GABA in mature neurons in adults, the excitatory effects of GABA in immature neurons have been demonstrated in a broad range of CNS regions [8, 9, 11, 12]. In early stages of brain development, GABA exhibits depolarizing actions due to the efflux of chloride ions mediated through $GABA_A$ receptors in immature neurons, due to a relatively higher intracellular versus extracellular concentration of chloride ions. The GABA-dependent neuronal excitation in the developing brain plays a fundamental role for trophic factors by influencing multiple developmental processes including neurite outgrowth, cell migration, and cell survival as well as instructive actions for the construction of neuronal circuits in the CNS [11, 13–17]. Taken together, dysfunction of GABAergic neurons such as the imbalanced generation of glutamatergic and GABAergic neurons is implicated as a cause of various neurodevelopmental disorders including epilepsy, hyperalgesia, and allodynia as well as seizures of the immature brain [11, 18–22].

The choice between the excitatory and inhibitory cell fates of progenitor cells is made by tightly controlled genetic programs. Elucidation of the mechanisms that control specific neuronal cell fate is fundamental for understanding how the central nervous system functions. Over the last decade, considerable progress has been made in defining the molecular mechanisms that control the balance of excitatory and inhibitory neuronal cell fate through recently developed mouse genetic lineage-tracing methods in addition to gene-transfer technologies. Progress in defining mechanisms underlying the development of cortical GABAergic interneurons has been well summarized in several recent reviews [23–25].

In this paper, we focus on the molecular mechanisms specifying GABAergic neuronal cell fate in the caudal part of CNS regions including the spinal cord, the cerebellum, and the cochlear nucleus, particularly from the viewpoint of transcriptional networks regulated by the homeodomain-type and proneural basic helix-loop-helix- (bHLH-) type transcription factors.

2. Specification of GABAergic Interneurons in the Dorsal Spinal Cord

In the dorsal spinal cord, association and relay neurons in the dorsal spinal cord play essential roles in integrating incoming sensory information, including pain, temperature, and mechanoception, and transducing these into signals for motoneurons or higher brain centers. Functionally, GABAergic neurons in the dorsal spinal cord are involved in modulating the strength of sensory input to the spinal cord by presynaptic inhibition of primary sensory afferents [26].

In the early developing neural tube during embryonic days 10–11.5 (E10–11.5), six distinct classes of deep dorsal interneurons (dI1–6) arise from six different progenitor domains (dP1–6), followed by the generation of two late-born neuronal subtypes of superficial laminae, dIL_A and dIL_B, from a common dorsal progenitor domain during E11–13 (Figure 1) [27, 28]. While postmitotic dorsal interneurons can be distinguished by the characteristic combinatorial expression of homeodomain (HD) transcription factors like ventral interneurons described below [29, 30], proneural bHLH transcription factors have a predominant role for establishing their progenitor domains with an almost complementary and nonoverlapping pattern in the spinal cord [27, 28, 31, 32].

GABAergic neurons in the dorsal spinal cord are composed of early-born dI4 and dI6 and late-born dIL_A neurons. These three classes of postmitotic interneurons express the HD transcription factor Lbx1, Pax2 and Lhx1/5 [29, 30]. A bHLH transcription factor Ptf1a plays a central role in the specification of these GABAergic inhibitory dorsal interneurons while suppressing the generation of excitatory glutamatergic interneurons [33, 34]. Mice lacking *Ptf1a* show a near complete loss of dI4 and dIL_A GABAergic interneurons while containing increased numbers of excitatory dI5 and dIL_B interneurons. In contrast, Ptf1a suppresses the HD factor Tlx3, which is an important postmitotic determinant for dorsal glutamatergic interneurons [35, 36]. Overexpression of Ptf1a in the chick neural tube can induce ectopic Pax2-positive inhibitory neurons at the expense of Tlx3-positive glutamatergic excitatory neurons dI5 and dIL_B [33, 34]. Another bHLH transcription factor, Ascl1, also participates in the specification of these neurons in a more complex way. Ascl1 and the HD factors Gsh1 and Gsh2 coordinately activate Tlx3 expression to promote the generation of dI5 glutamatergic interneurons in early developmental stages [37, 38]. In the late-born dIL populations, however, Ascl1 functions to antagonize Gsh1 and Gsh2 by upregulating Ptf1a expression and thus is necessary for the specification of dIL_A GABAergic interneurons in the dorsal horn [38, 39]. Furthermore, Ascl1 simultaneously activates Notch signaling in non-cell-autonomous manner that promotes dIL_B glutamatergic cell fate over a dIL_A cell fate. Pax2 is also an essential regulator for the differentiation of GABAergic inhibitory neurons, as demonstrated by *Pax2*-mutant mice in which GABAergic markers in the dorsal horn are drastically reduced [35]. Although Lhx1 and Lhx5 are not required for the initial specification of GABAergic neurons, these factors maintain Pax2 expression as well as inhibitory-neurotransmitter expression of genes such as Gad1 at later developmental stages [40]. Although Pax2 and Lhx1/5 seem to function downstream of Ptf1a, it remains to be determined whether these factors are direct or indirect downstream targets of Ptf1a. Recent studies have demonstrated that another bHLH transcription factor Neurog2 is a direct downstream target for Ptf1a [41].

3. Specification of GABAergic Interneurons in the Ventral Spinal Cord

In the ventral spinal cord, motor neurons and several types of interneurons assemble into local networks that contribute to the generation of the rhythmic output required for locomotion [42]. In the early development of the neural tube, an extrinsic factor Shh is released from the notochord and floor plate that induces the patterning of five distinct ventral progenitor domains (p0, p1, p2, pMN, and p3)

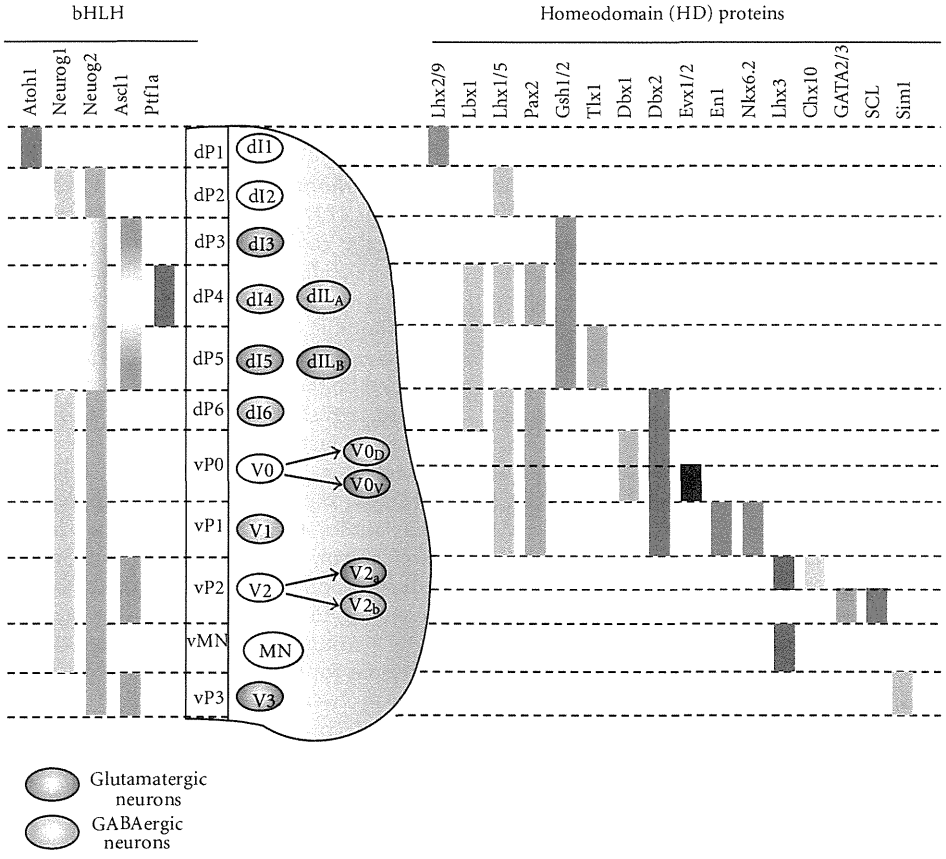


FIGURE 1: The specification of the spinal cord neurons directed by a combinatorial code of transcription factors. Schematic summary of the expression of bHLH transcription factors in the progenitor cells (left) and homeodomain (HD) transcription factors in the differentiating and differentiated neurons. Eleven early classes of postmitotic neurons (dI1~6, V0~3, and motoneurons (MN)) and two late-born dorsal interneurons (dIL$_A$ and dIL$_B$) are present in the embryonic spinal cord. The immature postmitotic V0 and V2 interneurons are further subdivided into two distinct interneuron subtypes indicated by arrows. The glutamatergic excitatory neurons and GABAergic inhibitory neurons are represented by red and blue circles, respectively.

in a concentration-dependent manner [43]. Four cardinal classes of interneurons (V0, V1, V2, and V3) and motor neurons are produced from each progenitor domain that can be distinguished by combinatorial transcription factor expression (Figure 1) [43–45].

Motor neurons are basically cholinergic [46]. Excitatory glutamatergic interneurons include V3 and a subset of the V2 and V0 interneuron population whereas inhibitory interneurons (GABAergic and glycinergic neurons) are generated from p0, p1, and p2 progenitor domains [42, 47].

While V0 populations, defined by the expression of Evx1/2, are commissural interneurons that extend axons contralaterally and rostrally for 2–4 spinal cord segments, V1 interneurons, marked by Engrailed-1 (En1) and Foxd3 expression, are inhibitory neurons that project axons ipsilaterally and rostrally [48–53]. The V1 interneurons are initially generated as a homogeneous GABAergic interneuron population in developing neural tubes [51] but subsequently differentiate into a range of inhibitory interneuron cell types, including Renshaw cells (RCs) and putative reciprocal Ia inhibitory interneurons [52, 54, 55].

V0 interneuron populations constitute heterogeneous neurotransmitter phenotypes. A majority of V0 interneurons derived from the ventral half of the p0 domain (described as V0$_D$ interneurons) express vesicular inhibitory amino acid transporter (VIAAT) and represent both GABAergic and glycinergic neurons whereas one-third of V0 interneurons derived from the dorsal p0 progenitor domain (V0$_V$ interneurons) show an excitatory neuronal phenotype that expresses VGLUT2, a marker of glutamatergic interneurons [49, 50, 56]. They are defined by the absence or presence of the HD factor Evx1 expression, respectively [56].

Progenitor cells of both V0 and V1 share a combinatorial expression of Pax6 and Dbx2 [57]. Dbx1 is, however, uniquely expressed in V0 progenitor cells and is considered an essential factor for the specification of V0 interneurons [50]. In mice lacking *Dbx1*, V0 interneurons are lost, and concomitantly V0$_D$ and V0$_V$ interneurons are respecified

into Lbx1-positive dI6 dorsal interneurons and En1-positive V1 interneurons, respectively.

In contrast, Nkx6.2 is expressed in V1 progenitor cells and is required for the specification of V1 neuronal fate, also it represses the generation of V0 interneurons [58]. *Nkx6.2*-mutant mice display an expansion of the Dbx1 domain into the ventral region where it is destined to be the V1 progenitor domain, followed by the loss of V1 interneurons and a concomitant increase of V0 interneurons. Therefore, Dbx1 and Nkx6.2 play an important role as molecular switches defining the progenitor domains for V0 and V1 interneuron subtypes.

V2 interneurons generated from a homogenous p2 progenitor domain are subdivided into two distinct subtype interneurons: excitatory V2a glutamatergic interneurons, marked by the expression of HD factors Lhx3 and Chx10, and inhibitory V2b GABAergic interneurons, which are characterized by the expression of GATA2/3 and a bHLH transcription factor SCL [59–65]. The asymmetry of V2a versus V2b interneuron fate is initiated by Notch-Delta signaling in immature postmitotic V2 progenitors [63, 66, 67]. Notch receptor ligand Dll4-expressing progenitors give rise to V2a interneurons, maintaining Lhx3 expression while repressing GATA2. These cells simultaneously activate the transcriptional pathways downstream of Notch signaling for the specification of V2b interneuron fate of Notch-expressing progenitors. Forkhead transcription factor Foxn4 acts as a key regulator of V2b interneuron specification [66, 68]. *Foxn4*-mutant mice show loss of Dll4 expression and subsequent cell fate change from V2b to V2a. As downstream factors of Notch signal, both GATA2 and SCL consolidate the transcription pathways to acquire V2b subtype identity [60, 62, 69]. Forced expression of GATA2 in the chick neural tube induces ectopic formation of V2b interneurons while suppressing the generation of other neurons including V2a interneurons. Mice lacking *SCL* exhibit downregulation of GATA2 and deficiency in V2b interneurons, accompanied by overproduction of V2a interneurons. LIM-only protein LMO4 functions as a nucleation factor by assembling a LIM complex with GATA2, SCL, and cofactor NLI, and this transcriptional complex promotes the GABAergic V2b interneuron identity [70].

4. GABAergic Neuron Specification in the Cerebellum

There are three major regions in the cerebellum: cortex, white matter, and nuclei. The cerebellar cortex includes several types of glutamatergic excitatory and GABAergic inhibitory neurons. Glutamatergic neurons are comprised of granule cells and unipolar brush cells (UBCs), while the GABAergic population includes Purkinje, Golgi, Lugaro, stellate, basket, and candelabrum cells. Cerebellar nuclei (CN) are comprised of three major types of neurons: large glutamatergic projection neurons (CN-Glu neurons), midsized GABAergic inhibitory projection neurons (CN-GABA-ION neurons), and small GABAergic interneurons (CN-GABA interneurons). CN-GABA-ION neurons extend their axons to the inferior olivary nucleus (ION) [71], while CN-Glu neurons send their axons to nuclei outside the cerebellum, including the red nucleus and the thalamus. These neurons mutually regulate each other's activity to achieve proper cerebellar function.

During development, the neuroepithelium of the alar plate of rhombomere 1 (r1) generates all types of cerebellar neurons [72–75]. The dorsal-most part of the neuroepithelium, the roof plate, of r1 does not generate neurons but produces cells of the choroid plexus [76]. Cerebellar neuron-producing neuroepithelium can be divided into two regions: the rhombic lip (RL) and the ventricular zone (VZ). These two regions can be morphologically discriminated by a notch located on their border.

In 1997, Ben-Arie et al. reported that a bHLH type transcription factor, Atoh1, is expressed in the rhombic lip and involved in cerebellar granule cell generation [77]. In contrast, our Cre-loxP recombination-based lineage tracing studies revealed that another bHLH type transcription factor, Ptf1a, is expressed in the cerebellar VZ, which produces most of the cerebellar GABAergic neurons including Purkinje, Golgi, basket, stellate cells, CN-GABA-ION neurons, and CN-GABA interneurons [78]. Ptf1a is required for GABAergic neuron production, as GABAergic neurons were not generated in *cerebelless*, Ptf1a loss-of-function mutants as well as Ptf1a-knockout mice. Furthermore, ectopic expression of Ptf1a by means of in utero electroporation caused ectopic production of GABAergic neurons from the dorsal telencephalic neuroepithelium. In addition, Pascual et al. reported that in the *Ptf1a*-null mutants, the fate of neurons produced from the VZ is changed to that of granule cells [79]. Moreover, a recent genetic fate mapping study using *Ascl1*^{CreER}-knock-in mice showed that minor cerebellar GABAergic neurons, such as Lugaro and candelabrum cells, are also derived from the cerebellar VZ [80]. These observations suggested that Ptf1a, expressed in the cerebellar VZ, determines GABAergic neuronal fate in the cerebellum. *PTF1A* was also identified as a causative gene for a human disease that exhibits permanent neonatal diabetes mellitus and cerebellar agenesis [81].

On the other hand, Fishell's and Zoghbi's groups reported a molecular fate map of the derivatives of *Atoh1*-expressing neuroepithelial cells in the cerebellar RL [82, 83]. They showed that not only granule cells but also some CN neurons are derived from the RL, although they did not discriminate between neuron types in the CN. In their studies, development of RL-derived CN neurons was shown to be disrupted in the *Atoh1* mutants. As GABAergic but not glutamatergic CN neurons were found to be derived from Ptf1a-expressing neuroepithelial cells in the VZ [78], this suggests that cerebellar glutamatergic neurons such as granule cells and CN-Glu neurons are derived from the RL. Accordingly, unipolar brush cells, which are glutamatergic, were also shown to emerge from the RL [84].

Together, these studies indicate the presence of two molecularly defined neuroepithelial areas in the cerebellum, the Atoh1-expressing RL and the Ptf1a-expressing VZ, which generate glutamatergic and GABAergic neurons, respectively.

Each bHLH transcription factor is involved in specifying the corresponding neuronal subtype in the cerebellum [85].

Although some clarification of the machinery governing GABAergic neuronal subtype specification by Ptf1a has been provided, molecular mechanisms to specify each GABAergic subtype (e.g., Purkinje, Golgi, basket, stellate cells and CN-ION, CN-interneurons) remain unclear. Birthdating studies using ^{3}H-thymidine and BrdU [86–90] as well as adenovirus [91] have revealed that each type of neuron is generated at distinct developmental stages.

With regard to GABAergic neurons, Purkinje cells are produced early (E10.5~13.5 in mice), Golgi cells a little later (E13.5~postnatal day P(0) in mice), and stellate/basket cells mainly perinatally [86–91]. The newest study by Sudarov et al. revealed that candelabrum cells are generated around P0, while GABAergic CN neurons arise at early stages (E10.5~11.5) [80]. In addition, somatic recombination-based clonal analyses suggested that Purkinje, Golgi, and basket/stellate cells as well as some CN neurons (probably GABAergic) belong to the same lineage [92, 93]. These data indicate that some temporal information in the neuroepithelium may be involved in specification of neuronal types in the VZ. However, the underlying molecular mechanisms have not yet been clarified.

Some scientists have attempted to divide the structure of the cerebellar primordium into several domains (Figure 2). Chizhikov et al. defined four cellular populations (denoted as c1–c4 domains) in the cerebellar primordium via the expression of a few transcription factors [76]. c1 corresponds to the Atoh1-expressing RL, and c2 is located just above the Ptf1a-expressing VZ (denoted as pc2), indicating that c2 cells mainly consist of GABAergic inhibitory neurons. Although c3 and c4 express Lmx1a and Lhx1/5, respectively, their neuronal subtypes are still unknown. This domain structure is disrupted when the roof plate is removed [76]. Furthermore, at the early neurogenesis stage (e.g., E12.5 in mice), Minaki et al. subdivided the c2 domain into dorsally (c2d) and ventrally (c2v) located subdomains that express corl2 (also called Skor2) and Pax2, respectively [94]. While corl2 is exclusively expressed in immature and mature Purkinje cells [94], Pax2 is expressed in GABAergic interneurons (e.g., Golgi, stellate, basket, and CN-GABA neurons) in the cerebellum [95, 96]. They also subdivided the Ptf1a-expressing neuroepithelial domain (pc2) into pc2d and pc2v, which strongly and weakly express E-cadherin, respectively. From the positions of the neuroepithelial and neuronal subdomains, they suggested that the pc2d neuroepithelial subdomain produces cells in the c2d domain, which give rise to Purkinje cells, while the pc2v subdomain generates cells in the c2v that become GABAergic interneurons [97]. As development proceeds, pc2d and pc2v subdomains contract and expand, respectively, and by E14.5 in mice, the Ptf1a-expressing pc2 domain comprises only the pc2v subdomain, which expresses E-cadherin weakly. This correlates with the fact that, at E14.5, Ptf1a-expressing neuroepithelium does not produce Purkinje cells but Pax2-positive interneurons [91, 95]. The expression of several other transcription factors in the cerebellar VZ during development has also been reported. For example, Zordan et al. described the

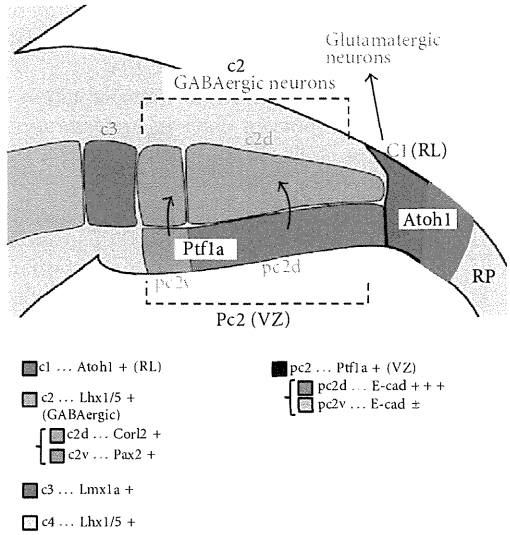


FIGURE 2: Domain structure of the cerebellar primordium. The c1 domain, expressing Atoh1, corresponds to the rhombic lip that produces all types of glutamatergic neurons in the cerebellum. The pc2 is the Ptf1a-expressing neuroepithelial domain that generates all types of GABAergic cerebellar neurons. At early neurogenesis stages, such as E12.5, the pc2 domain can be subdivided into pc2d and pc2v subdomains, which expresses E-cadherin strongly and weakly, respectively. The c2 domain, expressing Lhx1/5, consists of immature GABAergic neurons putatively generated from pc2 neuroepithelial domain. This domain can also be subdivided into two subdomains, c2d and c2v, corresponding to pc2d and pc2v, respectively. The c2d subdomain consists of corl2-expressing neurons or Purkinje cells, whereas the c2v subdomain includes Pax2-positive cerebellar GABAergic interneurons. Although c3 and c4 domains are Lmx1a and Lhx1/5 positive, respectively, cell types that consist these domains are unknown. The roof plate (RP) is located most dorsally and plays prominent roles in organizing this cerebellar domain structure.

expression patterns of proneural bHLH transcription factors, such as Ngn1, Ngn2, and Ascl1, in the cerebellar VZ [98]. It has also been reported that Pax2-positive neurons, but not Purkinje cells, are reduced in the *Ascl1*-null cerebellum [99], while Purkinje cells are reduced in *Ngn1*-null mice [100], suggesting that these bHLH transcription factors play distinct roles in cerebellar development.

In addition, several transcription factors have been reported to participate in the development of specific types of cerebellar neurons. Double knockout of *Lhx1* and *Lhx5* as well as the targeted disruption of their cofactor *Ldb1* resulted in lack of Purkinje cell production in the cerebellum although Pax2-positive interneurons did not seem to be affected. Because Lhx1 and Lhx5 are expressed in post-mitotic cells, this suggests that Lhx1, Lhx5, and Ldb1 are postmitotically involved in Purkinje cell specification [101]. It is recently suggested that corl2 is involved in Purkinje cell maturation from analyses of loss-of-function mutants of corl2 [102]. In addition, in the *cyclin D2* KO mice, the progenitor pool of GABAergic interneurons is precociously

exhausted and progenitor numbers are significantly reduced, leading to a remarkable decrease in the number of late-born interneurons, such as stellate cells [103, 104].

Heterotopic and heterochronic transplantation studies have also provided important clues to understanding cerebellar development [71]. When tissues from embryonic and postnatal cerebella were mixed and transplanted to the fourth ventricle of an adult mouse, the postnatal-derived cells differentiated only into interneurons such as granule, basket, and stellate cells, but not projection neurons, such as Purkinje cells, whereas the embryonic-derived cells were capable of becoming all types of cerebellar neurons [105]. It has also been shown that dissociated cells taken from cerebellar primordium at early neurogenesis stages can differentiate into all major types of cerebellar neurons, while those from postnatal cerebellum differentiated only to Pax2-positive interneurons [106]. These findings suggest that the differentiation competence of cerebellar progenitors becomes restricted as development proceeds. However, the molecular mechanisms underlying this fate restriction process have not yet been clarified. Interestingly, Leto et al. suggested that Pax2-positive interneurons, such as Golgi, stellate, basket cells, and CN-GABA interneurons, are derived from the same progenitor pool [89]. Leto et al. also clarified that, after leaving the VZ, progenitors for GABAergic interneurons continue to proliferate in the prospective white matter during late embryonic and postnatal development [107]. Their grafting studies showed that terminal commitment does not occur while precursors are still proliferating but occur postmitotically according to host-specific information, suggesting an instructive cue provided by the microenvironment of the prospective white matter.

5. GABAergic Neuron Specification in the Cochlear Nucleus

Sounds received in the ear are transmitted via the auditory nerve to the cochlear nucleus (CoN) of the mammalian hindbrain, where the auditory information is properly processed and relayed to the brain. The CoN is a very complex cell assembly that can be divided into two subregions, the ventral and dorsal cochlear nuclei (VCoN and DCoN), which differ in structure and feature. The DCoN exhibits a laminar and cerebellum-like architecture that includes a granule cell system whereas the VCoN does not have a laminar structure. Because of its importance in sound perception, the CoN has been intensely studied from anatomical, physiological, and histochemical points of view [108–110].

Histological observations have deduced that a portion of neurons generated from the dorsal hindbrain neuroepithelia migrate tangentially to give rise to CoN neurons [111, 112]. More directly, genetic fate mapping studies using transgenic mice confirmed that many CoN cells are derived from the dorsal region of the hindbrain neuroepithelia where the *Wnt1* promoter is active [113, 114]. As to the rostrocaudal axis, the origins of CoN neurons seem to differ between birds and mammals. Grafting studies revealed that bird CoN neurons are derived from a broader part of the hindbrain (r3∼r8), [115–117], while mouse genetic studies have suggested a more rostral and narrower origin (r2∼r5) [113].

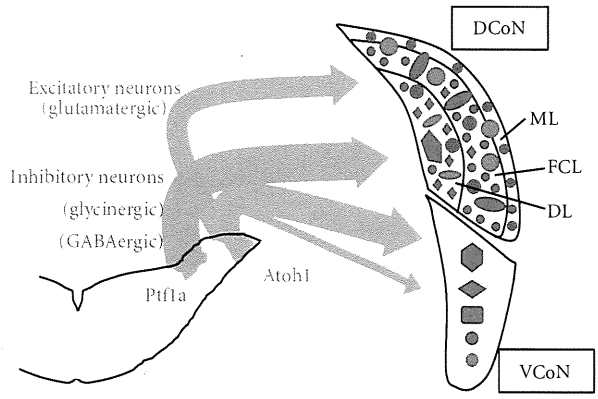
Very sophisticated genetic fate mapping studies were carried out by Farago et al. [113] using an FLP-FRT and Cre-loxP-based dual lineage tracing system. In addition to showing that CoN neurons are derived from r2∼r5, they also revealed that neurons in the anterior part of the VCoN (aVCoN), the posterior part of the VCoN (pVCoN), and the DCoN generally tend to be generated from rostral (∼r2, 3), middle (∼r3, 4), and caudal (∼r4, 5) parts of the CoN neuron-producing hindbrain (r2∼5), respectively, with some overlap.

The CoN contains a variety of neurons that have distinct features [108–110]. For example, the DCoN includes GABAergic neurons (e.g., Golgi and molecular layer (ML) stellate cells), glycinergic neurons (e.g., cartwheel and tuberculoventral cells), and glutamatergic neurons (e.g., granule, unipolar-brush, giant and fusiform cells). The VCoN consists of glutamatergic neurons (e.g., Octopus, globular-bushy, spherical-bushy, and T-stellate cells) and glycinergic neurons such as D-stellate cells.

In the neuroepithelium of the middle hindbrain (r2∼r5), Ptf1a and Atoh1 are expressed in distinct regions resembling the expression pattern in the cerebellum (Figure 3). Using Cre-LoxP-based genetic fate mapping studies, our group identified the origins of inhibitory and excitatory neurons of the cochlear nucleus; inhibitory (GABAergic and glycinergic) and excitatory (glutamatergic) neurons are derived from Ptf1a- and Atoh1-expressing neuroepithelial regions, respectively [118], and their development is dependent on the corresponding bHLH proteins. These findings suggest that Ptf1a and Atoh1 are involved in specifying inhibitory and excitatory neurons of the CoN, respectively, in a similar manner found in the cerebellum. However, little is known about the molecular machinery to generate distinct types of neurons with the same neurotransmitter, for example, Golgi and ML-stellate cells.

6. Conclusions and Future Perspectives

As described here, many recent studies have helped to clarify the molecular mechanisms controlling the specification of GABAergic neuronal cell fate in the hindbrain and spinal cord. While the patterning of the ventral spinal cord along the dorso-ventral axis is predominantly guided by combinatorial expression of HD transcription factors, in the hindbrain, including the cerebellum, the cochlear nucleus, and also the dorsal spinal cord, bHLH transcription factors play essential roles in not only patterning the progenitor domains but also specifying distinct neuronal subtypes. In the early developing dorsal spinal cord, distinct neuronal subtypes are defined by the specific expression of bHLH transcription factors including Atoh1, Neurog1/2, Ptf1a, and Ascl1 in their progenitor cells as well as the timing of their birth and different combinations of HD transcription factors. Among these factors, Ptf1a is a key molecule for the generation and specification of GABAergic interneurons



	Subtypes	Location
Dorsal cochlear nucleus		
Golgi cells	GABAergic	FCL and DL
ML-stellate cells		ML
Cartwheel cells	glycinergic	ML and FCL
Tuberculoventral cells		DL
Granule cells	glutamatergic	FCL
Unipolar-brush cells		FCL and DL
Giant cells		DL
Fusiform cells		FCL
Ventral cochlear nucleus		
Octopus cells	glutamatergic	mainly pVCoN
Globular-bushy cells		broadly VCoN
Spherical-bushy cells		mainly aVCoN
T-stellate cells		broadly VCoN
D-stellate cells	glycinergic	broadly VCoN

FIGURE 3: Lineages of excitatory and inhibitory neurons in the cochlear nucleus. (upper panel) Schematic of cochlear neuron lineages. Glutamatergic excitatory neurons are derived from the Atoh1-expressing RL whereas glycinergic/GABAergic inhibitory neurons are generated from Ptf1a-expressing neuroepithelial domain of the middle hindbrain (r2~5). (lower panel) Various cochlear nucleus neurons characterized by neurotransmitter subtype and location. DCoN: dorsal cochlear nucleus; VCoN: ventral cochlear nucleus; aVCoN: anterior VCoN; pVCoN: posterior VCoN; FCL: fusiform cell layer; ML: molecular layer; DL: deep layer.

among these factors. In the rostral (r1) and middle (r2~5) hindbrain, Ptf1a and Atoh1 are expressed in different neuroepithelial regions and participate in generating inhibitory and excitatory neurons, respectively. However, this rule is not applicable to the caudal (r6~r8) hindbrain. The Ptf1a neuroepithelial domain in the caudal hindbrain (r6~r8) produces not only inhibitory neurons (local circuit neurons) but also glutamatergic neurons (climbing fiber neurons) [119], while the Atoh1 domain generates glutamatergic mossy fiber neurons.

Despite the impressive progress in our understanding of the mechanisms controlling the balance of excitatory and inhibitory neuronal fate by these transcription factors in the hindbrain, many fundamental questions remain to be addressed. For example, although the requirement of Ptf1a for the appropriate balances of excitatory and inhibitory neurons in the hindbrain has been demonstrated, it remains unclear how Ptf1a diversifies the types of GABAergic inhibitory neurons generated from the common neuroepithelial regions during different developmental stages. Identification of downstream targets of Ptf1a will assist us in understanding the molecular mechanisms to specify each GABAergic neuronal subtype. In addition, we need to consider the regulation of bHLH function in

other mechanisms such as posttranslational modification of transcription factors or epigenetic control of gene expression in the diversification of GABAergic neurons.

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Research Report

Abnormal maturation and differentiation of neocortical neurons in epileptogenic cortical malformation: Unique distribution of layer-specific marker cells of focal cortical dysplasia and hemimegalencephaly

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TBR1

ABSTRACT

Focal cortical dysplasia (FCD) and hemimegalencephaly (HME) are major causes of intractable epilepsy in children. The probable pathogenesis of FCD and HMG is the abnormal migration and differentiation of neurons. The aim of the present study was to clarify the abnormal cytoarchitecture, based on neuronal immaturation. Tissue samples were obtained from 16 FCD and seven HME patients, aged between 2 months and 12 years, who had been diagnosed as typical FCD and HME, following surgical treatment for intractable epilepsy. Paraffin-embedded sections were stained with the antibodies of three layer-markers that are usually present only during the fetal period, namely SATB2 (expressed in the upper layer of the normal fetal neocortex), FOXP1 (expressed in the 5th layer), and TBR1 (expressed in the 6th layer). In FCD, SATB2-positive (+) cells located in the middle and deep regions of FCD Ia and Ib, but only in the superficial region of FCD IIa and IIb. FOXP1+ cells diffusely located in the neocortex, especially the upper layer of FCD IIa and IIb. TBR1+ cells mainly located in the middle and deep regions, and also white matter. In FCD IIb, TBR1+ cells were in the superficial region. In HME, SATB2+ and FOXP1+ cells were found diffusely. TBR1+ cells were in the middle and deep regions. On the basis of continued expression of fetal cortical layer-specific markers in FCD and HME brains, the

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abnormal neocortical formation in both is likely to be the result of disrupted neuronal migration and dysmaturation. The expression pattern is different between FCD and HME.

1. Introduction

Focal cortical dysplasia (FCD) and hemimegalencephaly (HME), rare epileptogenic brain malformations are usually accompanied by severe epilepsy and occasionally by mental retardation. The incidence of FCD epilepsy identified in surgical series varies between 12% and 40% (Blümcke et al., 1999; Nordborg et al., 1999; Prayson et al., 2002), while that of HME is lower. These diseases are of relatively high frequency in surgical epilepsy, but have a low morbidity rate (Prayson and Estes, 1995; Prayson, 2000). FCD and HME are usually diagnosed by neuropathological findings in specimens undergoing cortical resection for the treatment of refractory epilepsy. As common features, mental development problems sometimes remain, in spite of well-controlled epilepsy.

FCD was recognized as a pathologic substrate associated with epilepsy (Taylor et al., 1971). It is known that FCD has columnar and laminar disorganization with various cellular abnormalities, including dysmorphic neurons, giant neurons, and balloon cells (Prayson et al., 1996; Yamanouchi et al., 1996; Palmini et al., 2004; Alonso-Nanclares et al., 2005; Blümcke et al., 2011). On the other hand, HME mainly shows cortical laminar abnormality, such as polymicrogyria and neuronal heterotopia. Together with these pathological findings, it is thought that FCD and HME may result from erroneous migration, maturation, or cell death during ontogenesis (Crino and Eberwine, 1997; Cotter et al., 1999; Najm et al., 2007). However, a common pathogenesis remains unknown.

On the other hand, some molecules are useful to detect layer formation of human neocortex. We recently demonstrated that human malformed brains have unique layer patterns (Saito et al., 2010). In the present study, we seek to detect abnormal neuronal migration and differentiation in FCD and HME that will lead to greater understanding of the pathophysiology of the epileptogenic malformed brain.

2. Results

2.1. Histological distribution

Histopathological results were summarized in Table 1. We obtained 4 FCD Ia, 4 FCD Ib, 4 FCD IIa and 4 FCD IIb from the international classification (Blümcke et al., 2011), and seven HME. FCD Ic was relatively rare. Although we could not examine this subtype, it was enough to investigate FCD Ia and Ib for the aim of the present study because FCD Ic pathologically showed the combination of FCD Ia and Ib. All HME cases showed polymicrogyria and/or unlayered neocortex with neuronal heterotopia and mineralization.

2.2. Immunohistochemistry of FCD

Immunohistochemistry results were summarized in Table 2. SATB2+ cells were dominant in the middle and deep regions of the neocortex in FCD Ia and Ib, although they were widely distributed (Figs. 1 and 2, Table 2). Interestingly, SATB2+ cell distribution of FCD IIa and IIb was limited to the superficial region of the neocortex (Figs. 3 and 4, Table 2). FOXP1 immunoreactivity was diffusely intense (Table 2). Only FCD Ib revealed FOXP1+ cells in the white matter. FCD IIa and IIb demonstrated no FOXP1+ cells in the white matter, and FOXP1+ cells tended to appear in the superficial region of the neocortex. TBR1+ cells were the most prominent in the middle and deep regions of the neocortex (Fig. 4). Notably, TBR1+ cells exhibited a unique localization of the superficial region of the neocortex in FCD IIb (Fig. 4, Table 2). The immunopositive cells for SATB2+, FOXP1 and TBR1 were confirmed as neurons with NeuN-immunopositivity (data not shown). There were no significant differences in the marker expression patterns in each lobe.

2.3. Immunohistochemistry of HME

SATB2+ cells in HME were diffused in the neocortex, but relatively dense in the superficial region of the neocortex (Fig. 5). FOXP1 immunoreactivity also diffusely distributed, but was occasionally negative (Fig. 5, Table 2). TBR1+ cells were limited to the middle and deep regions of the neocortex (Fig. 5, Table 2). The immunopositive cells for SATB2+, FOXP1, and TBR1 in HME were also confirmed as neurons with NeuN-immunopositivity (data not shown). There were also no significant differences in the marker expression patterns in each lobe.

3. Discussion

In the normal developing cortex, the localization of SATB2, FOXP1, and TBR1 is restricted to specific cortical layers, and the expression of all three markers disappears in the postnatal brain (Saito et al., 2011). In the present series, SATB2, FOXP1, and TBR1 were diffusely expressed throughout the cortex in samples from all cases. The result indicates that FCD and HME consist of immature cells. Moreover, we identified that these layer-marker immunopositive cells were neurons by a neuron marker, NeuN. There was an observable tendency for SATB2+cells to be distributed in the middle and deep regions of the neocortex of FCD Ia and Ib, and limited to the superficial region of FCD IIa and IIb. It is quite interesting that SATB2+ and FOXP1+ cells in HME were diffusely distributed and TBR1+ cells were localized in the middle and deep regions of the neocortex. To evaluate the expression patterns of those specific markers in FCD subtypes or HME, we divided them into three regions of the neocortex in terms of thickness.

Although there is little evidence regarding the mechanisms responsible for human FCD, it has been reported that FCD neurons originate from abnormal migration, maturation, and

Table 1 – Clinicopathological profile of FCD and HME patients.

Case	Sex	Age at surgery	Age at seizure onset	Seizure	Intelligence	FCD location on imaging	Pathological findings	
							Main pathology	Others
FCD								
1	M	2 Y	2 m	CPS+GTC	100 (IQ)	P	FCD Ia	Mild gliosis
2	F	3 Y	3 m	CPS	50 (DQ)	F	FCD Ia	HN, gliosis
3	M	6 Y	4 m	CPS	33 (DQ)	F	FCD Ia	Mild gliosis
4	M	7 Y	11 m	CPS	58 (IQ)	F	FCD Ia	Mild gliosis
5	M	6 M	1 m	CPS	40 (DQ)	P	FCD Ib	Mild gliosis
6	M	2 Y	20 d	CPS	18 (DQ)	F	FCD Ib	HN, gliosis
7	F	3 Y	3 m	CPS	15 (DQ)	P	FCD Ib	HN, gliosis
8	F	12 Y	11 m	CPS	43 (IQ)	F	FCD Ib	Mild gliosis
9	F	3 Y	6 m	CPS+GTC	40 (DQ)	F+P	FCD IIa	HN, gliosis
10	F	5 Y	2 y 9 m	CPS	81 (IQ)	F	FCD IIa	HN, gliosis
11	M	6 Y	3 d	CPS	16 (DQ)	T+P+O	FCD IIa	HN, gliosis
12	M	7 Y	7 m	CPS	50 (IQ)	P	FCD IIa	HN, gliosis
13	M	10 Y	1 y 11 m	CPS+GTC	22 (IQ)	F+T+P	FCD IIb	HN, gliosis
14	M	3 Y	2y 9 m	GTC	15 (DQ)	T+P+O	FCD IIb	HN, gliosis
15	M	8 Y	3 m	CPS+GTC	30 (IQ)	F+P	FCD IIb	HN, gliosis
16	F	19 Y	9 m	CPS+GTC	25 (IQ)	P	FCD IIb	HN, gliosis
HME								
1	F	3 M	2 d	CPS+GTC	30 (DQ)	rt-hemisphere	Polymicrogyria	HN, M, gliosis
2	F	3 M	7 d	CPS+GTC	35 (DQ)	rt-hemisphere	DN, BC	HN, M, gliosis
3	M	3 M	14 d	CPS+GTC	30 (DQ)	lt-hemisphere	Polymicrogyria	HN, M, gliosis
4	M	6 M	7 d	CPS+GTC	50 (DQ)	lt-hemisphere	DN	HN, M, gliosis
5	M	3 M	14 d	EIEE	50 (DQ)	rt-hemisphere	DN, BC	HN, M, gliosis
6	F	4 M	1 d	EIEE	30 (DQ)	rt-hemisphere	Polymicrogyria	HN, M, gliosis
7	M	7 M	3 d	EIEE	20 (DQ)	lt-hemisphere	Polymicrogyria	HN, M, gliosis

M: male, F: female, M (m): month (s), Y (y): year (s), d: days, CPS: complex partial seizure, GTC: generalized tonic–clonic convulsion, EIEE: early infantile epileptic encephalopathy, IQ: intelligence quotient, DQ: development quotient, F: frontal lobe, P: parietal lobe, T: temporal lobe, O: occipital lobe, rt: right side, lt: left side, DN: dysmorphic neuron, BC: balloon cell, HN: heterotopic neuron, M: mineralization.

cell death during ontogenesis (Spreafico et al., 1998a; 1998b; Andres et al., 2005). Our results may support this theory, indicating the persistence of immature neurons in the white matter. A recent report shows that markers of neuronal immaturity were overexpressed to excess in FCD (Hanai et al., 2010). Moreover, the previous studies have also reported that FCD neurons exhibited various degrees of neuronal maturation, glial cells or a combination of neuronal and glial characteristics (Crino and Eberwine, 1997; Yamanouchi et al., 1998; Aronica et al., 2003; Fauser et al., 2004; Ying et al., 1999). FCD and HME may retain certain characteristics indicative of immaturity.

SATB2 is a DNA-binding protein that regulates chromatin organization and gene expression. In the developing brain, SATB2 is expressed in cortical projection neurons. In a previous study, SATB2 has expressed predominantly in the upper layer, and not in the deep layer, of the cortex (Britanova et al., 2008). This expression pattern suggests that SATB2 may be involved in the control of early aspects of upper layer neuron specification. Interestingly, the SATB2 expression pattern can be clearly divided into three types in the present study. The first pattern is SATB2 expression in the middle and deep regions of the neocortex and some in the white matter. The second is SATB2 expression in the superficial region of the neocortex. The third pattern is diffused SATB2 expression. The first was identified in FCD Ia and Ib, the second in FCD IIa and IIb, and the third in HME.

FOXP1+ cells are supposed to be projection neurons. FOXP1 is a member of a conserved family of genes that shares a common DNA-binding domain, namely the T-box (Tamura et al., 2004). The T-box genes encode transcription factors involved in the regulation of developmental processes. A similar protein that is highly expressed in the 4th and 5th layers has been reportedly disrupted in mice and shown to be critical for early cortical developmental processes (Takahashi

Table 2 – Summary of immunohistochemistry of FCD and HME patients.

Case	Pathological classification	SATB2				FOXP1				TBR1			
		Superficial	Middle	Deep	WM	Superficial	Middle	Deep	WM	Superficial	Middle	Deep	WM
FCD													
1	Ia	+	++	++	+	+	++	−	−	+	++	++	++
2	Ia	−	++	++	−	+	+	−	−		++	++	++
3	Ia	−	++	++	+	−	++	++	−	−	++	++	++
4	Ia	−	++	++	+	−	++	++	−	−	++	++	−
5	Ib	−	++	++	+	+	++	+	+	−	+	+	+
6	Ib	−	++	++	+	−	++	++	+	−	+	+	+
7	Ib	++	++	++	++	−	++	++	++	−	++	++	++
8	Ib	++	++	+	−	+	++	−	+	−	++	++	−
9	IIa	+	−	−	−	++	+	−	−	−	+	−	−
10	IIa	++	−	−	−	+	+	+	−	−	+	−	−
11	IIa	+	+	−	+	+	++	−	−	−	+	−	−
12	IIa	++	−	−	−	++	+	+	−	−	+	−	−
13	IIb	++	++	−	−	++	++	+	−	+	+	++	+
14	IIb	+	−	−	−	++	+	+	−	+	+	++	+
15	IIb	+	−	−	−	++	+	−	−	+	+	++	+
16	IIb	++	−	−	−	++	−	−	−	+	+	++	+
HOME													
1	rt-hemisphere	++	+	+	+	+	+	+	+	+	++	+	−
2	rt-hemisphere	++	+	+	+	+	+	+	+	+	++	+	+
3	lt-hemisphere	++	+	+	+	+	−	+	+	−	++	+	−
4	lt-hemisphere	++	+	+	+	−	+	−	−	−	++	+	−
5	rt-hemisphere	++	+	+	+	+	+	+	+	−	++	+	−
6	rt-hemisphere	++	+	+	+	+	+	+	+	−++	+	+	
7	lt-hemisphere	++	+	+	+	+	+	+	−	+	++	+	−
Control													
23–29 GW		+	++	−	−	−	−	++	−	++	++	+	−
1 M–8 Y		−	−	−	−	−	−	−	−	−	−	−	−

−: No immunopositive cells, +: less than 5 cells in 1 mm^2, ++: more than 5 cells in 1 mm^2.

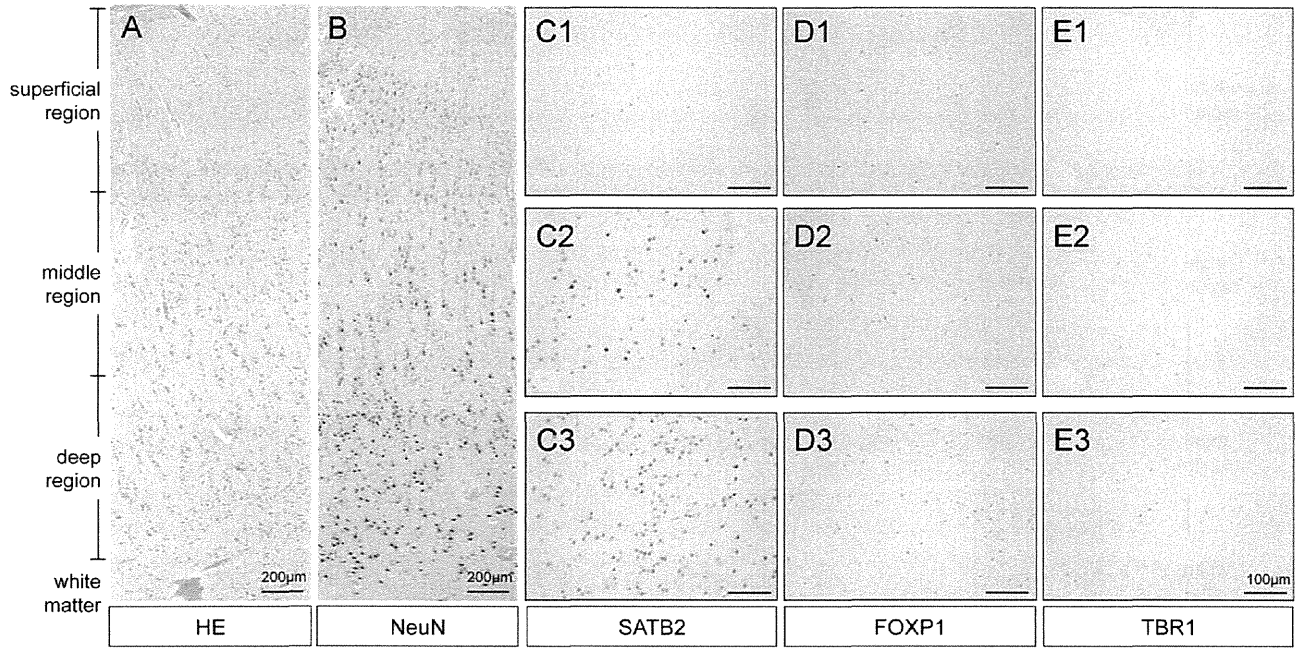

Fig. 1 – Histology and immunohistochemistry of FCD Ia. FCD Ia demonstrates abnormal radial lamination and abundant microcolumns of the neocortex (A, B). SATB2+ cells scattered in the superficial (C1), but at a high concentration in the middle (C2), and deep (C3) regions of the neocortex. FOXP1+ cells diffusely locate in the neocortex (D1–D3). TBR1+ cells diffusely locate in the neocortex (E1–E3) and those concentrations are low.
(A) Hematoxylin and eosin staining; (B) NeuN-immunostaining; (C) SATB2-immunostaining, superficial region (C1), middle region (C2) and deep region (C3); (D) FOXP1-immunostaining, superficial region (D1), middle region (D2), and deep region (D3); (E) TBR1-immunostaining, superficial region (E1), middle region (E2), and deep region (E3). Scales of A–B and C1–E3 indicate 200 μm and 100 μm, respectively.

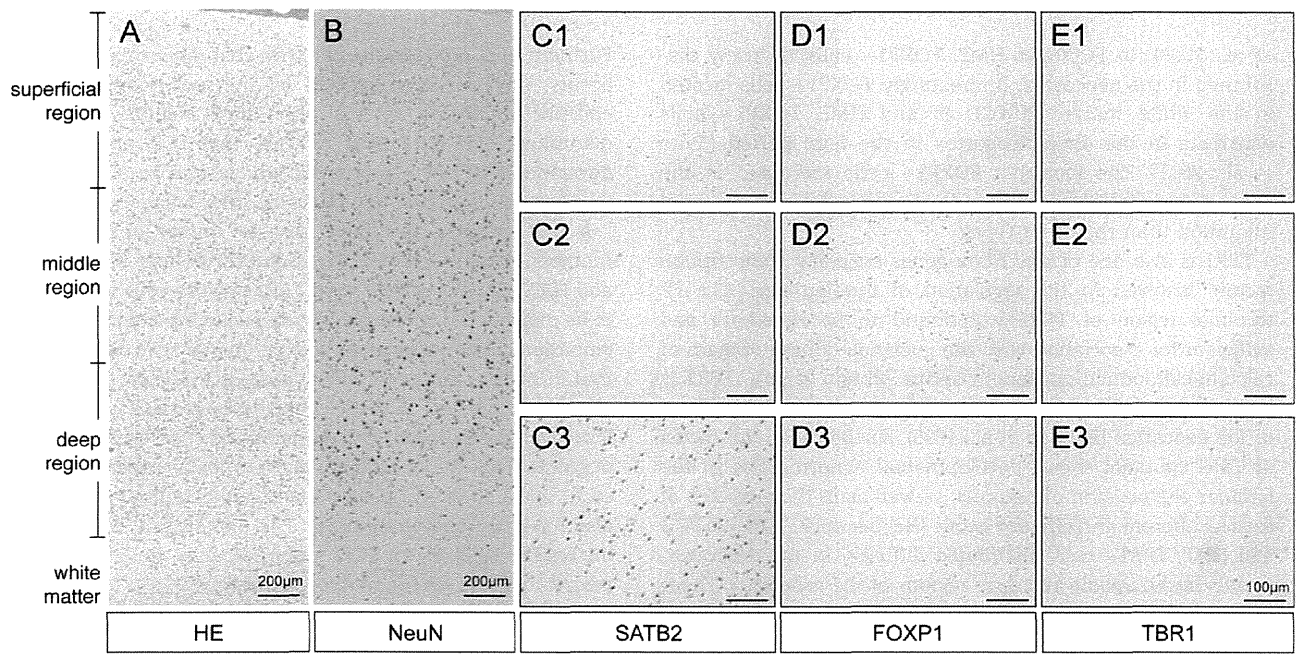

Fig. 2 – Histology and immunohistochemistry of FCD Ib. FCD Ib demonstrates abnormal tangential layer composition of the neocortex (A, B). SATB2+ cells scattered in the superficial (C1), but at a low concentration in the middle (C2) and a high concentration in the deep (C3) regions of the neocortex. FOXP1+ cells diffusely locate in the neocortex (D1–D3). TBR1+ cells diffusely locate in the neocortex, but those concentrations are low (E1–E3).
(A) Hematoxylin and eosin staining; (B) NeuN-immunostaining; (C) SATB2-immunostaining, superficial region (C1), middle region (C2), and deep region (C3); (D) FOXP1-immunostaining, superficial region (D1), middle region (D2), and deep region (D3); (E) TBR1-immunostaining, superficial region (E1), middle region (E2), and deep region (E3). Scales of A–B and C1–E3 indicate 200 μm and 100 μm, respectively.

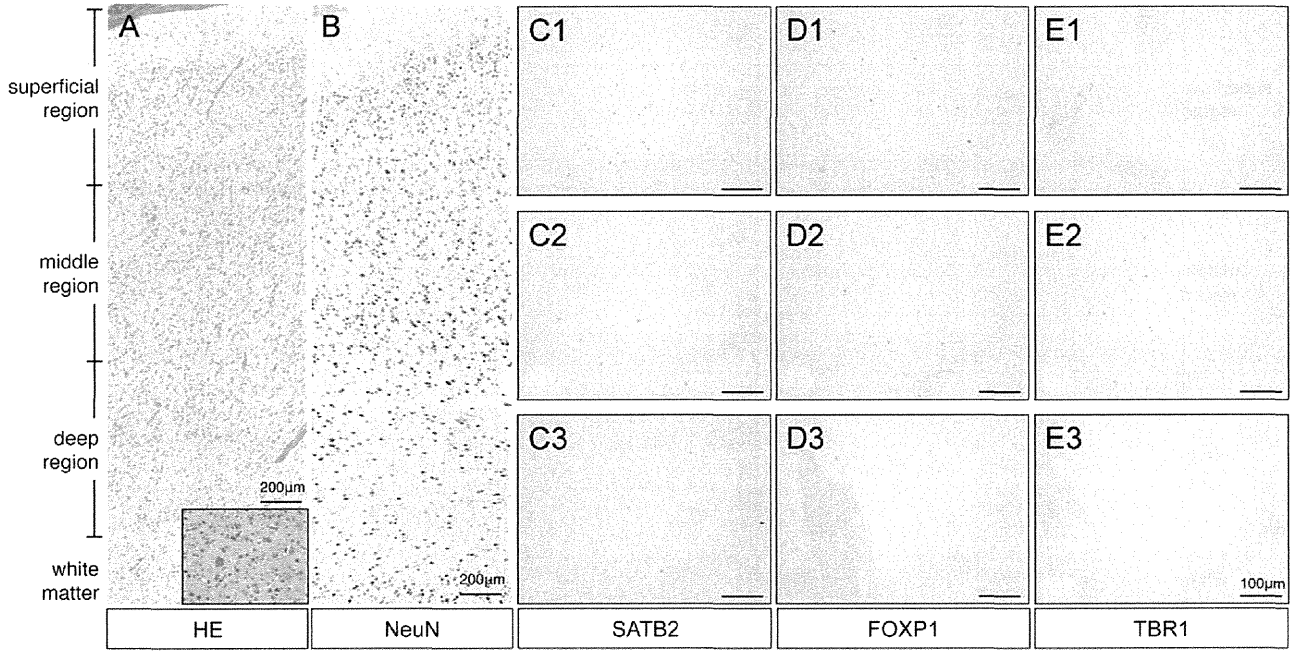

Fig. 3 – Histology and immunohistochemistry of FCD IIa. FCD IIa demonstrates unidentified layer-formation of the neocortex and a high neuronal concentration (A, B), and contains dysmorphic neurons (small window in A). SATB2+ cells scattered in the superficial (C1) and middle (C2) regions, but those concentrations are very low in the deep (C3) region of the neocortex. FOXP1+ cells diffusely locate in the neocortex (D1–D3), and evidence a relatively low concentration in the deep region (D3). TBR1+ cells diffusely locate in the neocortex, but those concentrations are low (E1–E3).
(A) Hematoxylin and eosin staining; (B) NeuN-immunostaining; (C) SATB2-immunostaining, superficial region (C1), middle region (C2), and deep region (C3); (D) FOXP1-immunostaining, superficial region (D1), middle region (D2), and deep region (D3); (E) TBR1-immunostaining, superficial region (E1), middle region (E2), and deep region (E3). Scales of A–B and C1–E3 indicate 200 μm and 100 μm, respectively.

et al., 2008). In FCD and HME, FOXP1+ cells diffusely distributed in the neocortex. Interestingly, FOXP1+ cells located in the white matter of FCD Ib and HME. FOXP1+ cells distribute in the deep neocortex in the fetal period (Saito et al., 2011). The fact that FOXP1+ cells remained in the postnatal white matter may indicate more delayed neuronal migration than the other types.

TBR1 is also one of the T-box genes encoding transcription factors involved in the regulation of development. The C-terminal region of TBR1 was found to be necessary and sufficient for association with the guanylate kinase domain of calcium/calmodulin-dependent serine protein kinase. TBR1 is highly expressed in early neurons of the preplate and deep layer of the neocortex (Bulfone et al., 1995). Furthermore, the cortex of TBR1 mutants shows developmental abnormalities in the laminar organization of neurons, as well as in the guidance of cortical afferent and efferent axons (Hevner et al., 2001). In FCD and HME, TBR1+ cells distributed diffusely in the neocortex, mainly in the middle and deep regions of the neocortex. These data may be supported by the previous study (Hadjivassiliou et al., 2010). Moreover, TBR1+ cells were observed in the superficial region of the neocortex of FCD IIb and in the white matter of FCD Ia and Ib.

SATB2, FOXP1, and TBR1 are normally expressed in immature neurons. Based on our results indicating the diffuse expression of all three markers throughout the neocortex of FCD and HME, it appears that immature cells are present in the cortex of both. Furthermore, our results confirm that abnormal migration occurs, and this is supported by the well-known fact that epileptic malformed brains often have neuronal migration disruption. Our data may indicate that FCD and HME are developmental brain disorders characterized by abnormalities in neuronal migration and crucially differentiation.

It is of considerable interest that the immature transcription markers of the neocortex are useful to identify FCD subtypes and HME. SATB1-, FOXP1-, and TBR1-positive cells are expected to be projection neurons, which use excitatory neurotransmitter glutamate. Electrophysiological experiments have demonstrated that immature and potentiated excited-GABAergic neurons are strongly related to FCD epileptogenesis (Cepeda et al., 2007). Moreover, dysmorphic neurons and balloon cells are thought to originate from the neocortical subventricular zone (Lamparello et al., 2007). From our data, glutaminergic neurons of FCD may also have neuronal immaturity. The pathological causes of the intractable seizures in FCD and HME may thus include the immaturity and dysfunction of neurons.

4. Experimental procedures

4.1. *Human tissue preparation*

Human neocortical tissues were obtained from 16 FCD and seven HME patients (Table 1) who underwent surgical treatment

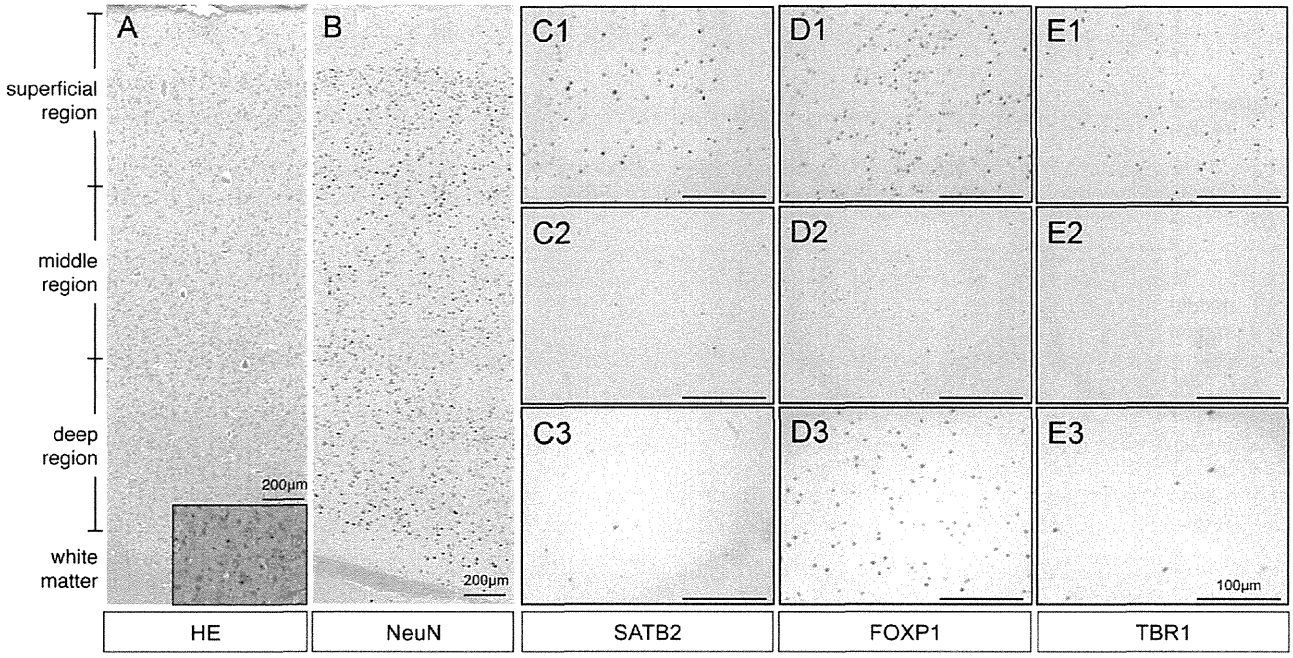

Fig. 4 – Histology and immunohistochemistry of FCD IIb. FCD IIb demonstrates unidentified layer-formation of the neocortex and high neuronal concentration (A, B), and contains balloon cells (small window in A). SATB2+ cells are at a high concentration in the superficial (C1), but scattered in the middle (C2) and deep (C3) region of the neocortex. The concentrations of FOXP1+ cells are high in the superficial (D1) and deep (D3) regions of the neocortex, and evidence a relatively low level in the middle region (D2). TBR1+ cells are at a relatively high concentration in the superficial (E1) and deep (E3) regions of the neocortex, but at a low concentration (E2) in the middle region. (A) Hematoxylin and eosin staining; (B) NeuN-immunostaining; (C) SATB2-immunostaining, superficial region (C1), middle region (C2), and deep region (C3); (D) FOXP1-immunostaining, superficial region (D1), middle region (D2), and deep region (D3); (E) TBR1-immunostaining, superficial region (E1), middle region (E2), and deep region (E3). Scales of A–B and C1–E3 indicate 200 μm and 100 μm, respectively.

of pharmacoresistant focal and/or general epilepsy after detailed examinations, including head magnetic resonance imaging (MRI), electroencephalogram (EEG), video monitoring EEG, single photon emission computed tomography (SPECT), positron emission tomography (PET), and magnetoencephalogram (MEG) at our hospital. The use of human tissue was approved by the Ethical Committee of our hospital and institute, and informed consent was obtained from the parents of all patients.

As control materials, we used six fetuses from 23 to 29 gestational weeks of age, and 10 children from 1 month to 8 years of age, in a previous study (Saito et al., 2011). All controls were autopsied. The average fixation time was within 14 days, and the time from death to starting fixation was within 12 h.

4.2. *Histological and immunohistochemistrical examination*

Fresh surgical tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and sliced at 6 μm thickness. Serial sections were evaluated histologically using hematoxylin and eosin and Klüver-Barrera stainings, as well as immunocytochemically using the antibodies against NeuN and three layer-markers. Three neuropathologists independently diagnosed with FCD, using the recent international classification (Blümcke et al., 2011) and HME, showing polymicrogyria, unlayered cortex and/or neuronal heterotopia.

Immunohistochemical examination was performed with the previously described methods. Briefly, after deparaffined, serial sections were autoclaved for antigen-retrieval, and endogenous peroxidase activity was blocked with 0.3% H_2O_2 and non-specific protein with 2% bovine serum albumin. They were then incubated with the four antibodies at 4 °C overnight. As the primary antibodies, we used NeuN, Neuronal nuclei (dilution of 1:100; Chemicon International Inc., Temecula, CA), SATB2, special AT-rich sequence-binding protein 2 (1:100; Bio Matrix Research, Japan), TBR1, T-box brain 1 (1:100; Abcam, UK), and FOXP1, Forkhead box P1 (1:100; Abcam). Previous studies demonstrate that SATB2, FOXP1, and TBR1 express only in the fetal neocortex, and distribute in the upper and middle layers, the deep layer and mainly the upper layer, respectively (Saito et al., 2011).

4.3. *Cell density analysis*

As FCD is histologically characterized by disorganization of cerebral neocortical layer-formation, it is difficult to determine separate layers in the neocortex. Therefore, we divided the neocortex thickness equally into three regions (superficial, middle, and deep) in order to evaluate the immunopositive cell distribution. This is because FCD and HME pathology usually

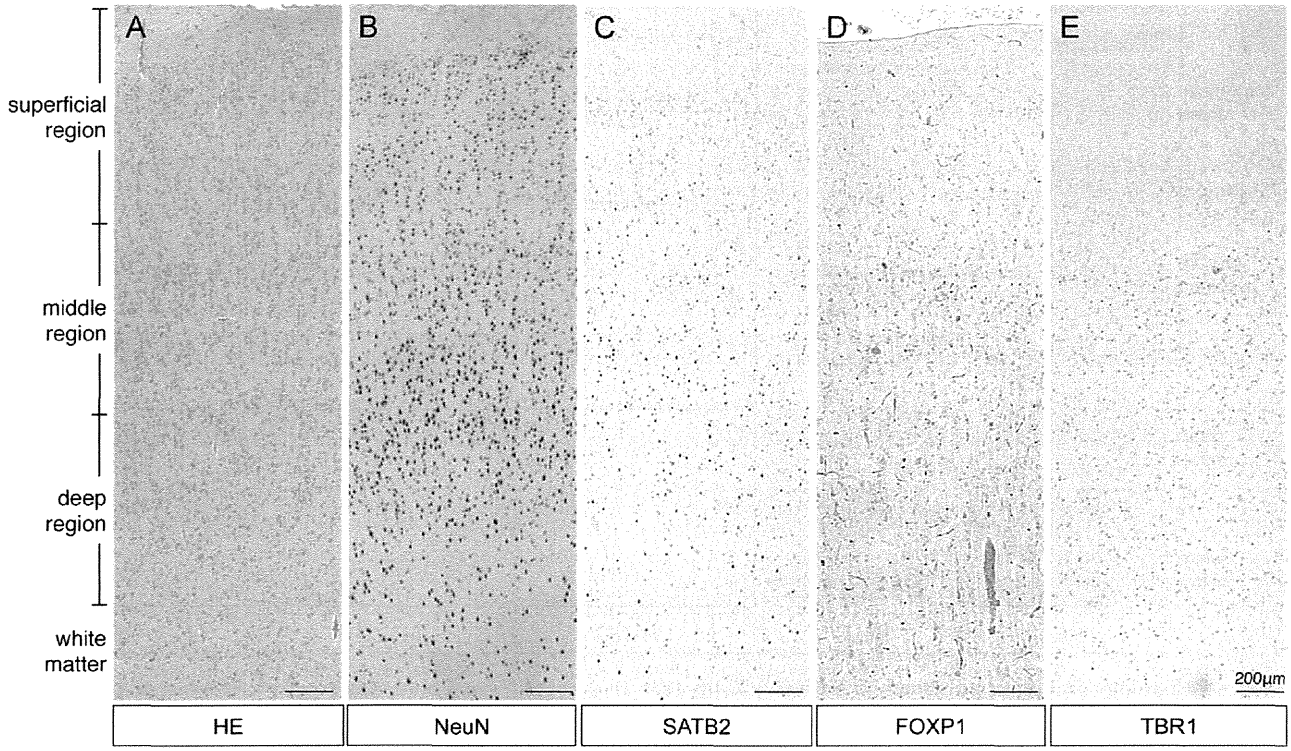

Fig. 5 – Histology and immunohistochemistry of HME. HME also demonstrates unidentified layer-formation of the neocortex, showing partially abundant microcolumns and abnormal tangential composition (A). NeuN+ cells are diffusely observed in the neocortex and white matter (B). SATB2+ cells show a high concentration in the superficial, middle and deep regions of the neocortex (C). FOXP1+ cells diffusely locate in the neocortex (D). TBR1+ cells diffusely locate in the middle and deep regions of the neocortex, but are absent in the upper region (E).
(A) Hematoxylin and eosin staining; (B) NeuN-immunostaining; (C) SATB2-immunostaining; (D) FOXP1-immunostaining; (E) TBR1-immunostaining. Scales indicate 200 μm.

show normal layer formation in the neocortex. The density of SATB2-, TBR1-, and FOXP1-positive (+) cells was determined in each brain region examined. The density of positively stained neurons was graded semiquantitatively as negative (–; no neurons stained), mild (+; <5 cells stained over an area of 1 mm^2 area), and marked (2+; >5 cells stained in a 1 mm^2 area) in each region. These layer-marker positive cells were confirmed as neurons with NeuN-immunostaining.

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The authors declare that they have no conflicts of interest. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with their guidelines.

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Imbalance of interneuron distribution between neocortex and basal ganglia: Consideration of epileptogenesis of focal cortical dysplasia

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ABSTRACT

Aim: The balance of excitation and inhibition of neurons and neuronal network is very important to perform complete neuronal function. Damage or loss of inhibitory γ-aminobutyric acid (GABA)-ergic interneuron is associated with impaired inhibitory control of cortical pyramidal neurons, leading to hyperexcitability and epileptogenesis. Ectopic neurons in the basal ganglia are to be one of the pathological features of epileptogenesis. In the present study, we investigated distribution of interneuron subtypes between neocortex and caudate nucleus.
Methods: We performed immunohistochemistry of GABA, glutamic acid decarboxylase (GAD), calretinin (CR), calbindin (CB), parvalbumin (PV) and neuropeptide. We used surgical materials of four focal cortical dysplasia (FCD) cases, having lesions of neocortex and caudate nucleus, and eight age-matched autopsy controls.
Results: The pathology showed three FCD IIa, containing dysmorphic neurons, and one FCD IIb, balloon cells. In the neocortex, the concentrations (each positive cell number/all cell numbers in the evaluated field) of GAD+, CR+ and CB+ cells were significantly lower in FCD than in controls. On the contrary, in the caudate nucleus those of CR+ and CB+ cells were significantly more in FCD than in controls.
Conclusion: The interneuron imbalance between the neocortex and basal ganglia may affect the epileptogenesis of FCD.

1. Introduction

To perform complete neocortical function, it is very important to have a balance between excitation and inhibition of neurons and neuronal network. Human neocortex mainly consists of projection neurons (excitatory) and interneurons (inhibitory), and glial cells. We know that approximately 35% of neocortical interneurons originate from the neocortical ventricular zone [1]. Also, 65% of them derive from the ganglionic eminence. Interneurons are characterized by the γ-aminobutyric acid (GABA) they contain and are divided by several specific markers, such as glutamic acid decarboxylase (GAD), calretinin (CR), calbindin (CB), parvalbumin (PV) and neuropeptide Y (NPY) [1].

It is thought that epilepsy results from molecular changes in glutamate and GABA receptors of aberrant neurons, causing a functional imbalance characterized by increased excitation and decreased inhibition [2,3]. Damage or loss of inhibitory GABAergic interneuron is associated with impaired inhibitory control of cortical pyramidal neurons, leading to hyperexcitability and epileptogenesis [3,4]. Malformations of cortical development (MCDs) are increasingly recognized as an underlying pathology in children with medically intractable epilepsy [5]. Focal cortical dysplasia (FCD), a distinct group of MCDs, is characterized mainly by disruption of the laminar architecture and/or the presence of specific abnormal cells [6,7]. The exact mechanism of epileptogenicity in FCD has not been elucidated so far. However, it may be based on the imbalance between the excitatory and inhibitory neuronal circuits, which seems to play an important role in the initiation and spread of epileptic seizures [8]. It is of considerable interest that the basal ganglia with dysmorphic neuron of FCD have been associated with epileptogenesis [9]. Recently, we reported that deep structure resection

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