #A-21141, RRID:AB_2535778) and anti-mouse IgG Cy3 (Sigma-Aldrich Cat. #C2181, RRID:AB_258785) secondary antibodies.

Immunohistochemistry, MGE primary culture and immunocytochemistry

For adult mice, perfusion, generation of 50 µm vibratome sections, immunofluorescent staining on free-floating sections and mounting in Vectashield with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA, Cat. #H-1200, RRID:AB_2336790) were performed as described previously (Schiff et al. 2020).
2011), but instead of fetal calf serum, 2% normal goat serum (Vector Laboratories, Cat. #S-1000) was added to the blocking buffer. PolySia and calbindin immunostaining of embryonic brain sections, as well as preparation and immunocytochemistry of MGE primary cultures were performed as described by Kröcher et al. (2014). Staining specificity was controlled by omission of primary antibodies. For the control of polySia staining, some sections were treated with 6 µg/mL endolysialase for 2 h at 37°C prior to antibody incubation, as described before (Tantra et al. 2014). This treatment completely abolished labeling with mAb 735.

To assess the rate of polySia biosynthesis, MGE primary cultures were treated with 20 ng/mL of endolysialase (Stummeyer et al. 2005), which reliably and with high specificity removes polySia from the surface of living cells and, after thorough washing, allows for monitoring the reappearance of polySia at the cell surface (Seidenfaden and Hildebrandt 2001).

**Image acquisition, cell counting and distance-calculations**

For analysis of interneuron distributions in the anterior cortex of adult mice, six equally spaced sections corresponding to bregma levels 1.10–1.94 according to Paxinos and Franklin (2001) were chosen for analysis. As specified in the figure legends, n = 5–13 animals were analyzed for each genotype. Double staining for parvalbumin and calbindin was conducted and images were acquired with an AxioObserver.Z1, equipped with an ApoTome device for near-confocal imaging by use of structured illumination and an AxioCam MRm digital camera. Optical sections of 1.10 µm (488 channel) and 5.54 µm (568 channel) were obtained using a 10 × Plan-Apochromat objective with 0.45 numerical aperture (Zeiss, Carl Zeiss Microimaging, Göttingen, Germany). Single images were stitched with Zen2012 (Zeiss). Regions of interest were identified by fitting a template based on the respective bregma levels (Paxinos and Franklin 2001). Deep layers (layer IV–VI or layer V–VI in the case of Cg1 lacking a distinct layer IV) were identified by inspection of nuclear counter-stain (4,6-diamidino-2-phenylindole) and calbindin immunostaining patterns. Cell counts at a given bregma level were averaged per animal for each area in both hemispheres and for all animals of a given genotype, mean values per area and bregma level were normalized relative to the values of the control (St8sia2+/−). Comparisons between the interneuron distributions over the anterior cortex of each of the St8sia2-deficient genotypes and the control were visualized by heatplots using MATLAB version R2016a (Mathworks). The analysis of the laminar distribution of interneurons was conducted by calculation of the shortest distance of each cell to the forcepts minor of the corpus callosum using MATLAB.

**Densitometric analysis**

Densitometric analysis of polySia and calbindin immunostaining was performed on ApoTome images acquired from at least three consecutive 50 µm sections. Using Zen 2012 software, mean gray values were obtained from three equal-sized frames placed either in the SVZ of the MGE or the LGE, or into the cortical plate of the neocortex, respectively. Values from both hemispheres were averaged. Per brain region, mean values obtained from at least three animals per genotype were compared.
branching observed for a given interneuron during the evaluated time period was registered.

Statistical analysis

SPSS 26 (IBM) was employed to test data for normality and for equality of variances using the Shapiro-Wilk and the Brown-Forsythe test, respectively, and to conduct three-way ANOVA including simple main effect analyses. Analyses by two-way (mixed) ANOVA as well as unpaired t-tests were performed with Prism 7.03 (GraphPad, San Diego, CA, USA). Sample calculation and tests for outliers were not performed and no values were excluded from analysis.

Availability of custom-made materials

Non-commercial reagents (mAb 735, endosialidase) and St8sia2f/f mice will be shared upon reasonable request.

Results

Interneuron-specific inactivation of ST8SIA2 leads to the loss of parvalbumin-positive, calbindin-negative (PV+CB-) interneurons

To address the question whether ST8SIA2 impacts cortical interneuron populations in a cell-autonomous or non-cell-autonomous manner, we generated mice with an MGE- or pallium-specific deletion of St8sia2. The fourth exon of the St8sia2 gene was flanked with loxp-sequences (Fig. 1a) and mice were crossbred with MGE-specific Lhx6-Cre (Fragkouli et al. 2009), with pallium-specific Emx1-Cre mice (Gorski et al. 2002) and with Zp3-Cre mice (Lewandoski et al. 1997) to generate St8sia2f/f mice. Recombination of St8sia2f/f alleles in Cre-expressing mice was analyzed by genomic PCR (Fig. 1b and c) and qPCR (Fig. 1d, e). Genomic PCR with DNA obtained from E13.5 MGEs confirmed
recombination of \textit{St8sia2} in \textit{Lhx6-Cre;St8sia2} mice, but bands indicating the presence of the recombined allele were relatively weak (Fig. 1b). Analysis of DNA obtained from E14.5 pallium confirmed successful recombination in all the conditional mutants used in the current study (Fig. 1c). In accordance with the small fraction of MGE-derived interneurons in the pallium, the band for the recombined allele was weaker in \textit{Lhx6-Cre;St8sia2} as compared to \textit{Emx1-Cre;St8sia2} or \textit{Lhx6-Cre;Emx1-Cre;St8sia2} embryos. Considering that the density of \textit{Lhx6}-positive cells in the MGE is many times higher than in the pallium (Liodis et al. 2007), the similar ratio of recombined to floxed alleles in MGE and pallium of \textit{Lhx6-Cre;St8sia2} embryos suggests a delayed onset of \textit{St8sia2} recombination affecting mostly cells that already migrated out of the MGE. \textit{St8sia2} transcripts in the MGEs of E14.5 \textit{Lhx6-Cre;St8sia2} and \textit{St8sia2} embryos were assessed by qPCR with primers binding to the exon 3 to exon 5 boundary, generated by CRE-mediated recombination, or to exon 4 of \textit{St8sia2} to detect the wild-type allele. Consistent with the weak signals for recombined \textit{St8sia2} obtained by genomic PCR, the levels of wild-type transcripts were not significantly reduced, but mRNA of recombined \textit{St8sia2} was readily detected in the MGE of \textit{Lhx6-Cre;St8sia2} embryos (Fig. 1d). Respective qPCR analyses of cortices obtained from E14.5 \textit{Emx1-Cre;St8sia2} and \textit{St8sia2} embryos revealed a clear decrease of wild-type transcripts (Fig. 1e).

We comparatively analyzed mice of overall five different genotypes (\textit{St8sia2} controls, \textit{St8sia2}–, \textit{Emx1-Cre;St8sia2}–, \textit{Lhx6-Cre;St8sia2}–, and \textit{double-deficient Lhx6-Cre;Emx1-Cre;St8sia2}– mice). To comprehensively assess interneuron changes in the mature cortex we evaluated not only the three-dimensional distribution of changes relative to the wild-type situation (Fig. 2c–f). This visualization shows drastic alterations of the PV+CB population in \textit{St8sia2}– mice, with a small increase of PV+CB cells in the caudal part of the analyzed regions and a gradual reduction of interneuron densities along a caudal to rostromedial gradient (Fig. 2f). \textit{Lhx6-Cre;St8sia2} animals displayed an overall similar pattern with the strongest alterations manifesting in the rostral regions of S1, M1 and M2 (Fig. 2d). In contrast, changes were less pronounced in \textit{Lhx6-Cre;Emx1-Cre;St8sia2}, and no major alterations were detected in \textit{Emx1-Cre;St8sia2} mice (Fig. 2c and e).

Analysis by three-way ANOVA revealed a significant main effect of genotype ($F_{(4,829)} = 55.38$, $p = 1.2e-40$), indicating differences between all genotypes, except for the comparisons between \textit{Lhx6-Cre;St8sia2} and \textit{St8sia2}–, as well as between \textit{St8sia2} and \textit{Emx1-Cre;St8sia2}. The main effects for region and bregma level were also significant ($F_{(3,829)} = 4.81$, $p = 0.003$; $F_{(5,829)} = 14.34$, $p = 2.2e-13$), as well as the two-way interactions of genotype × region and genotype × bregma level ($F_{(12,829)} = 2.93$, $p = 0.0006$; $F_{(20,829)} = 4.12$, $p = 6.07e-9$). Because of the non-significant three-way interaction ($F_{(60,829)} = 0.77$, $p = 0.9$) simple main effect analyses of the significant two-way interactions were performed. This disclosed that genotype effects on the PV+CB population are more pronounced in the three rostral bregma levels analyzed (Fig. 2g) and that the genotype × region interaction is mainly because of the prominent loss of PV+CB cells in the Cg1 of \textit{St8sia2}– mice (Fig. 2h, for interaction plots, see Figure S2; for pairwise comparisons, see Tables S1 and S2). Moreover, the pronounced loss of PV+CB in \textit{Lhx6-Cre;St8sia2} and \textit{St8sia2}– animals throughout the analyzed regions was confirmed. Notably,
in M2, M1 and S1 the deficits because of the Lhx6-Cre-mediated knockout of St8sia2 were indistinguishable from those observed in St8sia2−/− mice, while significant differences were found in the Cg1 (p = 0.0005, see Table S2). Despite a slight increase of PV+CB- cells in most of the analyzed regions of Emx1-Cre;St8sia2f/f mice (Fig. 2c, g, h; see also Figure S2b), there is no statistical evidence that these animals differ from St8sia2−/− mice, while significant differences were found in the Cg1 (p = 0.0005, see Table S2).

Despite a slight increase of PV+CB- cells in most of the analyzed regions of Emx1-Cre;St8sia2f/f mice (Fig. 2c, g, h; see also Figure S2b), there is no statistical evidence that these animals differ from St8sia2f/f controls (see Tables S1 and S2). Lhx6-Cre;Emx1-Cre;St8sia2f/f mice, however, showed an intermediate phenotype when compared to Emx1-Cre;St8sia2f/f and Lhx6-Cre;St8sia2f/f animals (Fig. 2c–e, g, h; Figure S2a, b; Tables S1 and S2). Compared to Emx1-Cre;St8sia2f/f, significant differences were found for bregma levels 1 and 2 (Table S1), as well as for Cg1 and M1 (Table S2), while differences to Lhx6-Cre;St8sia2f/f were detected for bregma levels 4 and 6 (Table S1) and for S1, with trends (0.1 > p > 0.05) for M2 and M1 (Table S2). This suggests that the overall reduction of PV+CB- interneurons in Lhx6-Cre;St8sia2f/f mice was partially reversed by additional knockout of St8sia2 in the cortical environment (Lhx6-Cre;Emx1-Cre;St8sia2f/f).

Examining the laminar distribution of PV+CB- cells in the most rostral sections of Lhx6-Cre;St8sia2f/f and St8sia2−/− mice revealed that the observed loss of interneurons was mostly confined to the upper bins analyzed (Fig. 3a). As for the regional distribution at this rostral level (Fig. 3b), the changes of the laminar distribution in M1 and S1 were very similar between St8sia2−/− and Lhx6-Cre;St8sia2f/f, but in M2, and particularly in Cg1, the effect was more pronounced in St8sia2−/− mice.
Taken together, the clear alterations of PV+ CB− interneurons in Lhx6-Cre;St8sia2f/f mice demonstrate a strong cell-autonomous impact of ST8SIA2 on interneuron distributions. However, a loss of St8sia2 in the cortical environment partially reversed the reduction of PV+ CB− cells caused by Lhx6-Cre-dependent knockout, pointing toward interactions between polySia on cortical interneurons and polySia in their environment.

Cell-autonomous effect of St8sia2 deletion on directional persistence of interneuron migration

Analyses of St8sia2+/+ and St8sia2−/− mice with GAD67-GFP labeled interneurons (St8sia2+/+;GAD67GFP+/+ and St8sia2−/−;GAD67GFP−/−) have shown that interneuron densities in the cortex of St8sia2-deficient mice are already reduced at P1 and therefore arise from defects during embryonic development (Kröcher et al. 2014). By live imaging of slice cultures obtained from GAD67GFP+/− embryos at E13.5 this study also demonstrated that acute loss of polySia because of enzymatic treatment with endosialidase reduces the velocity of interneuron migration within the cortex. By contrast, it remained unclear whether or not the loss of polysialylation by ST8SIA2 affects cortical interneuron migration, and if so, whether this occurs in a cell-autonomous or a non-cell-autonomous manner. We therefore sought to analyze migration in slice cultures from St8sia2+/+;GAD67GFP+/+ and St8sia2−/−;GAD67GFP−/− as compared to St8sia2+/+;GAD67GFP+/− and Lhx6-Cre;St8sia2+/+;GAD67GFP+/− embryos.

As a basic requirement for this analysis we examined where and when the conditional deletion of St8sia2 under the Lhx6 promoter affects the cell surface presentation of polySia. Consistent with the low level of recombinant St8sia2 detected in the MGE of Lhx6-Cre;St8sia2+/− embryos at E13.5 (see Fig. 1b), densitometric evaluation of polySia immunostaining in the MGE or the LGE of E13.5 and E15.5 Lhx6-Cre;St8sia2+/− and St8sia2−/−;St8sia4−/− embryos yielded no significant differences (Table 1a; see Figure S3 for representative micrographs of polySia immunostaining and of controls showing ablation of polySia immunoreactivity by endosialidase treatment). Assuming that compensation of polySia synthesis by the remaining expression of ST8SIA4 precludes the detection of altered polySia levels, St8sia4-negative Lhx6-Cre;St8sia2+/− animals (Lhx6-Cre;St8sia2+/+;St8sia4−/−) were generated and analyzed accordingly. Still, the polySia levels in MGE and LGE of Lhx6-Cre;St8sia2+/+;St8sia4−/− and St8sia2−/−;St8sia4−/− embryos at E15.5 were indistinguishable (Table 1b), suggesting that Lhx6-Cre-mediated inactivation of St8sia2 affects polySia only after these interneurons invade the pallium. In the pallium, however, only a minor fraction of polySia is produced by interneurons, and it is not possible to discriminate between polySia expressed on the surface of a migratory interneuron or on cells in its direct vicinity (Kröcher et al. 2014).

Therefore, the time-course of polySia expression on interneurons was monitored in MGE-derived primary cultures and thus independent of the cortical environment. After 24 h in vitro, almost all cells in these cultures were LHX6-positive interneurons and, consistent with previous findings for primary cultures from GAD67GFP+/− embryos (Kröcher et al. 2014), displayed strong polySia immunoreactivity (Fig. 4a). As expected from the assessment of polySia expression in situ, polySia immunoreactivity was not different between cultures obtained from St8sia2+/− or Lhx6-Cre;St8sia2+/− embryos. In order to assess if the rate of polySia biosynthesis is affected by Lhx6-Cre mediated recombination of St8sia2, polySia was removed by the application of endosialidase and the reappearance of polySia was monitored (Fig. 4b). Endosialidase treatment completely abolished polySia staining at the cell surface, but, as shown before (Kröcher et al. 2014), residual perinuclear spots in some of

Table 1 Densitometric evaluation of polySia signal intensities in medial ganglionic eminence (MGE) and LGE of immunostained sections of (a) St8sia2+/− and Lhx6-Cre;St8sia2+/− embryos at E13.5 and E15.5 and (b) St8sia2+/−,St8sia4−/− and Lhx6-Cre;St8sia2+/−,St8sia4−/− embryos at E14.5

| Age | Analyzed region | Genotype | Mean gray value | SEM  | n   | p valuea |
|-----|-----------------|----------|----------------|------|-----|---------|
| a   | E13.5 MGE       | St8sia2+/− | 22.9           | 0.7  | 3   | 0.83    |
|     |                 | Lhx6-Cre;St8sia2+/− | 21.7 | 5.2  |      |         |
|     |                 | St8sia2+/− | 23.4           | 1.2  | 3   | 0.86    |
|     |                 | Lhx6-Cre;St8sia2+/− | 22.4 | 5.4  |      |         |
|     | E15.5 MGE       | St8sia2+/− | 155.0          | 30.7 | 4   | 0.23    |
|     |                 | Lhx6-Cre;St8sia2+/− | 206.6 | 24.0 |      |         |
|     |                 | St8sia2+/− | 159.5          | 32.6 | 4   | 0.43    |
|     |                 | Lhx6-Cre;St8sia2+/− | 189.3 | 16.6 |      |         |
| b   | E14.5 MGE       | St8sia2+/−,St8sia4−/− | 70.3 | 15.2 | 3   | 0.93    |
|     |                 | Lhx6-Cre;St8sia2+/−,St8sia4−/− | 71.8 | 3.1  |      |         |
|     |                 | St8sia2+/−,St8sia4−/− | 65.2 | 11.5 | 3   | 0.46    |
|     |                 | Lhx6-Cre;St8sia2+/−,St8sia4−/− | 74.8 | 2.0  |      |         |

Unpaired Student’s t test.

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(a) 1div
Untreated

(b) 0
Re-expression [h after endo]

(c) 3div

(d) 2div

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the cells indicated the presence of newly synthesized polySia in the Golgi (arrowheads in Fig. 4b). In Lhx6-Cre;St8sia2f/f cultures these signals were notably weaker and the reappearance of polySia at the cell surface within 2.5 and 4.5 h after washing out the enzyme was considerably reduced (Fig. 4b). Without endosialidase treatment, however, polySia immunoreactivity was not detectably reduced in Lhx6-Cre;St8sia2f/f interneurons, even after 3 days in vitro (Fig. 4c). In contrast, in Lhx6-Cre;St8sia2fl;St8sia4–/– but not in St8sia2fl;St8sia4–/– cultures many of the LHX6-positive interneurons displayed a marked reduction of polySia immunoreactivity at the surface after 2–3 days in vitro (Fig. 4d, e), including the loss of polySia from cell processes, and first polySia-negative, LHX6-positive interneurons were detected after 3 days in vitro (Fig. 4e).

Together these findings indicate that polySia biosynthesis in Lhx6-Cre;St8sia2fl interneurons is reduced but the resulting changes are subject to a temporal delay and are too small to be detected by immunofluorescence methods under steady state conditions. It has to be assumed that this is owing to compensation by ST8SIA4 in combination with a slow turnover rate of polySia-NCAM at the cell surface. A late onset of polySia changes is consistent with the observation that, unlike prior findings in St8sia2–/– mice (Kröcher et al. 2014), no accumulations of calbindin-expressing precursor cells were detected in the MGE of Lhx6-Cre;St8sia2fl embryos at E13.5 or E15.5 (Table 2).

Considering (i) that MGE-derived interneurons need at least 48 h to reach the cortex (Denaxa et al. 2005; Miyoshi and Fishell 2011) and (ii) that a polySia reduction because of the Lhx6-Cre-mediated inactivation of St8sia2 is subject to a delay of at least the same order of magnitude, we hypothesized that MGE-derived interneurons in Lhx6-Cre;St8sia2fl mice should be affected to some point during their migration through the pallium. For the analysis of interneuron migration in slice cultures from Lhx6-Cre;St8sia2fl;GAD67GFP/+ embryos as compared to St8sia2fl;GAD67GFP/+ controls, we therefore evaluated migration velocity and directional persistence as well as leading process branching in two 50-µm-wide regions, one close to the subpallial–pallial boundary (proximal), the second at a distance of at least 150 µm further into the pallium (distal; Fig. 5a–c, Movies S1 and S2). For comparison, the same two regions were analyzed in live imaging sequences of slice cultures from St8sia2fl;GAD67GFP/+ and St8sia2–/–;GAD67GFP/+ embryos. As evident from Fig. 5d neither the full knockout nor the Lhx6-Cre-mediated deletion of St8sia2 affected the migration velocity. In contrast, the directional persistence was reduced in proximal and distal regions of St8sia2–/–;Gad67GFP/+ mice (Fig. 5e, upper). Notably, interneurons in slices from Lhx6-Cre;St8sia2fl;GAD67GFP/+ embryos also showed reduced persistence of directional migration, but only in the distal region (Fig. 5e, lower). Moreover, as evaluated in the distal region, a significantly larger fraction of interneurons displayed branched leading processes in slices from St8sia2–/– and Lhx6-Cre;St8sia2fl embryos as compared to the respective controls (Fig. 5f).

Together, the live imaging data demonstrate that the Lhx6-Cre-mediated deletion of St8sia2 affects interneuron migration to the same extent as the constitutive ablation, but, is consistent with the late onset of polySia reduction, only at some distance after entering the pallium.

**Discussion**

Here, we show that ST8SIA2 exerts a profound cell-autonomous impact on cortical interneuron development. A delayed disruption of polySia expression in MGE-derived interneurons results in migratory defects and causes reduced interneuron densities in confined rostral regions of the cortex. Focusing on the PV+ CB subpopulation of the deep cortical layers, we demonstrate that the loss of interneurons is not limited to the mPFC, as shown before (Kröcher et al. 2014), but that rostral aspects of the primary and secondary motor cortices as well as the primary sensory cortex are also affected. In more caudal regions, however, the reductions of interneurons are less prominent. The gradual decline of interneuron densities along a caudolateral to rostromedial gradient in St8sia2–/– mice is compatible with the assumption...
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(a) GAD67-GFP

(b) St8sia2
g; GAD67
g

(c) Lhx6-Cre; St8sia2
g; GAD67
g

(d) Velocity

(e) Directional persistence

(f) Branching

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Fig. 5 Constitutive and conditional knockout of ST8SIA2 affects interneuron migration in the pallium. (a) First frame of a representative live imaging sequence of a slice culture from a GAD67-GFP embryo at E13.5, to illustrate the evaluated regions, and of the live imaging sequences shown in (b) and (c) with the magnified areas indicated by red squares (inverted lookup table). As indicated in the left panel, two 50-μm-wide regions were evaluated (highlighted in yellow), the first (lower) close to the subpallial/pallial boundary (proximal), the second (upper) at a distance of at least 150 μm further into the pallium (distal). Red line, 150 μm. (b and c) Tracking of a migrating interneuron (red arrow) over 180 min in time lapse sequences of ST8SIA2-dependent changes on interneuron densities along parts of the cortex and suggests an increasing impact of a rostromedial migration of interneurons in the anterior cortex of mice with Lhx6-Cre-mediated and generic knockout of ST8SIA2. In contrast, an early loss of interneuron precursors in the MGE may account for the more pronounced interneuron deficits observed mainly in the Cg1 of ST8SIA2−/− as compared to Lhx6-Cre;St8sia2−/− mice. Minor differences between both genotypes could also be caused by defects of corticofugal or thalamocortical fibers. On one hand, both fiber systems are affected in polySia-negative ST8SIA2−/−; ST8sia4−/− double-knockout mice and contribute to the hypoplasia of the internal capsule, a phenotype that is also observed in ST8Sia2−/− mice (Hildebrandt et al. 2009; Schiff et al. 2011). On the other hand, corticofugal fibers can be used by interneurons as a scaffold for tangential migration (Denaxa et al. 2001; Denaxa et al. 2005) and disruption of thalamocortical innervation has been shown to affect the laminar distribution of cortical interneurons (Zechel et al. 2016). Defects of corticofugal fibers, however, should be reproduced in Emx1-Cre;St8sia2−/− mice, which show no alterations of PV+CB interneurons. In contrast, early pathfinding deficits of thalamocortical projections should be restricted to the ST8Sia2−/− genotype and therefore may contribute to the more pronounced changes of the laminar distribution in these mice.

The simultaneous ablation of St8sia2 in interneurons and in the cortical environment of Lhx6-Cre;Emx1-Cre;St8sia2−/− mice resulted in an intermediate phenotype. A possible explanation for this unexpected finding would be that Lhx6-Cre- and Emx1-Cre-mediated reductions of polySia synthesis enable heterophilic interactions of polySia-deficient NCAM, in which NCAM as a binding partner would either be presented on interneurons or on cells of their cortical environment. A simultaneous reduction in Lhx6-Cre;Emx1-Cre;St8sia2−/− mice may enable both of these heterophilic and, in addition, homophilic NCAM interactions. Indeed, as of a rostromedial migration of interneurons in the anterior parts of the cortex and suggests an increasing impact of ST8SIA2-dependent changes on interneuron densities along the migratory path. This pattern is largely recapitulated by Lhx6-Cre-mediated deletion of ST8SIA2 in MGE-derived interneurons, whereas the additional deletion of ST8SIA2 in pallial cells of the Emx1-expressing lineage had a small opposing effect. Furthermore, the analysis of the laminar distribution revealed that changes were more pronounced in the upper bins examined. Because MGE-derived interneurons populate the cortex in an inside-out pattern (Ang et al. 2003; Guo and Anton 2014), and because most, if not all, parvalbumin-positive interneurons in the deep cortical layers of the mature mouse PFC are basket cells (Miyamae et al. 2017), this indicates that early-born basket cells are less affected by loss of ST8SIA2.

A link between impairments during the late stages of developmental migration through the pallium and interneuron deficits in the cortex of adult ST8SIA2−/− mice is established by the finding that the Lhx6-Cre-mediated depletions of ST8SIA2 impacts migration of interneurons only after they invaded deep into the pallium. This is consistent with a late onset of polySia reductions because of Lhx6-Cre-mediated ST8SIA2 depletions, as inferred from the observation that it takes at least 2 days before MGE-derived interneurons in cultures from Lhx6-Cre;St8sia2−/−;St8sia4−/− embryos start to lose polySia. Together, these data lead to the conclusion that the Lhx6-Cre-mediated depletion dissects the consequences of ST8SIA2 deficiency on interneuron development in the pallium from those of an earlier loss. Hence, accumulation and apoptosis of precursors as observed in the MGE of ST8SIA2−/− animals (Kröcher et al. 2014) is circumvented in Lhx6-Cre;St8sia2−/− embryos and therefore not responsible for the loss of interneurons concurrently observed in the adult cortex of mice with Lhx6-Cre-mediated and generic knock-out of ST8SIA2. Two-way mixed ANOVA indicated no significant differences for (d), upper panel (interaction: $F_{(1,16)} = 0.02$, $p = 0.90$; region: $F_{(1,16)} = 0.28$, $p = 0.14$; genotype: $F_{(1,16)} = 1.05$, $p = 0.36$) and (d), lower panel (interaction: $F_{(1,16)} = 0.88$, $p = 0.40$; region: $F_{(1,16)} = 1.11$, $p = 0.35$; genotype: $F_{(1,16)} = 0.83$, $p = 0.41$), but significant differences for (e), upper panel (interaction: $F_{(1,16)} = 0.31$, $p = 0.60$; region: $F_{(1,16)} = 0.44$, $p = 0.54$; genotype: $F_{(1,16)} = 119.6$, $p = 0.0004$), (e), lower panel (interaction: $F_{(1,16)} = 18.77$, $p = 0.01$; region: $F_{(1,16)} = 19.38$, $p = 0.01$; genotype: $F_{(1,16)} = 4.44$, $p = 0.10$). Two-way ANOVA indicated significant differences in (f), upper panel (interaction: $F_{(1,16)} = 8.54$, $p = 0.001$; degree of branching: $F_{(1,16)} = 33.44$, $p < 0.0001$; genotype: $F_{(1,16)} = 7.7 \times 10^{-14}$, $p < 0.99$), and (f), lower panel (interaction: $F_{(1,16)} = 21.7$, $p < 0.0001$; degree of branching: $F_{(1,16)} = 146.7$, $p < 0.0001$; genotype: $F_{(1,16)} = 0$, $p = 0.99$). Sidak’s post hoc tests were applied and significant differences are indicated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).
shown for murine subventricular zone-derived neuronal precursors as well as in human neuroblastoma cell models, a loss of polySia on NCAM gives rise to potent heterophilic NCAM interactions that impact cell migration and differentiation (Seidenfaden et al. 2003; Röckle et al. 2008; Eggers et al. 2011). However, the observation that St8sia2+/− mice show the same reduction of PV+ CB− interneurons as NCAM-negative animals strongly argues against an involvement of NCAM interactions.

Another mechanism that could lead to a divergent impact of the Lhx6-Cre- and Emx1-Cre- mediated knockout of St8sia2, would be a competing interaction of polySia on migrating interneurons and polySia in their environment with soluble factors that drive interneuron migration, such as neurotrophin-4 or BDNF as motogenic factors (Polleux et al. 2002), or the chemokine CXCL12 (Stumm et al. 2003; Tiveron et al. 2006; Li et al. 2008; Lopez-Bendito et al. 2008). Functional and physical interactions between BDNF and polySia have been repeatedly reported (Muller et al. 2000; Vutskits et al. 2001; Burgess and Aubert 2006; Kanato et al. 2008; Sumida et al. 2015), but the unaltered velocity of migratory interneurons in slice cultures from Lhx6-Cre; St8sia2+/− and St8sia2−/− embryos speaks against an altered perception of purely motogenic cues. Moreover, unlike our results for St8sia2, a conditional ablation of the BDNF receptor TrkB in cortical interneurons did not affect the number or position of interneurons in the prefrontal cortex (Sanchez-Huertas and Rico 2011). In contrast, CXCL12 impacts the regional and laminar distribution of interneurons by directing intracortical migration (Li et al. 2008; Lopez-Bendito et al. 2008; Abe et al. 2014) including the regulation of leading process branching (Lysko et al. 2011; Lysko et al. 2014). CXCL12 inhibits the branching of leading processes by activation of the microtubule-associated protein doublecortin, which stabilizes the microtubule array by bundling of microtubules in the leading process (Lysko et al. 2014). Consistently, doublecortin knockout mice also show increased leading process branching of MGE-derived interneurons associated with reduced numbers and altered laminar distribution of interneurons in the cortex (Kappeler et al. 2006; Friocourt et al. 2007). These parallels to our findings in St8sia2-deficient mice and warrant future investigations of possible interactions between polySia and CXCL12, as well as CXCL12-dependent changes of leading process dynamics during cortical interneuron migration.

Despite a clear link between the formation of leading process branches and directional guidance during chemotactic migration (Martini et al. 2009), there is, to the best of our knowledge, no study that demonstrates a correlation between leading process branching and directional persistence of interneuron migration. However, a post-mitotic ablation of the cell adhesion molecule N-cadherin results in impaired polarization, leading process instability and abnormal cytoskeletal dynamics, which is associated with impaired directional persistence and causes delayed tangential and perturbed radial migration of MGE-derived interneurons (Luccardini et al. 2013). Similarly, altered cell adhesion by impaired presentation of polySia on interneurons could cause the cell-autonomous changes of migratory behavior in the pallium of St8sia2−/− and Lhx6-Cre;St8sia2+/− mice.

Over decades of research, the crucial role of polySia during development has been thoroughly examined by acute enzymatic removal of polySia and by conventional knockout of either the major polySia carrier Ncam1 or the polySia-producing enzymes. Focusing on the role of ST8SIA2 we now demonstrate that a delayed disruption of polySia biosynthesis by Lhx6-Cre-mediated deletion of St8sia2 in a subset of cortical interneurons is sufficient to cause migration defects within the pallium at embryonic stages as well as major alterations of interneuron distribution in defined areas of the rostral cortex in the adult. Therefore, even minor perturbations of polySia biosynthesis can be expected to affect cortical interneuron development. This could also apply to dysregulation of ST8SIA2 during human brain development, which may arise from genetic variations in ST8SIA2 that have been associated with neurodevelopmental psychiatric disorders, such as schizophrenia and autism. The current study, therefore, provides a mechanistic link between ST8SIA2 and alterations of parvalbumin-positive interneurons as observed in schizophrenia and autism.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Densities of parvalbumin-positive, calbindin-negative interneurons (PV+ CB−) in the deep layers of Cg1 of 30-day-old St8sia2−/− and Ncam1−/− mice (n = 3, each) compared to a control group consisting of one St8sia2+/− St8sia4+/− Ncam1+/+ and two St8sia2+/− St8sia4+/− Ncam1−/− mice, as described in Kröcher et al. 2014.

Figure S2. Graphical representations of the genotype × bregma level interaction (a) and genotype × region interaction (b and c) of
PV+CB- interneurons of the anterior cortex as part of the simple main effect analyses, to illustrate causes of interactions (i.e. diverging lines indicate interactions, parallel lines indicate no interactions).

**Figure S3.** Representative micrographs of polySia immunostaining at the subpallial–pallial border.

**Table S1.** Simple main effect analysis of the genotype × bregma interaction corresponding to the data shown in Fig. 2g.

**Table S2.** Simple main effect analysis of the genotype × region interaction corresponding to the data shown in Fig. 2h.

**Movie S1.** Live imaging of interneuron migration in slice cultures from St8sia2fl/fl; GAD67GFP/+ mice.

**Movie S2.** Live imaging of interneuron migration in slice cultures from Lhx6-Cre; St8sia2fl/fl; GAD67GFP/+ mice.

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