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Persistent cortical plasticity by upregulation of chondroitin 6-sulfation

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Cortical plasticity is most evident during a critical period in early life, but the mechanisms that restrict plasticity after the critical period are poorly understood. We found that a developmental increase in the 4-sulfation/6-sulfation (4S/6S) ratio of chondroitin sulfate proteoglycans (CSPGs), which are components of the brain extracellular matrix, leads to the termination of the critical period for ocular dominance plasticity in the mouse visual cortex. Condensation of CSPGs into perineuronal nets that enwrapped synaptic contacts on parvalbumin-expressing interneurons was prevented by cell-autonomous overexpression of chondroitin 6-sulfation, which maintains a low 4S/6S ratio. Furthermore, the increase in the 4S/6S ratio was required for the accumulation of Otx2, a homeoprotein that activates the development of parvalbumin-expressing interneurons, and for functional maturation of the electrophysiological properties of these cells. Our results indicate that the critical period for cortical plasticity is regulated by the 4S/6S ratio of CSPGs, which determines the maturation of parvalbumin-expressing interneurons.

Ocular dominance plasticity is a classic example that has been used to study the effects of experience on cortical circuits[1,2]. Brief monocular deprivation leads to a reduction in the responses of visual cortical neurons to the deprived eye and an increase in the responses to the nondeprived eye. Sensitivity to monocular deprivation is high in juvenile animals during a critical period in early life (postnatal days 19–32 (P19–32) in the mouse visual cortex), after which cortical neurons become less plastic[3]. Considerable evidence indicates that functional changes in the balance between excitation and inhibition control ocular dominance plasticity[2]. In particular, maturation of a subset of inhibitory interneurons expressing the calcium-binding protein parvalbumin has been proposed to be crucial for defining the timing of the critical period[2,4,5].

CSPGs consist of a core protein with covalently attached chondroitin sulfate glycosaminoglycan chains (**Fig. 1a**) and are major components of the brain extracellular matrix[6]. During postnatal development, CSPGs condense around the soma and proximal dendrites of parvalbumin-expressing interneurons, forming perineuronal nets (PNNs), a specialized extracellular matrix structure that interdigitates with synaptic contacts[7]. PNNs are composed of several CSPGs and hyaluronan, and the interaction between CSPGs and hyaluronan is stabilized by tenascin-R and cartilage link proteins[8]. Although the cellular origins of their components are not fully understood, both neurons and glial cells may contribute to the formation of PNNs[9]. Digestion of chondroitin sulfate chains with chondroitinase ABC disrupts PNNs and reactivates ocular dominance plasticity in adult animals after the closure of the critical period, indicating that the chondroitin sulfate moieties of CSPGs are responsible for PNN formation and control of the critical period plasticity[10]. It has been suggested that chondroitin sulfate chains present a physical barrier that limits the critical period plasticity by preventing rearrangements of synaptic connections because they inhibit axonal sprouting and regeneration after nerve injury[11]. However, it is unlikely that all chondroitin sulfate chains inherently inhibit plasticity, as chondroitin sulfate chains are very abundant, even in the juvenile brain during the critical period.

Recent studies have proposed that the functional information of CSPGs is encoded by the specific sulfation sequence of chondroitin sulfate chains[6,12–14]. Chondroitin sulfate chains are long linear polysaccharides that are composed of a repeating disaccharide unit that consists of glucuronic acid and *N*-acetylgalactosamine (GalNAc)[6]. During biosynthesis, GalNAc residues of the repeating disaccharide units are sulfated at C6 and C4 by chondroitin 6-sulfotransferase-1 (C6ST-1) and chondroitin 4-sulfotransferase-1 (C4ST-1), respectively (**Fig. 1a**). Chondroitin sulfate chains can act as both inhibitory molecules for axonal growth and neuritogenic molecules depending on their sulfation status[6,13]. Previously, we found that the neuritogenic effect of particular chondroitin sulfate chains was mediated by specific interaction with a cell adhesion molecule, contactin-1 (ref. 13). Despite increasing *in vitro* evidence that chondroitin sulfate chains act in a sulfation pattern–dependent manner, the role of CSPG sulfation patterns in cortical plasticity has garnered little attention. Specifically, the relationship between sulfation patterns of chondroitin sulfate and functional maturation of inhibitory interneurons has not been addressed. Moreover, the importance of specific sulfation patterns of chondroitin sulfate to cortical plasticity may have been overlooked in previous studies of chondroitin sulfate and plasticity because chondroitinase ABC treatment destroys all chondroitin

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sulfate chains, irrespective of sulfation status. The 4S/6S ratio changes during several biological processes[15,16]; thus, we hypothesized that sulfation patterns of chondroitin sulfate regulate the critical period plasticity. To test this hypothesis, we generated transgenic mice that overexpress human C6ST-1, a sulfotransferase catalyzing the 6-sulfation of chondroitin sulfate chains, under the control of a chicken β-actin promoter.

RESULTS

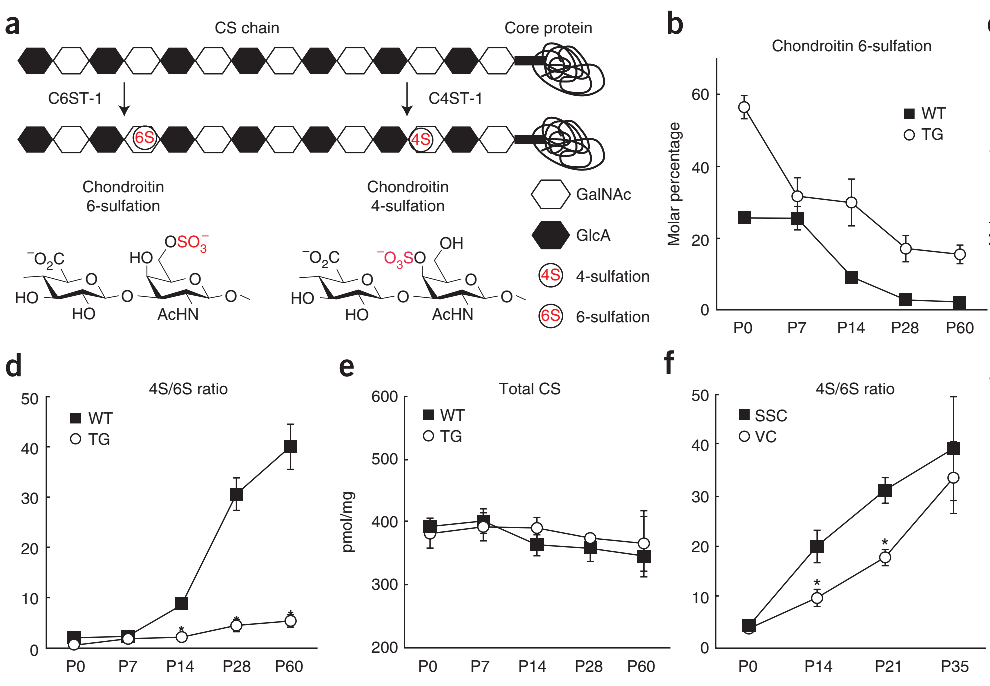
Sulfation patterns of chondroitin sulfate in mouse brain

We initially examined sulfation patterns of chondroitin sulfate in the developing mouse brain. Chondroitin sulfate chains isolated from mouse brain at different ages were digested with chondroitinase ABC and the resultant disaccharides were analyzed by high-performance liquid chromatography (**Supplementary Table 1**). In wild-type mice, the proportion of chondroitin 6-sulfation gradually decreased, whereas that of chondroitin 4-sulfation progressively increased (**Fig. 1b**,**c**), which resulted in a sharp increase in the 4S/6S ratio during development (**Fig. 1d**). In adult wild-type brain, chondroitin sulfate chains were mostly composed of chondroitin 4-sulfation, with chondroitin 6-sulfation only accounting for 2% of the total chondroitin sulfate. *C6ST-1* (also known as *CHST3*) transgenic mice showed an increased proportion of chondroitin 6-sulfation and a decreased proportion of chondroitin 4-sulfation throughout development relative to control mice. The 4S/6S ratio in *C6ST-1* transgenic mice was substantially lower than the ratio in control mice from P14 onward. Although the sulfation patterns of chondroitin sulfate changed markedly, the total amount of chondroitin sulfate remained constant during development and did not differ between *C6ST-1* transgenic and wild-type mice (**Fig. 1e**).

We also found that the developmental shift of the 4S/6S ratio correlated with the maturation timing of cortical regions. The 4S/6S ratio shift in the visual cortex occurred after that in the somatosensory cortex (**Fig. 1f**), which is consistent with the later maturation of the visual cortex[17]. Raising animals in complete darkness (dark rearing) delays the onset and termination of the critical period for ocular dominance plasticity in the visual cortex[2]. However, the 4S/6S ratio in the visual cortex was not affected by dark rearing (control, $n = 4$, 4S/6S ratio = 16.8 ± 0.39; dark rearing, $n = 4$, 4S/6S ratio = 15.9 ± 0.30; $P > 0.1$), suggesting that the temporal shift of the 4S/6S ratio is developmentally programmed, but is not dependent on visual experience. On the basis of immunohistochemical analysis using CS56 antibody that recognizes chondroitin 6-sulfation–related structures[18], we found that the shift in the sulfation patterns of chondroitin sulfate in *C6ST-1* transgenic mice was delayed relative to that in wild-type mice (**Fig. 1g**), which is consistent with the results of our chemical disaccharide composition analysis. These results indicate that the sulfation patterns, but not the amount of chondroitin sulfate chains, changed during the critical period and that *C6ST-1* transgenic mice retain immature sulfation patterns of chondroitin sulfate throughout life.

C6ST-1 transgenic adults retain ocular dominance plasticity

To investigate whether the sulfation patterns of chondroitin sulfate influence the onset of the critical period, we assessed ocular dominance



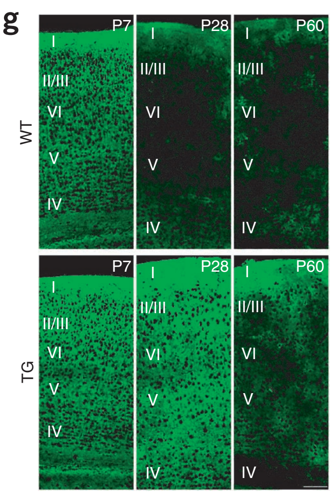


Figure 1 Developmental changes of sulfation patterns of chondroitin sulfate in mouse brain. (**a**) Schematic of the sulfation of the chondroitin sulfate (CS) chain. During biosynthesis, most of the repeating disaccharide units consisting of glucuronic acid (GlcA) and GalNAc are sulfated by C6ST-1 or C4ST-1. (**b**–**e**) The proportion of chondroitin 6-sulfation (**b**), chondroitin 4-sulfation (**c**), the ratio of 4S/6S (**d**), and the total amounts of chondroitin sulfate chains (**e**) in wild-type (WT, filled square) and *C6ST-1* transgenic (TG, open circle) mice during development. Molar percentages in **b** and **c** were calculated by dividing the amount of chondroitin 6- and 4-sulfation, respectively, by the total amount of disaccharides. The 4S/6S ratio was calculated by dividing the molar percentage of chondroitin 4-sulfation by that of chondroitin 6-sulfation. (**f**) The ratio of 4S/6S in the somatosensory cortex (SSC, filled square) and visual cortex (VC, open circle) in wild-type mice. Error bars represent s.d. ($n = 3$ for each point). $^*P < 0.05$ (Student's t test) between the two groups at the same time point. (**g**) *C6ST-1* transgenic mice showed a delayed decrease in CS56 staining in the developing visual cortex. Cortical layers are indicated with Roman numerals on the left. Scale bar represents 100 μm.

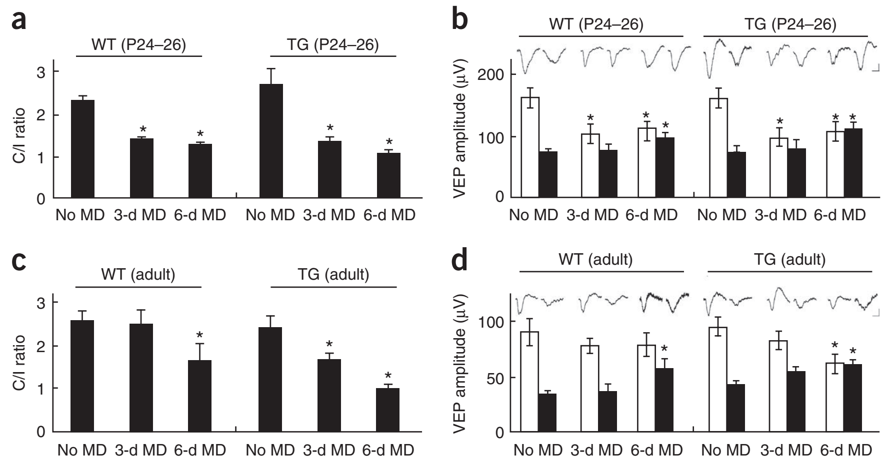


Figure 2 Sulfation patterns of chondroitin sulfate participate in the termination of the critical period. (**a**) Monocular deprivation periods of 3 or 6 d beginning at P24–26 resulted in a significant decrease in the contralateral/ipsilateral (C/I) ratio in both wild-type and *C6ST-1* mice as compared with nondeprived (No MD) mice. (**b**) In both groups, 3-d monocular deprivation depressed responses to the deprived eye (open bars) and 6-d monocular deprivation potentiated responses to the nondeprived eye (filled bars) (No MD: wild type $n = 12$, *C6ST-1*, $n = 10$; 3-d monocular deprivation: wild type $n = 18$, *C6ST-1*, $n = 14$; 6-d monocular deprivation: wild type $n = 12$, *C6ST-1*, $n = 11$). (**c**) In adulthood (P60–90), 3-d monocular deprivation did not affect the contralateral/ipsilateral ratio in wild-type mice, but did induce a significant contralateral/ipsilateral shift in *C6ST-1* mice. 6 d of monocular deprivation caused a significant contralateral/ipsilateral shift in both groups. (**d**) In wild-type mice, 6-d monocular deprivation induced potentiation of the responses to the nondeprived eye (filled bars), but no change in the responses to the deprived eye (open bars). *C6ST-1* mice showed depression in the responses to the deprived eye and potentiation of the responses to nondeprived eye (No MD: wild type $n = 11$, *C6ST-1*, $n = 12$; 3-d monocular deprivation: wild type $n = 10$, *C6ST-1*, $n = 11$; 6-d monocular deprivation: wild type $n = 8$, *C6ST-1*, $n = 14$). $^*P < 0.05$ (Student's *t* test) from No MD groups. Error bars represent s.e.m. Representative waveforms of VEPs are shown at the top of **b** and **d**. Scale bars represent 100 ms and 100 µV.

plasticity by recording visual evoked potentials (VEPs) from the binocular zone of the primary visual cortex. In both groups, the baseline ratio of contralateral eye VEP amplitude to ipsilateral eye VEP amplitude (the contralateral/ipsilateral ratio) was approximately 2, reflecting the contralateral eye dominance (**Fig. 2a**). We found that a 3- or 6-d monocular deprivation from P24–26 (the peak of the critical period) resulted in a significantly decreased contralateral/ipsilateral ratio in both groups ($P < 0.05$; **Fig. 2a**). Comparison of VEP amplitudes before and after monocular deprivation revealed two temporally distinct mechanisms for ocular dominance shift, as have been reported previously[19]. In both groups, the decrease in the contralateral/ipsilateral ratio was mediated by rapid depression of the responses to the deprived eye and by delayed potentiation of the responses to the nondeprived eye (**Fig. 2b**). Eye-specific projections from the retina to the dorsal lateral geniculate nucleus (dLGN) are known to be established in the first postnatal week[20]. *C6ST-1* transgenic mice had normal eye-specific segregation of retinogeniculate axon terminals in the dLGN (**Supplementary Fig. 1**). Together, these results suggest that the initial construction of visual pathway and the onset of the critical period occur normally in *C6ST-1* transgenic mice.

We next explored the effect of an altered sulfation pattern of chondroitin sulfate on the termination of the critical period. A 3-d monocular deprivation period did not alter the contralateral/ipsilateral ratio in wild-type adults as it had in juvenile mice (**Fig. 2c**). However, *C6ST-1* transgenic adults did experience a significant contralateral/ipsilateral shift ($P = 0.02$) following 3 d of monocular deprivation imposed after the critical period. Although recent studies have suggested that a longer period of monocular deprivation can induce a weak shift in the contralateral/ipsilateral ratio in adult animals, the nature of juvenile and adult ocular dominance plasticity is markedly different[21]. In wild-type adults, 6 d of monocular deprivation caused a mild, but statistically significant ($P < 0.05$), contralateral/ipsilateral shift that was mainly mediated by increased responses to the nondeprived eye, rather than by decreased responses to the deprived eye (**Fig. 2d**). This indicates that depression in responses to the deprived eye in wild-type mice is restricted to the critical period. In contrast, the contralateral/ipsilateral shift in *C6ST-1* transgenic adults caused by 6 d of monocular deprivation resulted from both depression in responses to the deprived eye and potentiation of responses to the nondeprived eye, which is similar to that observed in juvenile mice. These results suggest that *C6ST-1* transgenic adults retain juvenile-like ocular dominance plasticity.

C6ST-1 transgenic mice have reduced PNNs formation

We next examined whether the sulfation patterns of chondroitin sulfate affected formation of PNNs using a well-established marker, *Wisteria floribunda* agglutinin (WFA)[22]. During the first postnatal week, CSPGs were diffusely distributed and no PNN was observed in the visual cortex (**Fig. 3**). Formation of WFA^+ PNNs began around P14, gradually increased after the critical period and displayed a time course similar to the shift in the 4S/6S ratio (**Fig. 3a**,**b**). In the visual cortex of wild-type adults, most parvalbumin-expressing interneurons (90%) were surrounded by WFA^+ PNNs. Notably, *C6ST-1* transgenic mice showed a significantly reduced ($P = 0.002$ for P28, $P = 0.0005$ for adults) number of WFA^+ PNNs during and after the critical period (**Fig. 3a**,**c**); however, the number of parvalbumin-expressing interneurons was similar in wild-type and *C6ST-1* transgenic mice (**Fig. 3a**,**d**). This indicates that developmental changes in sulfation patterns of chondroitin sulfate are required for normal formation of PNNs.

Notably, some parvalbumin-expressing interneurons (10%) in *C6ST-1* transgenic mice were enwrapped by a distinct PNN-like structure that was recognized by CS56 antibody (**Fig. 3e**). These $CS56^+$ PNNs rarely colocalized with conventional WFA^+ PNNs in *C6ST-1* transgenic mice and were not observed in wild-type controls (**Fig. 3f**). Thus, the lack of WFA staining does not necessarily mean the absence of PNNs in *C6ST-1* transgenic mice. Instead, subpopulation of parvalbumin-expressing interneurons was surrounded by chondroitin 6-sulfation–enriched PNNs. Western blot analysis showed that the overall quantity of various core proteins, including aggrecan, a major CSPG in PNNs[23], were not altered in *C6ST-1* transgenic mice (**Supplementary Fig. 2**), indicating that abnormal formation of PNNs in *C6ST-1* transgenic mice is not a result of the reduced expression of PNN components. These results indicate that overexpression of C6ST-1 reduces the formation of conventional WFA^+ PNNs and increases the number of $CS56^+$ PNNs rich in chondroitin 6-sulfation around parvalbumin-expressing interneurons.

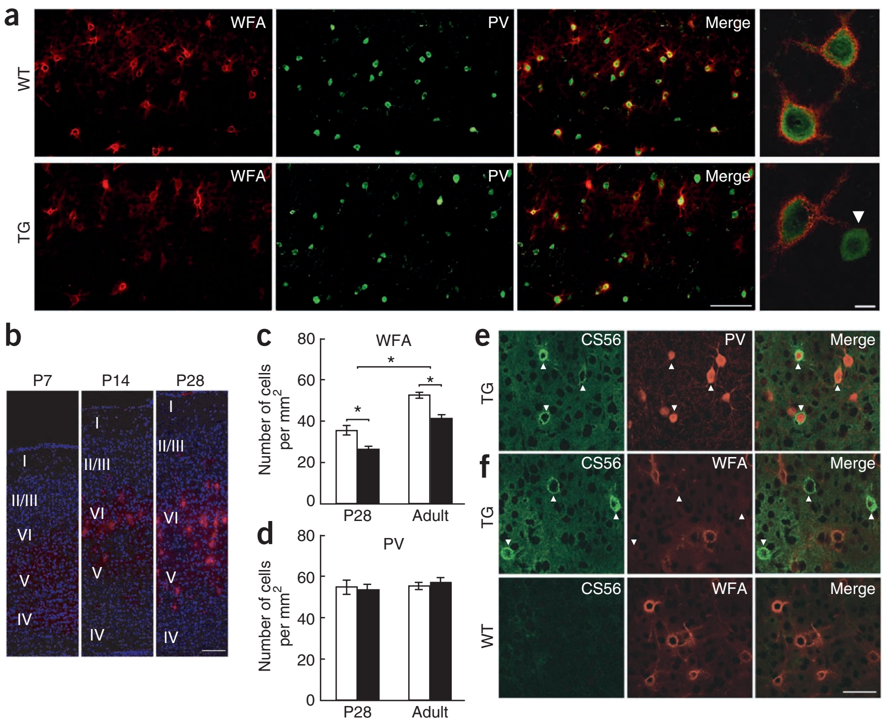


Figure 3 Impaired PNNs formation in *C6ST-1* mice. (**a**) Adult *C6ST-1* mice showed decreased formation of WFA+ PNNs relative to controls. Right, high-magnification images of parvalbumin (PV)-expressing interneurons with or without (arrowhead) WFA+ PNNs. (**b**) WFA+ PNNs (red) first appeared at P14 in the visual cortex of wild-type mice. Nuclei were counterstained with Hoechst (blue). Cortical layers are indicated with Roman numerals on the left. (**c**,**d**) The number of WFA+ PNNs (**c**), but not parvalbumin-expressing interneurons (**d**) in *C6ST-1* mice (closed bars) was significantly less than controls (open bars) throughout life. $^*P < 0.005$ (Student's *t* test) between control versus *C6ST-1* transgenic for P28 and adult (P60–90), and P28 versus adult for control and *C6ST-1* transgenic. Error bars represent s.e.m.; $n = 4$–9. (**e**) Subpopulation of parvalbumin-expressing interneurons were surrounded by chondroitin 6-sulfation–enriched CS56+ PNNs (arrowheads) in *C6ST-1* mice. (**f**) CS56+ PNNs (arrowheads) were not colocalized with conventional WFA+ PNNs in *C6ST-1* mice and were not observed in wild-type mice. Scale bars represent 100 μm (left panels in **a**,**b**), 10 μm (right panels in **a**) and 50 μm (**e**,**f**).

Sulfation patterns of PNNs regulate accumulation of Otx2

How could the sulfation patterns of chondroitin sulfate in PNNs influence critical period plasticity? The Otx2 homeoprotein was recently shown to regulate ocular dominance plasticity via its effects on maturation of parvalbumin-expressing interneurons[5]. Previous data suggest that Otx2 protein produced in the retina and dLGN is transported to the visual cortex via thalamocortical axons, secreted from thalamocortical terminals and internalized by postsynaptic parvalbumin-expressing interneurons[5]. However, because less than 20% of all thalamocortical synapses contact inhibitory interneurons, including parvalbumin-expressing interneurons[24], it is unclear how Otx2 selectively accumulates in parvalbumin-expressing interneurons. Thus, we tested whether the sulfation patterns of PNNs around parvalbumin-expressing interneurons affect the incorporation of Otx2. In the visual cortex of wild-type mice, Otx2 protein first became evident in parvalbumin-expressing interneurons during the critical period, and the number of Otx2+ cell was substantially increased by adulthood (**Fig. 4a**,**b**). Notably, there were significantly fewer ($P = 0.001$ for P28, $P = 0.0002$ for adults) Otx2+ cells in *C6ST-1* transgenic mice than in wild-type mice (**Fig. 4a**,**b**). Otx2 was mostly detected in parvalbumin-expressing interneurons surrounded by WFA+ PNNs, but was not observed in parvalbumin-expressing interneurons surrounded by CS56+ PNNs (**Fig. 4c**), suggesting that the accumulation of Otx2 was dependent on the sulfation patterns of chondroitin sulfate in PNNs. Closer observation revealed that Otx2 was distributed in a punctate pattern in the soma and proximal dendrites of parvalbumin-expressing interneuron (**Fig. 4d**). Otx2-immnopositive puncta did not overlap with, but were closely apposed to, WFA+ PNNs. The expression of *Otx2* mRNA in the retina and dLGN was indistinguishable between the two groups, excluding the possibility that overexpression of C6ST-1 affects expression of *Otx2* transcripts (**Supplementary Fig. 3**). Neither wild-type nor *C6ST-1* transgenic mice expressed detectable levels of *Otx2* mRNA in the visual cortex.

We further analyzed the spatial relationship between PNNs and thalamocortical synaptic contact sites in layer 4, which receives most of the thalamocortical input. Three-dimensional reconstruction of WFA+ PNNs revealed a distinct meshwork surrounding soma and proximal dendrites of parvalbumin-expressing interneurons in wild-type mice (**Fig. 5a**). We selectively labeled thalamocortical boutons using antibody against vesicular glutamate transporter 2 (VGluT2)[24]. The holes in the meshwork of WFA+ PNNs were evidently the areas occupied by thalamocortical boutons. In contrast, CS56+ PNNs showed sparse dot-like particles rather than a meshwork structure, and thalamocortical boutons projecting onto parvalbumin-expressing interneurons in *C6ST-1* transgenic mice were not surrounded by PNNs (**Fig. 5a**). Because both WFA lectin and CS56 antibody recognize the chondroitin sulfate moiety of CSPGs, we stained WFA+ and CS56+ PNNs with an antibody recognizing the core protein portion of aggrecan. This confirmed the meshwork structure of WFA+ PNNs in controls and the diffusely spread and less condensed morphology of CS56+ PNNs in *C6ST-1* transgenic mice (**Fig. 5b**). These results indicate that diffuse PNNs that are rich in chondroitin 6-sulfation may be unable to tightly surround thalamocortical synaptic contacts, leading to diffusion and reduced accumulation of Otx2 at these contacts.

Overexpression of C6ST-1 in inhibitory interneurons

To address the cellular origin of the abnormal PNNs formation in *C6ST-1* transgenic mice, we carried out two series of experiments. First, we assessed the possible contribution of astrocytes to the formation of CS56+ PNNs using a heterologous neuron and astrocyte co-culture system. We found that parvalbumin-expressing interneurons were surrounded by CS56+ PNNs when cortical neurons from *C6ST-1* transgenic, but not wild type, mice were grown on an astrocyte monolayer, regardless of the origin of the astrocytes (**Supplementary Fig. 4**). This suggests that overexpression of C6ST-1 in neurons, but not in astrocytes, is required for the formation of CS56+ PNNs.

Second, we sought to directly determine whether cell-autonomous upregulation of chondroitin 6-sulfation in parvalbumin-expressing interneurons induces the formation of CS56+ PNNs *in vivo*. In contrast with excitatory pyramidal neurons that are born in the

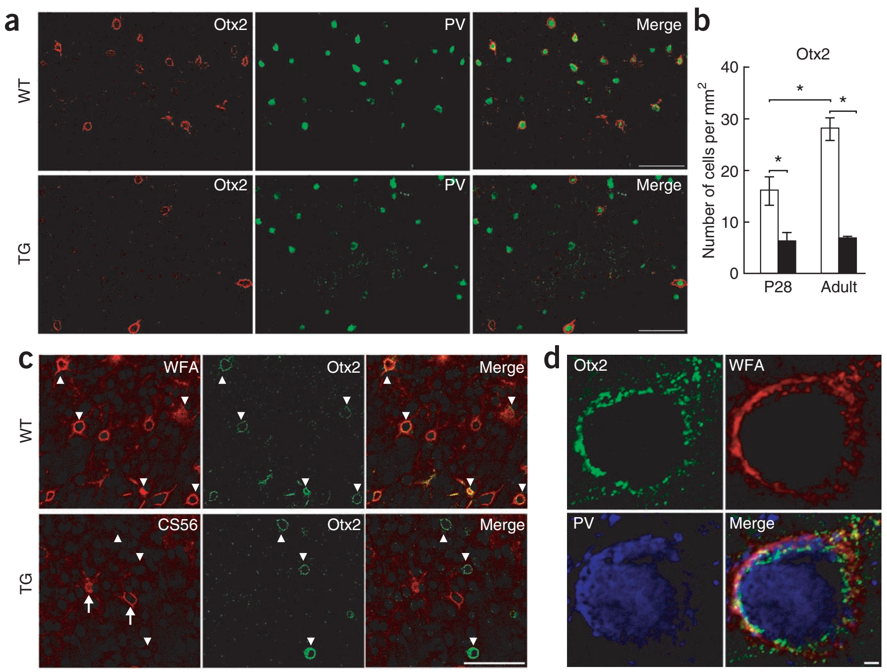


Figure 4 Sulfation patterns of chondroitin sulfate–dependent accumulation of Otx2 homeoprotein in parvalbumin-expressing interneurons. (**a**) In adult visual cortex, *C6ST-1* mice had reduced accumulation of Otx2 in parvalbumin-expressing interneurons as relative to controls. (**b**) Quantitative analysis of the number of Otx2$^+$ cells in wild-type (open bars) and *C6ST-1* (closed bars) mice. $*P < 0.05$ (Student's *t* test) between P28 versus adult for control and control versus *C6ST-1* transgenic for P28 and adult. Error bars represent s.e.m. ($n = 3$–6). (**c**) Otx2 (arrowheads) accumulated in parvalbumin-expressing interneurons surrounded by WFA$^+$ PNNs in controls, but not in parvalbumin-expressing interneurons surrounded by CS56$^+$ PNNs (arrows) in *C6ST-1* mice. (**d**) Otx2-immnopositive puncta in parvalbumin-expressing interneurons closely apposed to WFA$^+$ PNNs. Scale bars represent 100 μm (**a**,**c**) and 2 μm (**d**).

cortical proliferative zones, inhibitory interneurons, including parvalbumin-expressing interneurons, originate from the ganglionic eminences and migrate tangentially into the cortex[25]. Thus, to achieve interneuron-specific gene expression, we performed ganglionic eminence–directed *in utero* electroporation[26,27] with *C6ST-1*–expressing and *yellow fluorescence protein* (*YFP*)-expressing plasmids at embryonic day 12.5 (E12.5; **Fig. 6a**). Immunohistochemical analysis of the postnatal cortex at P30 showed that 26% of transfected neurons ($n = 24$ of 91 YFP-positive neurons) were immunoreactive for parvalbumin (**Fig. 6b**). Notably, in contrast with untransfected control parvalbumin-expressing interneurons, most C6ST-1–transfected parvalbumin-expressing interneurons were surrounded by CS56$^+$ PNNs (88%, $n = 21$ of 24 YFP and parvalbumin double-positive neurons; **Fig. 6b**). As observed in *C6ST-1* transgenic mice, Otx2 was not accumulated in parvalbumin-expressing interneurons surrounded by CS56$^+$ PNNs (0%, $n = 0$ of 20 YFP and CS-56 double-positive neurons; **Fig. 6c**). No CS56$^+$ PNNs were formed when C6ST-1 was overexpressed in excitatory pyramidal neurons (**Fig. 6d**). These results indicate that PNNs formation and Otx2 accumulation were locally affected by parvalbumin-expressing interneuron autonomous production of chondroitin 6-sulfation. In addition, these results suggest that sulfation patterns of chondroitin sulfate are involved in the uptake of Otx2 at the postsynaptic level, but not at the presynaptic level (for example, transport from subcortical to cortical areas and secretion from presynaptic terminals).

Electrophysiological properties of visual cortical neurons

We then asked what kinds of changes are produced in the electrophysiological properties of parvalbumin-expressing interneurons by overexpression of C6ST-1 using slice preparations of visual cortex at P30–34, after the peak of ocular dominance plasticity. Whole-cell recordings were conducted from nonpyramidal neurons located in or near layer 4 with patch pipettes containing biocytin in the current-clamp mode. We analyzed fast-spiking cells, as parvalbumin-expressing interneurons are practically equivalent to fast-spiking cells[28]. We encountered cells showing spike discharges that were characteristic of fast-spiking cells in *C6ST-1* transgenic, as well as in wild-type, mice. A relative lack of spike-frequency adaptation during repetitive firing, a characteristic

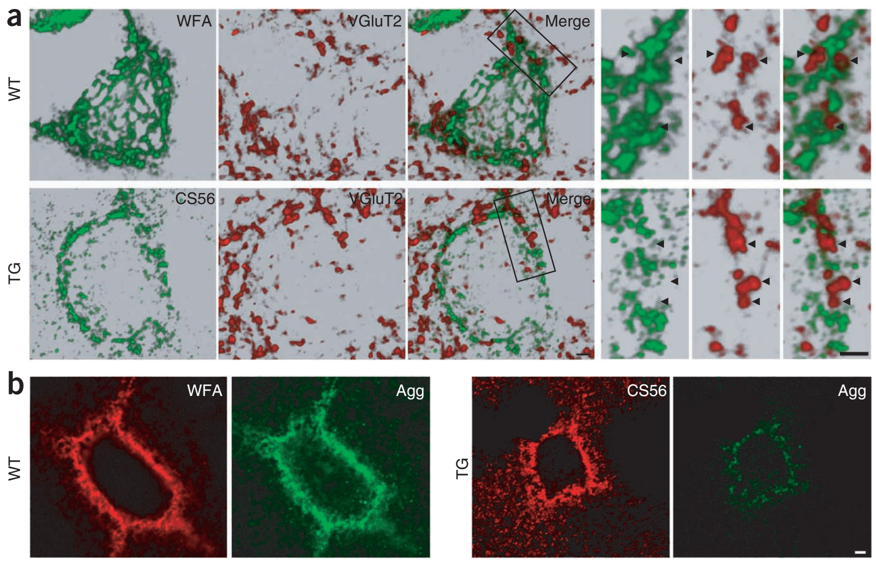


Figure 5 Chondroitin 6-sulfation–enriched PNNs show a diffuse structure and are unable to tightly surround thalamocortical synaptic contacts. (**a**) Three-dimensional reconstruction revealed a distinct meshwork of WFA$^+$ PNNs in controls. In contrast, CS56$^+$ PNNs in *C6ST-1* mice were sparse dot-like structures. Right, magnification of boxed regions in the left panels. VGluT2-labeled thalamocortical boutons (arrowheads) were embedded in the meshwork of WFA$^+$ PNNs in controls, whereas these boutons were not surrounded by CS56$^+$ PNNs in *C6ST-1* mice. (**b**) Staining with antibody to aggrecan (Agg) revealed a well-formed meshwork and almost the same pattern as that with WFA lectin in controls. In contrast, aggrecan staining of CS56$^+$ PNNs in *C6ST-1* mice appeared diffuse and less condensed over the soma. Scale bars represent 2 μm.

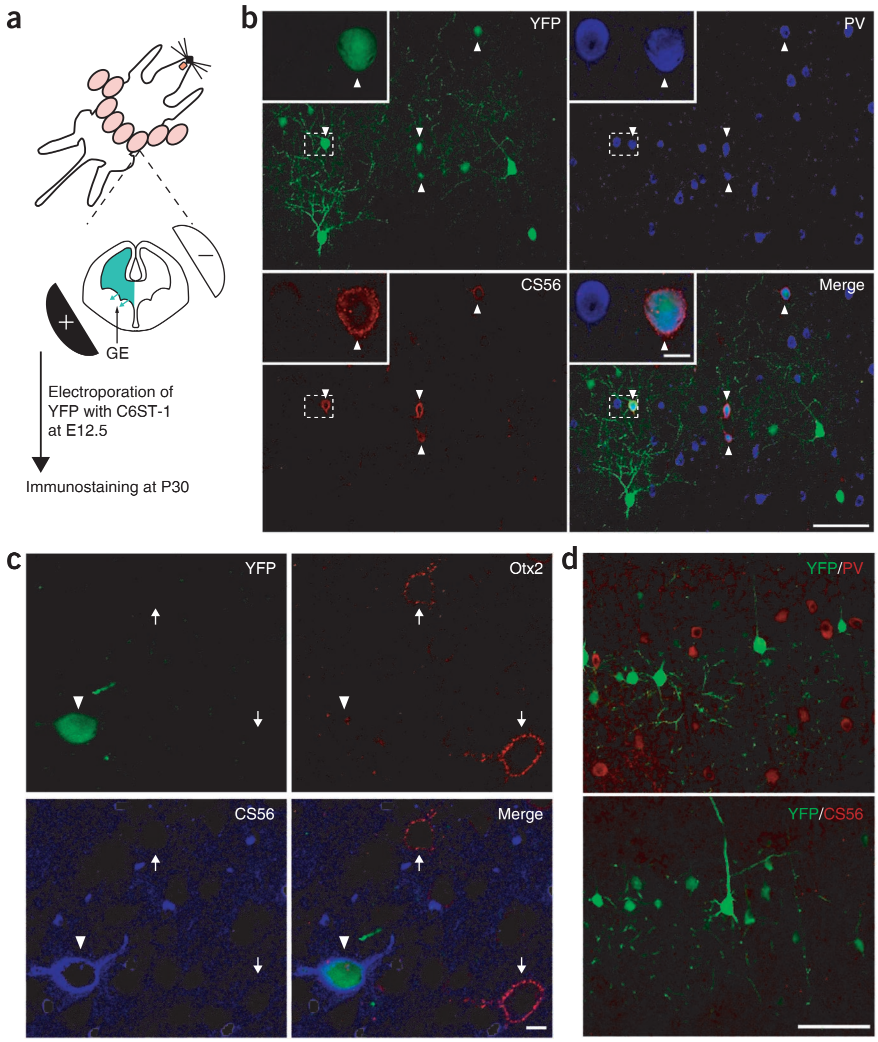


Figure 6 Overexpression of C6ST-1 in parvalbumin-expressing interneurons cell-autonomously affects PNN formation. (**a**) Experimental model for ganglionic eminence (GE)–directed electroporation. Wild-type interneurons were co-electroporated with *C6ST-1* and *YFP* at E12.5 and analyzed at P30. (**b**) CS56$^+$ PNNs were formed around co-transfected parvalbumin-expressing interneurons (arrowheads). Insets, magnification of boxed regions. Note that the transfected (right), but not the neighboring untransfected (left), parvalbumin-expressing interneurons were surrounded by CS56$^+$ PNNs. (**c**) Otx2 signal was not found in transfected parvalbumin-expressing interneurons surrounded by CS56$^+$ PNNs (arrowheads). Arrows indicate Otx2 accumulation in untransfected parvalbumin-expressing interneurons. (**d**) Overexpression of C6ST-1 in excitatory pyramidal neurons resulted in no YFP signal in parvalbumin-expressing interneurons and CS56$^+$ PNNs. Scale bars represent 100 μm (**b**–**d**) and 10 μm (insets in **b**).

feature of fast-spiking cells[29,30], was commonly found in both groups (**Fig. 7a**). No significant difference was found in the ratio of the last interspike interval to the second interspike interval (wild type, $n = 5$ neurons, 1.4 ± 0.15; *C6ST-1* transgenic, $n = 6$ neurons, 1.4 ± 0.18; $P > 0.7$). Furthermore, both groups showed another feature of fast-spiking cells: short-duration action potentials followed by fast afterhyperpolarizations[29,30] (**Fig. 7a**). Although no significant difference was found in the amplitude (wild type, $n = 5$ neurons, 16 ± 0.38 mV; *C6ST-1* transgenic, $n = 6$ neurons, 17 ± 0.49 mV; $P > 0.2$) or duration (wild type, $n = 5$ neurons, 26 ± 2.8 ms; *C6ST-1* transgenic, $n = 6$ neurons, 28 ± 2.9 ms; $P > 0.6$) of the afterhyperpolarization, the action potentials were significantly wider ($P < 0.009$) in *C6ST-1* transgenic mice than in wild-type mice (**Fig. 7b**). In addition, the resting membrane potential was significantly more depolarized ($P < 0.03$; **Fig. 7c**) in *C6ST-1* transgenic mice, although no difference was found in input resistance (wild type, $n = 5$ neurons, 145 ± 22.1 MΩ; *C6ST-1* transgenic, $n = 6$ neurons, 166 ± 20.7 MΩ; $P > 0.3$). Parvalbumin immunoreactivity was detected in all of the fast-spiking cells (four cells in wild-type mice and five cells in *C6ST-1* transgenic mice) in which we successfully conducted immunohistochemical examinations (**Fig. 7a**). A recent study on parvalbumin-expressing interneurons found that a shortening of spike width and a hyperpolarization of resting membrane potential proceeded in parallel with changes in the other membrane properties during postnatal development[31]. Thus, the overexpression of C6ST-1 prevented the maturation of some of the electrophysiological properties of parvalbumin-expressing interneurons.

In *C6ST-1* transgenic mice, parvalbumin-expressing interneurons may more easily initiate action potentials in response to excitatory inputs, as a result of their depolarized membrane potentials, and therefore inhibit their target cells more frequently than in wild-type mice. The shortening of spike width results in a reduction of transmitter release probability and short-term depression at the nerve terminals

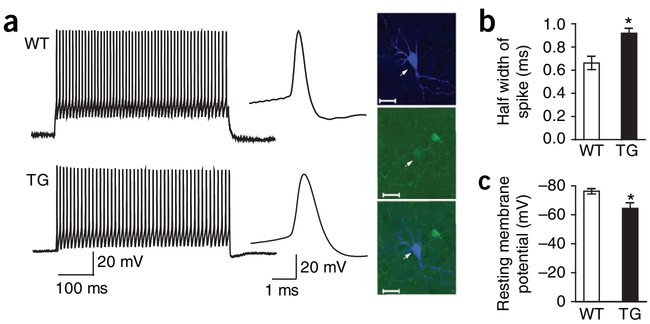


Figure 7 Overexpression of C6ST-1 prevents the maturation of the membrane properties of fast-spiking cells. (**a**) Example traces of spike discharges in response to a depolarizing current pulse (500-ms duration) in fast-spiking cells sampled from wild-type (current amplitude, 400 pA; top left) and *C6ST-1* mice (300 pA; bottom left). Middle, expanded traces show the second spike evoked by the current pulse. Calibrations were common to the two cells. Right, confocal images showing parvalbumin expression in a fast-spiking cell (arrows) sampled from a mouse. Staining for biocytin (top) and parvalbumin (middle) is shown together with a merged image (bottom). Scale bars represent 10 μm. (**b**) Half width of action potentials. (**c**) Resting membrane potential. *$P < 0.05$ (Student's t test) between *C6ST-1* and wild-type mice, and the numbers of fast-spiking cells were 5 (wild-type mice) and 6 (*C6ST-1* mice). Error bars represent s.e.m.

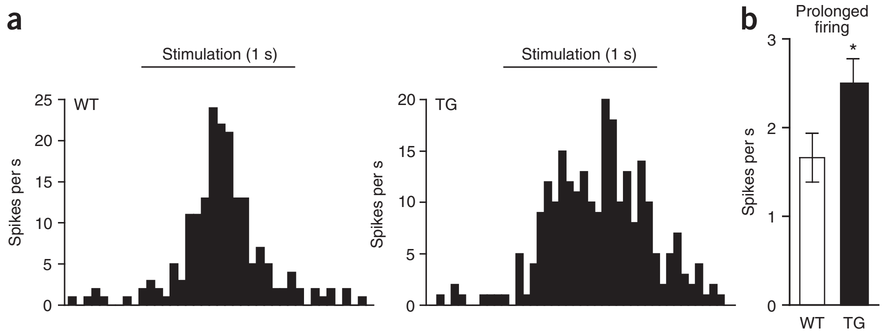


Figure 8 Comparison of visual responsiveness in visual cortical neurons between *C6ST-1* and wild-type mice. (**a**) Examples of post-stimulus time histograms for two cells, each of which was sampled from wild-type (left) and *C6ST-1* mice (right). Bars indicate the time (1 s) of presenting drifting gratings. (**b**) Mean firing rate during a 0.5-s period after the presentation of gratings. Values were calculated after subtraction of spontaneous firing rate. The number of cells was 40 (wild type) and 47 (*C6ST-1*). *$P < 0.05$ (Mann-Whitney test). Error bars represent s.e.m.

of parvalbumin-expressing interneurons[31]. Thus, individual action potentials in mature parvalbumin-expressing interneurons can inhibit their target cells effectively even when the firing frequency is high. In *C6ST-1* transgenic mice, short-term depression may remain stronger and the inhibitory effects of individual action potentials may therefore be weaker when parvalbumin-expressing interneurons fire frequently, as compared with wild-type mice. Thus, it is difficult to predict the overall influence of C6ST-1 overexpression on the inhibitory effects of parvalbumin-expressing interneurons.

To test whether the overexpression of C6ST-1 increased or decreased the inhibitory effects of parvalbumin-expressing interneurons, we examined prolonged firing by extracellular unit recordings from adult visual cortex. It is known that when the inhibitory effects of parvalbumin-expressing interneurons are reduced, visual cortical neurons showed prolonged firing after visual stimulus leave their receptive fields[5,32]. Visual responses were evoked repetitively by stimulation of the contralateral eye with sinusoidal gratings drifting for 1 s. On the whole, the visual responsiveness in *C6ST-1* transgenic mice was very similar to that in wild-type mice. No significant difference was found in orientation selectivity (wild type, $n = 40$ neurons, 0.51 ± 0.068 ; *C6ST-1* transgenic, $n = 47$ neurons, 0.50 ± 0.044; $P > 0.9$), mean firing rate during the optimal stimulation (wild type, $n = 40$ neurons, 7.8 ± 1.5 spikes per s; *C6ST-1* transgenic, $n = 47$ neurons, 6.5 ± 0.71 spikes per s; $P > 0.4$) or spontaneous firing rate (wild type, $n = 40$ neurons, 1.6 ± 0.18 spikes per s; *C6ST-1* transgenic, $n = 47$ neurons, 1.8 ± 0.18 spikes per s; $P > 0.3$) between the two groups. However, we noticed a tendency for prolonged firing after the end of the visual stimulus in *C6ST-1* transgenic mice (**Fig. 8a**), although the degree of prolonged firing seemed weaker than that found in mice deficient for a GABA-synthesizing enzyme[32]. We confirmed this tendency by comparing the mean firing rate during a 0.5-s period after the end of each drifting stimulus between *C6ST-1* transgenic and wild-type mice. The mean firing rate was significantly higher ($P < 0.003$) in *C6ST-1* transgenic than in wild-type mice (**Fig. 8b**). Thus, we conclude that overexpression of C6ST-1 maintains the inhibitory effects of parvalbumin-expressing interneurons in a slightly reduced state until adulthood, mainly as a result of a failure in spike shortening.

DISCUSSION

It has been proposed that there are two classes of molecular brakes that limit plasticity in adulthood[33]. Functional brakes regulate the balance between excitation and inhibition in local circuits. For example, Lynx1, a protein that inhibits nicotinic acetylcholine receptor signaling, restricts plasticity in adulthood through its influence on the balance between excitation and inhibition[34]. On the other hand, structural brakes, such as CSPGs and myelin-related proteins, are thought to limit plasticity by acting as physical barriers based on the mechanism by which they inhibit axonal growth[10,35]. However, our findings suggest an alternative model, in which specific sulfation patterns of chondroitin sulfate chains regulate the maturation of parvalbumin-expressing interneurons through the incorporation of Otx2. Because of the reduced accumulation of Otx2 in parvalbumin-expressing interneurons, *C6ST-1* transgenic mice had a reduced cortical inhibitory tone that did not reach the normal adult level; thus, these mice retained juvenile-like plasticity as adults. Originally, depletion of Otx2 was reported to prevent the initiation of the critical period[5]. Our data suggest that Otx2 also participates in the termination of the critical period. This is not surprising, as the maturation of cortical inhibitory interneurons is crucial for both the onset and termination of the critical period[2]. The onset and termination of the critical period can be accelerated by prematurely enhancing inhibitory transmission[2]. Conversely, reducing intracortical inhibition in adult animals reactivates ocular dominance plasticity after the critical period[36]. The cortical inhibition level has been proposed to cross two thresholds during development[37]. In this model, the critical period is initiated when the inhibition level reaches the first threshold. As development proceeds, the inhibition level increases further and terminates the critical period once it crosses the second threshold. Our data suggest that developmental changes in sulfation patterns of chondroitin sulfate may be required to cross the second threshold, but not the first.

Previous data showed that the number of parvalbumin-immunopositive cells and the intensity of parvalbumin staining were reduced by Otx2 knockdown[5]. We did not find such changes in *C6ST-1* transgenic mice, in spite of the impaired maturation of membrane properties of parvalbumin-expressing interneurons in these mice. Thus, expression levels of parvalbumin do not always correlate with the electrophysiological properties of parvalbumin-expressing interneurons. This idea is supported by the fact that parvalbumin deficiency dose not substantially affect PNN formation and the basic electrophysiological properties of parvalbumin-expressing interneurons[38]. Our results also suggest that the promotion of parvalbumin expression by Otx2 does not require the internalization of Otx2 into the neurons. Instead, a temporal interaction with some signaling molecules on the surface of neurons may be enough to promote parvalbumin expression. How Otx2 regulates parvalbumin-expressing interneuron function is currently unknown. We found that the reduced accumulation of Otx2 prevented the maturation of membrane properties of parvalbumin-expressing interneurons in *C6ST-1* transgenic mice; thus, Otx2 may be required for upregulation of K^+ channels of the K_v3 subfamily underlying brief action potentials[39] and other types of K^+ channels contributing to the hyperpolarization of resting membrane potentials of parvalbumin-expressing interneurons[31].

How the temporal shift of the 4S/6S ratio is regulated remains unknown. We investigated whether reducing cortical inhibition levels, which reactivate the critical period plasticity in adult mice[36], also reduces the 4S/6S ratio by means of intracortical microperfusion of 3-mercaptopropionic acid (MPA), an inhibitor of GABA synthetic enzyme. However, the 4S/6S ratio in the visual cortex was not affected by MPA perfusion (**Supplementary Fig. 5**). The temporal shift of the 4S/6S ratio may be developmentally programmed and independent or upstream of visual experience or inhibition levels. Expression of several plasticity-associated genes, such as *cartilage link protein-1* (*Crtl1*) and *insulin-like growth factor-1*, is regulated by visual experience[40,41]. On the other hand, expression of myelin-related proteins is independent of visual experience[35]. PNNs formation partially depends on visual experience[10], suggesting that expression of some of these components is regulated by vision. Expression of Crtl1, which is required to stabilize PNNs formation, is downregulated by dark rearing, and *Crtl1*-deficient mice have attenuated PNN formation[40]. Thus, both experience-dependent (for example, Crtl1 expression) and experience-independent (for example, the 4S/6S ratio of chondroitin sulfate chains) factors seem to cooperate for PNN formation. Dysregulation of one of these factors may result in impaired PNN formation and persistent cortical plasticity.

The cellular origin of PNNs has been controversial. The glial origin of PNNs is supported by the fact that they are closely associated with glial processes and that some of their components are synthesized by glial cells[7–9]. In contrast, *in vitro* construction of PNNs in dissociated neuronal culture without glial cells suggests that they originate from neurons[42]. Our findings support the latter possibility, as it was possible to manipulate local PNN formation by parvalbumin-expressing interneuron autonomous elevation of chondroitin 6-sulfation. We also found that C6ST-1 overexpression in non–parvalbumin-expressing interneurons (that is, glial cells and pyramidal neurons) did not affect PNN formation around parvalbumin-expressing interneurons. This may be explained by the fact that the expression patterns of CSPG core protein vary among cell types. For example, brevican is produced in both neurons and glial cells[9], whereas aggrecan seems to be synthesized selectively by parvalbumin-expressing interneurons[43]. Our results indicate that sulfation patterns of CSPGs produced in parvalbumin-expressing interneurons largely contribute to PNN formation. According to a recent model of PNN formation, secreted CSPGs bind to both cell surface hyaluronan and tenascin-R to form massive macromolecules in the pericellular space[8]. Thus, the interaction between CSPGs with other PNN components may depend on the sulfation patterns of chondroitin sulfate. PNNs are very heterogeneous, probably because of differences in the glycan structure of CSPGs[23]. Our data may provide genetic evidence that the heterogeneity of PNNs results, at least in part, from differential sulfation patterns of chondroitin sulfate, which in turn differentially modulate parvalbumin-expressing interneuron function by regulating incorporation of Otx2.

PNN formation is not restricted to the visual cortex and can be found in many brain regions in which Otx2 is not found. Outside the visual cortex, PNNs may facilitate accumulation of other molecules that are secreted from presynaptic terminals. One such candidate is neuronal activity–regulated pentraxin, a secreted synaptic protein that binds to AMPA receptors at excitatory synapses on parvalbumin-expressing interneurons and regulates synaptic plasticity[44]. In the hippocampal neuronal culture, disruption of PNNs with chondroitinase ABC reduces accumulation of neuronal activity–regulated pentraxin on parvalbumin-expressing interneurons[44]. Dysfunction of parvalbumin-expressing interneurons has been implicated in not only cortical plasticity, but also some psychological disorders, such as schizophrenia[45]. It was recently reported that the number of PNNs was reduced in individuals with schizophrenia[46]. Thus, it will be fascinating to assess the contribution of sulfation patterns of chondroitin sulfate in models of these diseases.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

S.M., Y.Y., Y.K. and H.K. designed and performed the research, analyzed the data and wrote the manuscript. S.M. and H.K. conceived the idea. C.T. produced *C6ST-1* transgenic mice.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Generation of *C6ST-1* transgenic mice. Full-length human *C6ST-1* cDNA was amplified by reverse transcription PCR (RT-PCR) using a human placenta cDNA library and cloned into the EcoRI site of a pCAG vector, which drives transgene expression using a chicken β-actin promoter and CMV enhancer[47] Plasmid DNA was injected into C57BL6 embryos, which were placed into pseudopregnant females to produce transgenic offspring. Transgenic mice were identified by Southern blot using tail DNA and were mated with C57BL6 wild-type mice. Mice were kept under specific pathogen–free conditions in an environmentally controlled clean room at the Institute of Laboratory Animals, Kobe Pharmaceutical University. All experiments were conducted according to institutional ethics guidelines for animal experiments and safety guidelines for gene manipulation experiments. All animal procedures were approved by the Kobe Pharmaceutical University Committee on Animal Research and Ethics.

Chondroitin sulfate disaccharide composition. Brains were removed and cortical regions were dissected according to a mouse brain atlas and homogenized in cold acetone. The air-dried powder was exhaustively digested with actinase E (Kaken Pharma) at 60 °C for 24 h. The digest was treated with 5% trichloroacetic acid (wt/vol) and the acid-soluble fraction was extracted with diethyl ether. The aqueous phase was neutralized and subjected to gel filtration on a PD-10 column (GE Healthcare). The flow-through fractions were collected, evaporated dry and dissolved in water. An aliquot of the sample was digested with chondroitinase ABC (Seikagaku) at 37 °C for 2 h. The digests were derivatized with a fluorophore, 2-aminobenzamide, and then analyzed by anion-exchange high-performance liquid chromatography (SLC-10A, Shimadzu) on a PA-03 column (YMC). Identification and quantification of the resulting disaccharides were achieved by comparison with chondroitin sulfate–derived authentic unsaturated disaccharides (Seikagaku), as described previously[48].

Monocular deprivation. Monocular deprivation began at P24–26 for critical period mice and P60–P90 in adult mice. The eyelid margins of the right eye were trimmed and sutured under intraperitoneal pentobarbital anesthesia and deprivation continued for 3 or 6 d until the recording. Mice were monitored daily to be sure that the sutured eye remained shut and uninfected. Animals whose eyelids did not fully seal shut were excluded from further experiments.

VEP recordings. Mice were anesthetized with intraperitoneal fentanyl and droperidol and then placed in a stereotaxic frame. A local anesthetic, lidocaine, was administered at pressure points caused by the stereotaxic frame. Body temperature was maintained at about 37 °C. A large portion of the skull overlying the visual cortex was carefully drilled and removed leaving the dura intact. Microelectrodes (300–500 kΩ, Frederick Haer & Co) were inserted into the binocular visual cortex (3 mm lateral of lambda) and advanced 450 μm into the cortex. At this depth, VEPs had their maximal amplitude. Stimuli consisted of full-field sine-wave gratings of 0% and 100% contrast (mean luminance 25 cd m^{-2}, area = 30 × 50 cm), reversing at 1 Hz, and were presented at 0.05 cycles per degree. These stimuli were generated by a VSG4/2 card (Cambridge Research System) and displayed on the face of a monitor (FlexScan T962, Eizo Nanao), which was placed in front of the animal (distance, 28.5 cm). VEPs were elicited by horizontal gratings. VEP amplitude was quantified by measuring the negative peak amplitude of average responses, as described previously[49].

Unit recordings. Adult mice (P60–90) were anesthetized with urethane (1.1 g per kg of body weight, intraperitoneal) supplemented by the sedative chlorprothixene (9 mg per kg of body weight, intramuscular). Unit recordings were conducted from the binocular zone of the primary visual cortex using a tungsten-in glass electrode (3–5 MΩ; Frederick Haer & Co). Cells were recorded across all layers. We analyzed spikes that originated from single neurons using an off-line spike-sorting procedure. Visual stimuli consisted of a full-field drifting sinusoidal grating at a temporal frequency of 2 Hz were applied to the eye contralateral to the recording hemisphere. These stimuli were generated in the Psychophysics Toolbox extensions of MATLAB (Mathworks) and displayed on the face of a monitor (Flexscan T962, Nanao, 160-Hz refresh rate), which was placed in front of the animal (distance, 28.5 cm). To characterize visual responsiveness, we varied the direction of gratings between 0–360° (12 steps) at the spatial frequency between 0.01 and 0.32 (six steps). The orientation selective index was calculated as the ratio of $(R_{pref} - R_{orth})/(R_{pref} + R_{orth})$ at the optimal spatial frequency in each cell, where R_{pref} was the response to the preferred direction and R_{orth} was the mean response of the two directions orthogonal to the preferred direction.

Whole-cell recordings from slice preparations. Coronal slices (300 μm) of primary visual cortex were prepared from mice at P30–34 under deep anesthesia with isoflurane. Slices were cut and recovered for 1 h in oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 3 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$ and 10 mM glucose at 33 °C. Then slices were kept in the ACSF at 24–25 °C. During recording experiments, slices were perfused with ACSF at 24–25 °C. An infrared Olympus DIC microscope with a 40×, 0.8 NA water-immersion lens was used to visualize and target recording electrodes to nonpyramidal neurons located in and near layer 4. Patch pipettes (4–6 MΩ) were filled with an internal solution containing 130 mM potassium gluconate, 8 mM KCl, 1 mM $MgCl_2$, 0.6 mM EGTA, 10 mM HEPES, 3 mM Na^+-ATP, 0.5 mM Na^+-GTP, 10 mM sodium phosphocreatine and 0.3% biocytin (wt/vol) (pH 7.3 adjusted with KOH). Recordings were made using a Multiclamp 700B (Molecular Probes) in the current-clamp mode. For analysis, we selected cells with series resistance less than 20 MΩ and did not compensate the resistance. To assess the membrane properties of recorded cells, we stimulated cells with current pulses (duration, 500 ms) starting from −200 pA and increasing up to 700 pA in 100-pA steps. After recording, the recorded cells were visualized using Alexa Fluor 405–conjugated streptavidin (Molecular Probes).

Immunohistochemistry. Mice were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS (wt/vol). Brains were removed and post-fixed overnight. Coronal sections (30 μm thick) were cut with a vibratome (Leica). Sections were permeabilized with 0.2% Triton X-100 (vol/vol) in PBS, blocked with 2% bovine serum albumin (wt/vol) in PBS, and incubated overnight at 25 °C with primary antibodies to parvalbumin (Swant, mouse monoclonal IgG, 1:5,000), CS56 (Seikagaku, mouse monoclonal IgM, 1:200), Otx2 (Santa Cruz Biotech, goat polyclonal IgG, 1:50 or Abcam, rabbit polyclonal IgG, 1:100), aggrecan (Millipore, rabbit polyclonal IgG, 1:400) or VGluT2 (Millipore, guinea pig polyclonal IgG, 1:500). TRITC-labeled WFA lectin (EY laboratories, 5 μg ml^{-1}) was used for staining perineuronal nets. Sections were incubated with the appropriate Alexa 488/568/647-labeled (Invitrogen) or Cy-5–labeled (Millipore) secondary antibodies for 1 h at 25 °C. After washing with PBS, sections were stained with Hoescht 33342 (2 μg ml^{-1}) to identify cell nuclei. Images were captured with an LSM 710 laser-scanning confocal microscope using a 10× objective (Carl Zeiss). For quantification of the number of WFA-, CS56-, parvalbumin- and Otx2-immunopositive cells, labeled cells were counted in a 1.0 × 1.2 mm area spanning all cortical layers of the primary visual cortex. For three-dimensional reconstruction, we randomly selected PNNs in layer 4 from both wild-type and *C6ST-1* transgenic mice. Images at 0.35-μm steps were acquired using a 63× objective and processed using ZEN software (Carl Zeiss).

***In utero* electroporation.** Ganglionic eminence–directed *in utero* electroporation was performed on E12.5 wild-type mice as described pereviously[32,33]. Briefly, 2–3 μl of a mixture of *pCAG-C6ST1* (1 μg μl^{-1}) and *pCAG-YFP* (1 μg μl^{-1}) plasmid solution was injected into the lateral ventricle using a pulled glass capillary. The head was clasped with a pair electrode (CUY650P2 or CUY650P5; NEPA Gene) at an angle of 30–60° from the horizontal plane. Square electric pulses (30 V for 50 ms, five times in 950-ms intervals) were passed using an electroporator (NEPA21, NEPA Gene).

Immunoblotting. Brains were homogenized in M-PER buffer (Thermo) containing protease inhibitor cocktail (Nacalai) and incubated on ice for 30 min. After centrifugation, protein concentrations of supernatants were determined by BCA assay kit (Thermo). Protein (200 μg) was digested with 5 milliunits of chondroitinase ABC for 2 h at 37 °C. Protein (10 μg) was separated by 5% acrylamide gel electrophoresis, transferred onto PVDF membrane (GE Healthcare), and incubated overnight at 4 °C with primary antibodies to aggrecan (Millipore, rabbit polyclonal IgG, 1:2,000), phosphacan (Developmental Studies Hybridoma Bank, mouse monoclonal IgG, 1:200) or brevican (BD Biosciences, mouse monoclonal IgG, 1:2,000). The blot was incubated with the appropriate horseradish peroxidase–labeled secondary antibodies for 1 h at 25 °C and developed with the ECL detection system (GE Healthcare). The relative abundance of CSPG core proteins was quantified as the density of the band using ImageQuant TL software (GE Healthcare).

Labeling of retinogeniculate axons at dLGN. Mice under pentobarbital anesthesia received intravitreous injections of Alexa Fluor 488– and Alexa Fluor 594–conjugated cholera toxin B subunits (Invitrogen, 2.5 μl of 2 mg ml^{-1} in PBS) into the left and right eyes, respectively, with a 33 G needle. The needle was gently pulled out after being held in place for about 1 min. Mice were perfused at 24 h after injection. Brains were dissected, post-fixed and cut coronally into 100-μm-thick serial sections with a vibratome. Images of the dLGN at the largest cross-section were captured with a LSM 710 laser-scanning confocal microscope.

Quantitative real-time RT-PCR. Total RNA from the retina, dLGN and visual cortex was isolated with the RNeasy mini kit (Qiagen). cDNA was synthesized from 300 ng of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega). We used primer sequences specific for *Otx2* (forward, 5′-CTCGACGTTCTGGAAGCTCT-3′; reverse, 5′-ACTGGCCACTTGTTCCACTC-3′) and *β-actin* (forward, 5′-AGAGGGA AATCGTGCGTGAC-3′; reverse, 5′-CAATAGTGATGCCTGGCCGT-3′). Quantitative real-time RT-PCR was performed using FastStart DNA Master plus SYBR Green I (Roche Applied Science) in a LightCycler ST300 (Roche Applied Science).

Primary cell culture. For primary astrocyte culture, dissociated cerebral cortical cells from P0 wild-type or *C6ST-1* transgenic mice were plated in 10-cm^2 dishes and grown in DMEM (Wako) containing 10% fetal bovine serum (vol/vol) and antibiotics until they reached confluence. Cells were split with trypsin and re-plated in 24-well dishes at a density of 100,000 cells per well (1.9 mm^2) on glass coverslips coated with poly-L-lysine (10 μg ml^{-1}). Medium was changed every 3 d until the cells reach confluence. At that time, the culture consisted mostly of astrocytes. Dissociated neuronal cultures were prepared from cerebral cortices of E18 wild-type or *C6ST-1* transgenic mice and plated onto the astrocyte monolayer at a density of 200,000 cells per well (1.9 mm^2). Cells were maintained up to 18 d in Neurobasal Medium (Gibco) supplemented with 2% B-27 (vol/vol) plus (Miltenyi Biotec), 2 mM glutamax I (Gibco) and antibiotics. Half of the culture medium was replaced every 3–4 d. The cultures were fixed with 4% paraformaldehyde and stained with various antibodies as described above.

Intracortical microperfusion of MPA. Osmotic minipumps (model 1007D, rate = 0.5 μl h^{-1}; Alzet) containing MPA (100 μM in saline) were connected to a cannula (gauge 30) and implanted stereotaxically into the left hemisphere (3 mm lateral to the midline, 2 mm anterior to lambda) of adult mice. MPA was continuously infused for 1 week into the left visual cortex. The left (MPA treated) and the right (control) visual cortex was dissected and homogenized in ice-cold acetone. Chondroitin sulfate disaccharide composition analysis was performed as described above.

Statistical analysis. Statistical significance was determined by using unpaired two-tailed Student's *t* test. Differences were considered to be significant with a *P* value less than 0.05.

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