および橋核も発生の途中で失われる．それぞれ生後2日目，5日目までは正常に発生しているようにみえるのであるが，その後の2〜3日間のうちにアポトーシスによって完全に失われるのである．下オリーブ核を構成する登上線維神経細胞および橋核に含まれる苔状線維神経細胞は，それぞれ小脳皮質のPurkinje細胞および顆粒細胞へと投射することが知られている．セレベレスにおいて下オリーブ核および橋核の神経細胞が消失する時期は神経投射の時期よりも後である．一般的に多くの種類の神経細胞ではその生存が神経投射に依存していることが知られているので，セレベレスの下オリーブ核と橋核の神経細胞は，投射先を失ったために二次的に失われているという可能性が考えられる．

セレベレスの原因遺伝子 *Ptf1a*

上述のように，セレベレスはトランスジェニックマウス作製の過程で得られたのであり，またその表現型は何代交配させてもつねにトランスジーンと連鎖していた．さらに，セレベレスのヘテロ接合体にはまったく異常は認められなかった．これらの状況証拠から考えて，セレベレスの表現型はトランスジェニックマウス作製の過程でトランスジーンがマウスゲノム上に挿入される際に，その周辺のゲノムDNAが傷つけられ，ある特定の遺伝子が破壊されたせいでもたらされたのであろうと推定された．

トランスジェニックマウス作製の過程で，トランスジーンのゲノム挿入部位の近傍の遺伝子が損傷されて突然変異体を生じることは少なからずある．実際に老化・カルシウム代謝異常モデルマウスとしてよく知られている“*Klotho*(クロトー)”変異体も，もともとは著者がかつて所属した研究室でトランスジェニックマウス作製の過程で偶然に得られたものである[3]．クロトーの場合には，トランスジーンの挿入部位の近傍でクロトー遺伝子の発現調節のためのゲノム領域が欠失しており，その結果としてこの遺伝子の発現が著しく低下していることが判明している．セレベレスの場合には第二染色体のゲノム上の1カ所にトランスジーンが約400コピーも挿入されており，またトランスジーン近傍の約31万3千塩基ものゲノム領域が失われていることがわかった．

著者らは，そのゲノム欠失領域の一端から外側へ6万塩基離れたところに，セレベレスの原因遺伝子として*Ptf1a*(*Pancreatic transcription factor 1a*，膵転写因子1a)を同定した．この遺伝子は神経系における機能はなにも知られてはいなかったが，もともと膵の発生に必要とされることが知られていた．*Ptf1a*遺伝子のノックアウトマウスでは膵がほとんど形成されず，出生後すぐに死んでしまう．しかし，セレベレス変異体では膵は正常に形成され，野生型と同じくらい(約2年)長生きする．実はセレベレスでは，*Ptf1a*遺伝子の発現が小脳原基ではほとんど失われているが，膵では保たれていることが判明した．このことから，セレベレス変異体では*Ptf1a*遺伝子の小脳原基での発現に関与するエンハンサー配列が失われている(おそらく31万3千塩基の欠失領域に含まれている)が，膵での発現に関与するエンハンサーは保存されているのではないかと推定される．そのおかげで膵は正常発生し，その個体は成体まで成長できるのであろう．しかし，小脳原基でのこの遺伝子の発現は失われるため，小脳の発生は異常となる．結果として小脳皮質を完全に欠失しながらも成体まで成長する，世界で唯一の突然変異マウスが得られたということになる．

小脳神経細胞の発生機構

発生途上の小脳原基には神経細胞を生み出す神経上皮領域が2種類ある．もっとも背側の菱脳唇とそれよりやや腹側にある脳室帯である．*Ptf1a*は小脳原基の脳室帯の神経上皮細胞で発現するが，そこから誕生する分裂を終えた神経細胞や菱脳唇の神経上皮細胞では発現しない．それではさまざまな種類の小脳神経細胞のうち，どの神経細胞が*Ptf1a*を発現する神経上皮細胞から生みだされるのであろうか．

この課題を解くために，著者らは*Ptf1a*遺伝子にCreリコンビナーゼをコードしたcDNAをノックインすることによってつくられたマウス系統(*Ptf1a*^{cre})を用いて遺伝的細胞系譜追跡実験を行った．このマウスを，あるレポーター系統

(*Rosa26R-loxP-lacZ*：以下，R26R と略)と交配することによって，*Ptf1a* を発現する神経上皮細胞から生みだされた細胞が将来どのような種類の神経細胞になるのかを *lacZ* 遺伝子の発現によって調べることができる．この細胞系譜追跡実験を $Ptf1a^{Cre}$ ノックインマウスのヘテロ接合体バックグラウンド($Ptf1a^{cre/+}$；R26R：これは正常に発生する)で行うことによって，すべての種類の小脳の抑制性 GABA 作動性神経細胞(Purkinje 細胞，Golgi 細胞，バスケット細胞，星状細胞，そして GABA 作動性小脳核神経細胞)が *Ptf1a* を発現する小脳脳室帯の神経上皮由来であり，逆に興奮性のグルタミン作動性小脳核神経細胞や顆粒細胞はその細胞系譜にはないことが明らかになった(図 3)．さらに，セレベレス(あるいは *Ptf1a* ノックアウトマウス)において小脳脳室帯神経上皮における *Ptf1a* の発現が失われると，すべての GABA 作動性ニューロンが生みだされなくなることから，「*Ptf1a* が小脳脳室帯の神経上皮で発現し，GABA 作動性ニューロンの誕生に必須な働きをしていること」が明らかになった[1]．

著者らの上記の報告の後に別のグループから，菱脳唇神経上皮から生みだされる神経細胞についての報告がなされた[4,5]．彼らは菱脳唇で bHLH 型転写因子 Atoh1(Math1 ともよばれる)が発現することを利用して遺伝学的細胞系譜追跡実験を行い，従来報告されていた顆粒細胞だけではなく，(おそらくは興奮性の)小脳核神経細胞が菱脳唇の神経上皮由来であることを示した(図 3)．彼らはその小脳核神経細胞の発生が *Atoh1* ミュータントマウスでは異常になることも示している．

すると，小脳全体において「グルタミン酸作動性の興奮性神経細胞はすべて菱脳唇由来であり，GABA 作動性の抑制性神経細胞はすべて脳室帯由来である」というように単純化して考えることができる．実際に，小脳皮質の神経細胞としては比較的マイナーであまり研究がなされてこなかったグルタミン酸作動性の unipolar brush cells (UBCs)も菱脳唇由来であることがその後報告された[6]．

以上から，小脳の各種神経細胞の発生について実にシンプルなモデルが描ける．すなわち，「小脳の連続した神経上皮は背側の領域が Atoh1 を発現することによって菱脳唇としての性質を与えられ，そこからグルタミン酸作動性神経細胞が生みだされる一方，より腹側に位置する神経上皮が Ptf1a を発現することによって脳室帯としての性質をもち，そこから GABA 作動性神経細胞が生みだされる」という「bHLH 型転写因子による神経上皮の領域化」モデルである[7](図 3)．

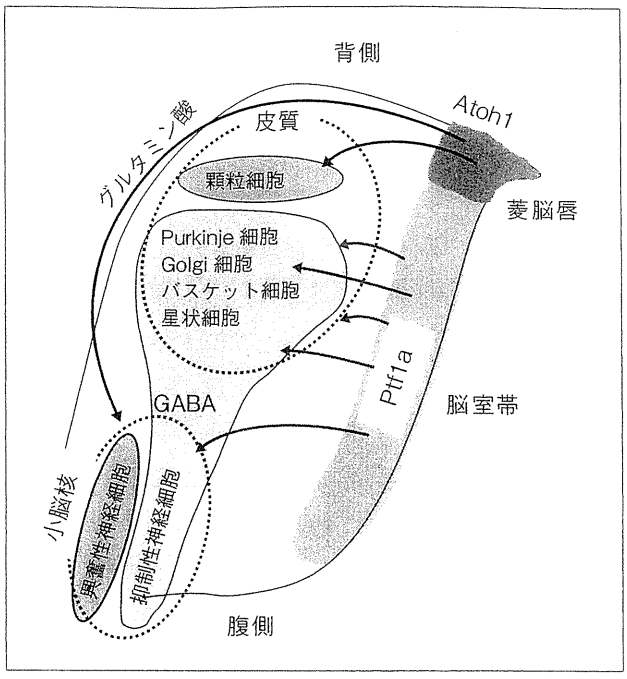

図 3　小脳原基神経上皮からの神経細胞の発生
小脳原基の連続した神経上皮は背側の領域(菱脳唇)で Atoh1 を，やや腹側の脳室帯で Ptf1a を発現する．それぞれの領域からグルタミン酸作動性および GABA 作動性神経細胞が生みだされる．セレベレス変異体においては脳室帯での Ptf1a の発現が失われるために，すべての種類の GABA 作動性抑制性神経細胞が生みだされない．

Ptf1a 遺伝子の変異とヒト疾患

このようにしてセレベレスの解析から，小脳発生における *Ptf1a* 遺伝子の果たす役割について明らかになってきたが，この遺伝子とヒト疾患との関連も報告されている[8,9]．*PTF1A* 遺伝子に突然変異が入り機能的な PTF1A 蛋白質が産生されない症例では，小脳が失われ重度な運動失調を呈し，また膵が形成されないことによって重度な糖尿病が生じる(図 4-A, B)．残念ながらこれらの症例ではおそらくは重症糖尿病のせいであろうが，生後 1 年以内に死亡するケースが多い．また，こ

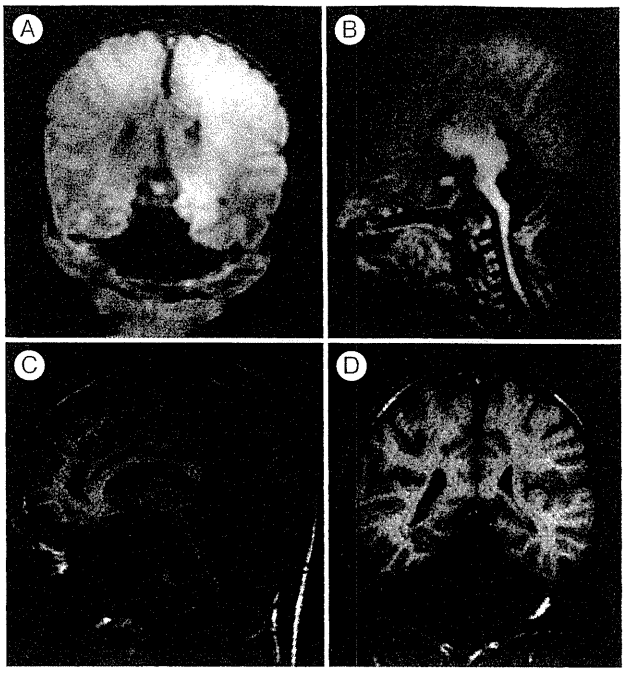

図 4　小脳無形成症の症例

A, B：*Ptf1a* 遺伝子の異常によって引き起こされる，重症糖尿病を伴う小脳無形成症[8]．生後 3 日目の MRI 像．小脳はほとんど認められない．この論文が出た時点では原因遺伝子は不明であったが，その後同定された[9]．

C, D：イタリアの小脳無形成の一症例の MRI 像[12]．17 歳の少年．中等度の協調運動障害と軽い精神遅滞はあるが，普通の学校に自転車で通うなど，かなり症状は軽い．

れらの症例は小脳および膵をともに欠失するという点において，*Ptf1a* 遺伝子のノックアウトマウスの表現型に近い．もしもヒトにおいて *PTF1A* 遺伝子の小脳における発現制御領域のみに異常をきたした場合には，セレベレスのように小脳を失いつつも膵機能は正常でその後も生存しつづけるであろうと考えられるが，そのようなヒト症例はいままで報告されてはいない．この遺伝子の異常によるヒトの症例についてはすこしずつ報告が蓄積してきてはいるものの[10,11]，大規模な研究はまだこれからであり，今後のさらなる研究が待たれているところである．

上述したように，セレベレスのホモ接合体では小脳皮質を完全に欠失するにもかかわらず，成体まで成長し，正常マウスと同等の寿命まで生きる．雄は運動失調症状のためうまく交配ができないためか不妊であるが，雌には妊性がある．これは，餌と水が十分に与えられる環境にあれば，すくなくともこの突然変異マウスにおいては小脳が生存のためには必須でないということを示している．しかし，一般的に小脳が生存のために必須な働きをしていないかどうかについて結論を出すのは早計である．セレベレスでは発生段階から小脳形成の異常があるために，脳の他の領域が小脳の機能を代償するように適応した発生が行われている可能性があるからである．

一方，ヒトにおいては一般的に小脳無形成・低形成症は重篤であり，予後の悪いものが多い．しかし，MRI において小脳の完全欠損が認められるにもかかわらず，日常生活にはさほど支障がない（自転車に乗って普通の学校に通っているほどである）比較的症状の軽い症例も報告されている（図 4-C, D）[12]．この症例では，胚発生期の早い段階で脳の可塑性によって機能の代償が起こっているのではないかと考えられる．しかし，セレベレスにしても，このヒト症例にしても，脳のどの領域が小脳機能を代償しているのかについてはまったくわかっていない．それゆえに，セレベレスのようなモデルマウスやあるいはヒト症例を調べることによって，小脳の機能とその代償システムについてのあらたな知見が得られるかもしれない．

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Review Article

Neuronal subtype specification in the cerebellum and dorsal hindbrain

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In the nervous system, there are hundreds to thousands of neuronal cell types that have morphologically, physiologically, and histochemically different characteristics and this diversity may enable us to elicit higher brain function. A better understanding of the molecular machinery by which neuron subtype specification occurs is thus one of the most important issues in brain science. The dorsal hindbrain, including the cerebellum, is a good model system to study this issue because a variety of types of neurons are produced from this region. Recently developed genetic lineage-tracing methods in addition to gene-transfer technologies have clarified a fate map of neurons produced from the dorsal hindbrain and accelerated our understanding of the molecular machinery of neuronal subtype specification in the nervous system.

Key words: cerebellum, cochlear nucelus, hindbrain, precerebellar system, subtype specification.

Introduction

The dorsal hindbrain, or alar plate of the hindbrain, represents a good model system to investigate the molecular machinery to specify neuronal subtypes, as many different types of neurons are generated from this region. In mammals and avians, the hindbrain consists of eight rhombomeres (r1–r8). Previous grafting studies as well as detailed precision anatomical studies have suggested that the alar plate of r1 produces all cerebellar neurons, the alar plate of the middle hindbrain (r2–r5) generates neurons in the cochlear nucleus, and the alar plate of the caudal hindbrain (r6–r8) produces precerebellar neurons, although some slight differences exist between rodents and birds (Altman & Bayer 1987; Tan & Le Douarin 1991; Cambronero & Puelles 2000; Cramer *et al.* 2000; Farago *et al.* 2006). Furthermore, recently developed genetic lineage-tracing methods in addition to gene-transfer technologies have clarified a fate map of neurons produced from the dorsal hindbrain and accelerated our understanding of the molecular machinery of neuronal subtype specification in the dorsal hindbrain.

Neuron subtype 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) comprise three major types of neurons: large glutamatergic projection neurons (CN-Glu neurons), mid-sized 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) (Carletti & Rossi 2008), 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 (Millet *et al.* 1996; Wingate & Hatten 1999; Chizhikov & Millen 2003; Zervas *et al.* 2004). The dorsal-most part of the neuroepithelium, the roof plate, of r1 does not generate neurons but produces

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cells of the choroid plexus (Chizhikov *et al.* 2006). 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.

Although studies on the cerebellum have a very long history (Cajal 1909), the molecular machinery through which cerebellar neuron subtype specification occurs is still unclear. In 1997, Ben-Arie *et al.* reported that a basic-helix-loop-helix type (bHLH) transcription factor, Atoh1 (also called Math1), is expressed in the rhombic lip and is involved in cerebellar granule cell generation (Ben-Arie *et al.* 1997). At that time, it was believed that only granule cells are generated from the rhombic lip, while the remaining neurons are produced from the cerebellar VZ. Although several studies had clarified the specification machinery for granule cells, how other types of cerebellar neurons developed remained elusive until three breakthrough papers were published in 2005.

During the generation of some transgenic mice, our group obtained a mutant line ''*cerebelless*'' in which homozygotes exhibited uncoordinated locomotion, ataxic gait and tremors. Surprisingly, adult mice were found to lack the entire cerebellar cortex. In this mutant, no GABAergic inhibitory neurons are produced from the cerebellar primordium. In contrast, glutamatergic populations are initially generated, but glutamatergic granule cells are lost secondarily at postnatal stages. Eventually, the entire cerebellar cortex is lost in this mutant (Hoshino *et al.* 2005). The responsible gene was identified as *pancreatic transcription factor 1a* (*Ptf1a*), a bHLH transcription factor known to participate in pancreatic development. This gene is expressed in the neuroepithelium of the VZ but not in the RL and its expression is lost in the *cerebelless* mutants. Cre-loxP recombination-based lineage tracing analysis revealed that most types of cerebellar GABAergic neurons (Purkinje, Golgi, basket, stellate cells and GABAergic neurons in the CN) are derived from Ptf1a-expressing neuroepithelial cells in the VZ, but glutamatergic neurons, such as granule cells and CN-Glu neurons, are not. Loss of *Ptf1a* expression in *cerebelless* as well as in *Ptf1a*-knock out mice resulted in inhibition of the production of GABAergic neurons in the cerebellar primordium. Furthermore, ectopic introduction of *Ptf1a* by means of *in utero* electroporation resulted in the abnormal production of neurons with GABAergic characteristics from the dorsal telencephalon: the dorsal telencephalon only produces glutamatergic neurons under normal conditions. In addition, Pascual *et al.* (2007) reported that in the *Ptf1a*-null mutants, the fate of neurons produced from the VZ is changed to that of granule cells. Moreover, a recent genetic fate mapping study using *Ascl1*^{CreER} knock-in mice showed that other cerebellar GABAergic neurons, such as Lugaro and candelabrum cells, are also derived from the cerebellar VZ (Sudarov *et al.* 2011). 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 (Sellick *et al.* 2004).

Just after our report, Zoghbi's and Fishell's groups reported a molecular fate map of the derivatives of *Atoh1*-expressing neuroepithelial cells in the cerebellar RL (Machold & Fishell 2005; Wang *et al.* 2005). They showed that not only granule cells but also, at least some DCN 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 (Hoshino *et al.* 2005), 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 (Englund *et al.* 2006).

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 producing the corresponding neuronal subtype in the cerebellum. These facts suggest a model in which regionalization of the cerebellar neuroepithelium into two distinct regions, the VZ and the RL, is mediated by the two bHLH transcription factors, Atoh1 and Ptf1a (Hoshino 2006). During embryonic development, the ventral cerebellar neuroepithelium expresses Ptf1a, leading to cerebellar VZ characteristics and the ability to generate GABAergic neurons, while the dorsal cerebellar neuroepithelium expresses Atoh1 and becomes the cerebellar RL, producing glutamatergic neurons. In the telencephalon, similar regionalization by bHLH transcription factors takes place. Glutamatergic neurons emerge from dorsal neuroepithelium expressing Neurogenin 1/2 (Ngn 1/2) and GABAergic neurons are produced from ventral neuroepithelium expressing Ascl1 (also called Mash1) (Wilson & Rubenstein 2000).

How are these neuroepithelial areas formed? In general, the roof plate can affect the dorsal structure of

the neural tube (Lee *et al.* 2000; Millonig *et al.* 2000). Chizhikov *et al.* (2006) revealed that the roof plate plays an important role in the formation of the cerebellar dorso-ventral domain formation by analyzing cerebellar mutants that lack the roof plate. Moreover, it has been suggested that bone morphogenetic proteins (BMPs) secreted from the roof plate as well as Notch signaling are involved in the formation of the RL and the VZ (Machold *et al.* 2007). An *in vitro* study that induced Purkinje cells from ES cells suggested that loss of sonic hedgehog signaling may provide dorso-ventral spatial information of the cerebellar VZ to the cerebellar neuroepithelium, which eventually leads to the expression of Ptf1a (Muguruma *et al.* 2010).

Although some clarification of the machinery governing GABAergic and glutamatergic neuronal subtype specification by transcription factors has been provided, molecular mechanisms to specify each GABAergic (e.g. Purkinje, Golgi, basket, stellate cells and CN-ION, CN-interneurons) or glutamatergic (e.g. granule, unipolar brush cells and CN-Glu neurons) subtype remain unclear. Birthdating studies using ^{3}H-thymidine and BrdU (Chan-Palay *et al.* 1977; Batini *et al.* 1992; De Zeeuw & Berrebi 1995; Sultan *et al.* 2003; Leto *et al.* 2006) as well as adenovirus (Hashimoto & Mikoshiba 2003) have revealed that each type of neuron is generated at distinct developmental stages.

With regard to GABAergic neurons, Purkinje cells are produced early (embryonic day (E) 10.5–13.5 in mice), Golgi cells a little later (E13.5–postonatal day (P) 0) and stellate/basket cells mainly perinatally. 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). As to glutamatergic neurons, in addition to the above studies, molecule-based lineage tracing analyses (Machold & Fishell 2005; Wang *et al.* 2005; Englund *et al.* 2006) have clarified that CN-Glu neurons leave the cerebellar RL at early stages (E10.5–12.5) and granule cells and unipolar brush cells at middle to late stages (granule cell; approximately E12.5~perinatal, ubc: approximately E12.5-E18.5). 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 (Mathis *et al.* 1997; Mathis & Nicolas 2003). These data indicate that some temporal information in the neuroepithelium may be involved in specification of neuronal types in the RL and VZ, respectively. 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 (Fig. 1). Chizhikov *et al.* (2006) defined four cellular populations (denoted c1–c4 domains) in the cerebellar primordium via the expression of a few transcription factors. c1 corresponds to the Atoh1-expressing RL and c2 is located just above the Ptf1a-expressing VZ (denoted 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 was removed (Chizhikov *et al.* 2006). Furthermore, at the early neurogenesis

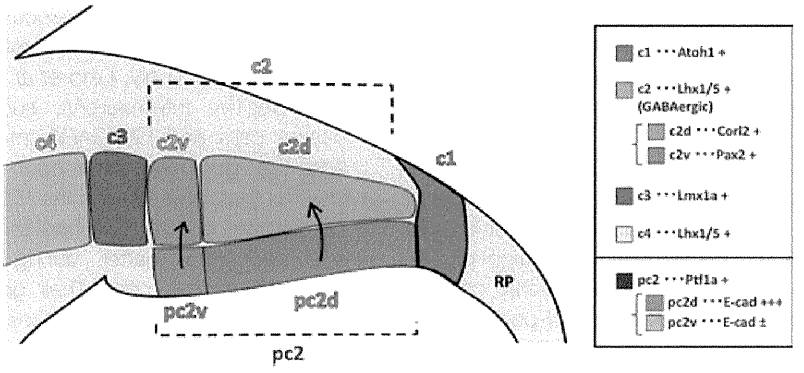

Fig. 1. Domain structure of the cerebellar primordium. The c1 domain, expressing Atoh1, corresponds to the rhombic lip that produces all types of glutamatergic neurons in the cerebellum. The pc2 is the Ptf1a-expressing neuroepithelial domain that generates all types of GABAergic cerebellar neurons. At 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 Puarkinje 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 which make up these domains are unknown. The roof plate (RP) is located most dorsally, and plays prominent roles in organizing this cerebellar domain structure.

stage (e.g. E12.5 in mice), Minaki *et al.* (2008) subdivided the c2 domain into dorsally (c2d) and ventrally (c2v) located subdomains that express corl2 and Pax2, respectively. While corl2 is exclusively expressed in immature and mature Purkinje cells (Minaki *et al.* 2008), Pax2 is expressed in GABAergic interneurons (e.g. Golgi, stellate, basket, CN-GABA neurons) in the cerebellum (Maricich & Herrup 1999; Weisheit *et al.* 2006). 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 (Mizuhara *et al.* 2010). 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 (Maricich & Herrup 1999; Hashimoto & Mikoshiba 2003). The expression of several other transcription factors in the cerebellar VZ during development have also been reported. For example, Zordan *et al.* (2008) described the expression patterns of proneural bHLH transcription factors, such as Ngn1, Ngn2 and Ascl1 in the cerebellar VZ although their function in cerebellar development is still unclear. It has also been reported that Pax2-positive neurons, but not Purkinje cells, are reduced in the *Ascl1*-null cerebellum (Grimaldi *et al.* 2009), while Purkinje cells are reduced in *Ngn1*-null mice (Lundell *et al.* 2009), 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 a 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 post-mitotically involved in Purkinje cell specification (Zhao *et al.* 2007). 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 (Huard *et al.* 1999; Leto *et al.* 2011).

From the RL, several types of glutamatergic neurons, such as CN-Glu neurons, granule cells and unipolar brush cells, are generated (Machold & Fishell 2005; Wang *et al.* 2005; Englund *et al.* 2006). CN-Glu neurons leave the RL early during neurogenesis. Some transcription factors, such as Tbr1, Irx3, Meis2, Lhx2 and Lhx9 are expressed in post-mitotic progenitors of CN-Glu neurons, but their roles have not been clarified (Morales & Hatten 2006). Other molecules, such as Zic1 (Aruga *et al.* 1998), have been reported to play important roles in the migration, maturation and survival of granule cells, but the molecular machinery underlying the specification of granule cell identity is unknown. Although unipolar brush cells strongly express Tbr2, its function remains elusive.

Heterotopic and heterochronic transplantation studies have also provided important clues to understanding cerebellar development (Carletti & Rossi 2008). 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 (Jankovski *et al.* 1996). 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 (Carletti *et al.* 2002). 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.* (2006) suggested that Pax2-positive interneurons, such as Golgi, stellate, basket cells and CN-GABA interneurons are derived from the same progenitor pool. Leto *et al.* (2009) 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. 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.

Neuron subtype 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.

Because of its importance in sound perception, the CoN has been intensely studied from anatomical, physiological and histochemical points of view (Osen 1969; Ryugo & Willard 1985; Hackney *et al.* 1990). Histological observations have deduced that a portion of neurons generated from the dorsal hindbrain neuroepithelia migrate tangentially to give rise to CoN neurons (Pierce 1967; Ivanova & Yuasa 1998). 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 (Farago *et al.* 2006; Nichols & Bruce 2006). As to the rostro-caudal axis, the origins of CoN neurons seem to differ between avians and mammals. Grafting studies revealed that avian CoN neurons are derived from a broader part of the hindbrain (r3–r8), (Tan & Le Douarin 1991; Cambronero & Puelles 2000; Cramer *et al.* 2000), while mouse genetic studies have suggested a more rostral and narrower origin (r2–r5) (Farago *et al.* 2006).

Very sophisticated genetic fate mapping studies were carried out by Farago *et al.* (2006) using an FLP-FRT and Cre-loxP-based dual lineage tracing system. In addition to showing that CoN neurons are derived from r2 to 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 (Osen 1969; Ryugo & Willard 1985; Hackney *et al.* 1990). For example, the DCoN includes Golgi, molecular layer (ML)-stellate, cartwheel, tuberculo-ventral, unipolar brush, giant, and fusiform cells, while the VCoN is comprised of octopus, globular-bushy, spherical-bushy, T-stellate and D-stellate cells.

In the neuroepithelium of the middle hindbrain (r2–r5), distinct transcription factors are expressed constituting several molecularly-defined domains (Fig. 2, upper panel). Using Cre-LoxP-based genetic fate mapping studies, our group identified the origins of inhibitory and excitatory neurons of the cochlear nucleus; inhibitory (glycinergic and GABAergic) and excitatory (glutamatergic) neurons are derived from Ptf1a- and Atoh1-expressing neuroepithelial regions, respectively (Fujiyama *et al.* 2009) and their development is dependent on the corresponding bHLH proteins.

Subtype specification of precerebellar neurons

There are two types of precerebellar afferent systems; mossy fiber (MF) and climbing fiber (CF) systems. MF neurons are located in several nuclei throughout the brain stem and extend their glutamatergic projections to granule cells conveying peripheral and cortical information to the cerebellum. Four major nuclei containing MF neurons are the pontine gray nucleus (PGN), the reticulotegmental nucleus (RTN), the lateral reticular nucleus (LRN) and the external cuneate nucleus (ECN) in the hindbrain (Altman & Bayer 1987). Some MF neurons are also located in the spinal trigeminal nucleus (Sp5) in the hindbrain and Clarke's column in the spinal cord. In contrast, CF neurons reside exclusively in the inferior olive nucleus (ION), which receives input from the cerebral cortex, the red nucleus, spinal cord and other brain stem nuclei, and sends glutamatergic projections to Purkinje cells (Ruigrok *et al.* 1995). Both types of precerebellar neurons also send branch axons to the neurons in the cerebellar nucleus. These precerebellar systems are thought to transmit both external and internal information to the cerebellar cortex to modulate cerebellar function, including regulation of movement.

Previous birthdating studies in mice revealed that CF neurons are generated at relatively early neurogenesis stages (E9.5–11.5) while MF neurons are produced at slightly later stages (E10.5–16.5) (Pierce 1973). Along the rostrocaudal axis, both MF and CF neurons in the hindbrain are generated from the caudal hindbrain, around rhombomeres 6–8, as suggested by avian grafting studies as well as mammalian fate map analyses (Ambrosiani *et al.* 1996; Cambronero & Puelles 2000; Farago *et al.* 2006; Kawauchi *et al.* 2006). By contrast, MF neurons in the Clarke's nucleus are generated in the spinal cord (Bermingham *et al.* 2001). Classic anatomical and immunohistochemical studies have suggested that these precerebellar nuclei neurons in the hindbrain emerge from the dorsal part of the hindbrain and migrate tangentially or circumferentially to their final loci (Bloch-Gallego *et al.* 1999; Yee *et al.* 1999; Kyriakopoulou *et al.* 2002). However, they take slightly different paths from each other; MF and CF neurons move extramurally and intramurally, respectively. Introduction of a GFP-expressing vector into the embryonic dorsal hindbrain allowed the dramatic visualization of migrating precerebellar nuclei neurons during development (Kawauchi *et al.* 2006; Okada *et al.* 2007).

Many groups have reported transcription factors that are expressed within the dorsal neuroepithelium of the caudal (r6–8) hindbrain during embryonic development,

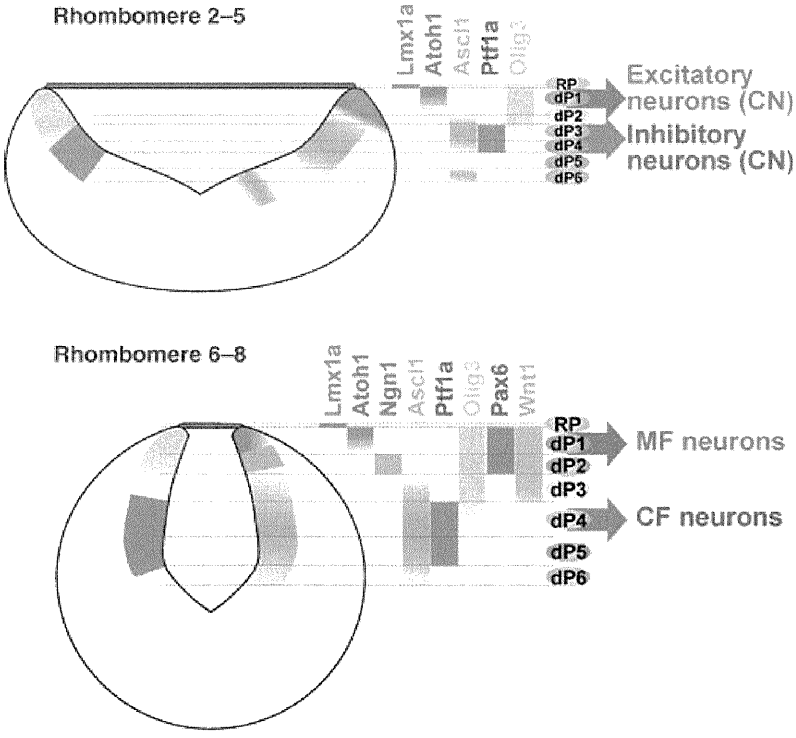

Fig. 2. In the middle (r2–5, upper panel) and caudal (r6–8, lower panel) hindbrain, several transcription factors are expressed within the dorsal neuroepithelium during embryonic development. The dorsal-most part, the roof plate (RP), expresses Lmx1a. Other than the roof plate, the dorsal neuroepithelium can be divided into six domains (dP1–dP6) according to the expression pattern of indicated transcription factors. (upper panel) As to the neurons in the cochlear nucleus, glutamatergic excitatory neurons and GABAergic/glycinergic inhibitory neurons are derived from dP1 and dP3/dP4, respectively. (lower panel) While mossy fiber (MF) neurons are derived from the dP1 domain expressing Atoh1, climbing fiber (CF) neurons are generated from the dP4 domain expressing Ptf1a and Olig3. Ngn1 is expressed in r7 and r8 but not in r6.

trying to define domains along the dorsoventral axis. The dorsal-most part expressing Lmx1a corresponds to the roof plate, which gives rise to the choroid plexus (Chizhikov *et al.* 2006). Other than the roof plate, the dorsal neuroepithelium can be divided into six domains (dP1–dP6) according to the expression pattern of the transcription factors, such as Atoh1, Ngn1, Ascl1, Ptf1a and Olig3 (Fig. 2, lower panel). As for the precerebellar nuclei neurons, a series of studies have tried to clarify the precise origins of MF and CF neurons by genetic lineage tracing methods.

By analyzing genetically engineered mice that express *lacZ* or *Cre recombinase* under the control of the endogeous or exogenous *Atoh1* promoter, MF neurons of PGN, RTN, LRN and ECN were shown to emerge from the Atoh1-expressing neuroepithelial domain (dP1, Ben-Arie *et al.* 2000; Rodriguez & Dymecki 2000; Landsberg *et al.* 2005; Wang *et al.* 2005). Targeted disruption of the Atoh1 gene resulted in loss of these MF neurons, suggesting an involvement of Atoh1 in the MF neuron development.

Atoh1 regulates the expression of the transcription factor Barhl1 (Mbh2) that is expressed in MF neurons. Loss of Barhl1 expression resulted in a decrease of MF neurons, leading to a decrease in the size of MF precerebellar nuclei (Li *et al.* 2004). In addition, Flora *et al.* (2007) reported that Tcf4, an E-protein, interacts with Atoh1 and regulates differentiation of a specific subset (PGN, RTN) of MF neurons.

Landsberg *et al.* also performed lineage trace analysis by using two variants of FLP (Flippase recombinase) with different recombinase activities that were expressed under the control of the *Wnt-1* promoter whose strength is the highest at the dorsal-most part and gradually decreases ventrally. They demonstrated that CF neurons are derived from the neuroepithelial region where *Wnt-1* is very weakly expressed, whereas MF neurons emerge from the strong Wnt1-expressing region (Landsberg *et al.* 2005). In addition, Nichols & Bruce (2006) generated transgenic mice carrying a *Wnt-1*-enhancer/lacZ transgene and observed that MF neurons but not CF neurons were labeled by β-gal in

those mice. These findings suggested that CF neurons are generated from the neuroepithelial region ventral to the Atoh1-expressing domain.

By Cre-loxP-based lineage trace analysis, our group showed that all CF neurons in the ION are derived from the Ptf1a-expressing neuroepithelial region (Yamada *et al.* 2007). Loss of the *Ptf1a* gene resulted in the fate change of some CF neurons to MF neurons, suggesting that Ptf1a plays a critical role in fate determination of CF neurons. We also showed an involvement of Ptf1a in migration, differentiation and survival of CF neurons. It has been reported that both MF neurons and CF neurons are derived from the Olig3-expressing neuroepithelial region that broadly expands within the dorsal hindbrain (Storm *et al.* 2009) by Cre-loxP-based linage tracing. Targeted disruption of the *Olig3* gene caused the disorganized development of MF neurons and complete loss of CF neurons (Liu *et al.* 2008; Storm *et al.* 2009). Moreover, the ectopic co-expression of Olig3 and Ptf1a induced cells expressing a CF neuron marker in chick embryos (Storm *et al.* 2009). These findings suggest that CF neurons emerge from the Ptf1a/Olig3-expressing neuroepithelial domain (dP4) and that Ptf1a and Olig3 are cooperatively involved in the development of CF neurons. The domain structure of the dorsal neuroepithelium in the caudal hindbrain region is shown in the lower panel of Figure 2.

Conclusions

Various types of neurons are generated from the dorsal hindbrain. As described above, the dorsal neuroepithelium of the rostral hindbrain (r1) produces all types of cerebellar neurons, while the dorsal regions of the caudal hindbrain (r6–r8) generate neurons that include the precerebellar system neurons, such as MF and CF neurons. In addition, the dorsal part of the middle hindbrain (r2–r5 in mice) produces neurons of the cochlear nucleus, where auditory information is processed and relayed to the brain.

There are dorso-ventral domain structures defined by several transcription factors, which are longitudinally expressed throughout the hindbrain. In particular, two bHLH transcription factors, Atoh1 and Ptf1a seem to play important roles in specifying distinct neuronal subtypes. These two proteins are expressed in different neuroepithelial regions throughout the hindbrain (Fig. 3). In both the rostral (r1) and middle hindbrain (r2–r5 in mice), Atoh1 and Ptf1a participate in generating excitatory and inhibitory neurons, respectively. However, this rule is not applicable to the caudal hindbrain. The Ptf1a neuroepithelial domain in the caudal hindbrain (r6–r8) produces not only inhibitory neurons (local circuit neurons) but also glutamatergic neurons (CF neurons) (Yamada *et al.* 2007), while the Atoh1 domain generates glutamatergic MF neurons. This

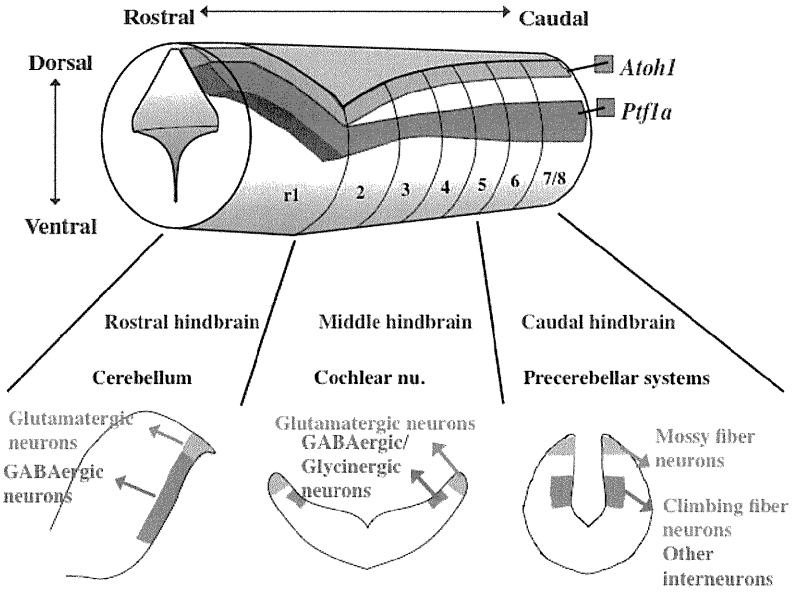

Fig. 3. Neurons produced from the dorsal hindbrain. Atoh1 and Ptf1a are expressed in distinct neuroepithelial regions throughout the rhombomeres 1–8 (r1–8). Each number represents the rhombomeric number. Upper side is dorsal, lower is ventral. Left side is rostral, right side is caudal. Neuronal subtypes generated from the dorsal neuroepithelium of the rostral, middle and caudal hindbrain regions are shown.

raises the possibility that the rostral/middle (r1–r5) and caudal (r6–r8) hindbrain subregions have distinct characteristics. Overall, throughout the hindbrain regions, transcription factors, such as Atoh1 and Ptf1a, seem to define neuroepithelial domains along the dorsoventral axis and participate in specifying distinct neuronal subtypes according to the rostrocaudal spatial information (Fig. 3).

In the spinal cord, Atoh1 and Ptf1a are also expressed in the dorsal neuroepithelium in a non-overlapping manner, defining specific neuroepithelial domains, although Ptf1a seems to be transiently expressed in immature postmitotic neurons. Ptf1a is involved in producing inhibitory neurons in the dorsal spinal cord (Glasgow *et al.* 2005). Atoh1 is known to participate in generating some types of neurons in the dorsal spinal cord, including commissural neurons (Helms & Johnson 1998; Bermingham *et al.* 2001; Miesegaes *et al.* 2009). These neurons in the Atoh1-lineage are believed to include glutamatergic populations, although the neurotransmitter subtypes have not been well studied. Thus, from the viewpoint of transcription factors and neurotransmitter subtypes, the dorsal spinal cord has similar characteristics of those of rostral (r1) and middle (r2–r5) hindbrain but not caudal hindbrain (r6–r8), which emphasizes the complexity of the hindbrain.

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Late-onset mental deterioration and fluctuating dystonia in a female patient with a truncating *MECP2* mutation

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ABSTRACT

A 26-year-old woman with psychomotor developmental delay since late infancy showed rapid deterioration of her psychomotor abilities at the 11 years of age. She had gained the ability to verbally express herself and perform motor activities such as running and dancing in early childhood, but she lost the ability to verbally communicate and was unable to walk independently after this period. She also presented with dystonia in the right extremities, which markedly fluctuated with a periodicity of hours to months. Sleep disturbance and epileptic seizures also emerged during adolescence. Frontal lobe atrophy and hypoperfusion of the left cerebral hemisphere were noted on neuroimaging examinations. Analysis of the *MECP2* gene revealed a late truncating mutation of c.1196_1200delCCACC (p.P399QfsX4) near the 3′-terminal of the coding region. The phenotype of this patient corresponds to the rare, unestablished variant of "late childhood deterioration" in MECP2-related disorders. For the first time, *MECP2* mutation was confirmed to be the genetic basis of this condition.

1. Introduction

Rett syndrome (RS) is characterized by normal development during infancy followed by developmental and motor regressions between the ages of 1 and 3 years that result in severe cognitive impairments, stereotyped hand movement, and motor disabilities [1]. However, atypically mild phenotypes of RS, such as late childhood deterioration or preserved verbal communication skills through adolescence or adulthood, had been delineated before the gene for methyl CpG binding protein 2 (*MECP2*) was identified as the causative gene for majority of the cases of this disorder [2,3]. Additional recent studies on the genotypes and phenotypes of *MECP2* mutants have revealed that certain missense or truncating mutations are preferentially formed in the preserved speech variant of RS [4].

MECP2 is a transcriptional repressor protein that is known to possess a methyl binding domain (MBD), a transcription repression domain (TRD) and a nuclear localization signal. MECP2 can act as both an activator and repressor of the transcription of some downstream genes, including *BDNF*, *DLX5* and *IGFBP* 3 [5], but how these genes contribute to the RS phenotype is still unknown [6].

In this report, we describe a female patient who had a truncating, 5-bp deletion at the 3′-terminal region of *MECP2* gene. She presented with exceptionally delayed onset and deterioration even compared with atypical cases that have been described in the literature [2–4]. She also presented with dystonia in the right-side extremities, the intensity of which markedly fluctuated with time.

2. Case report

The patient was a 26-year-old woman who was uneventfully born with a body weight of 2928 g (+0.2 SD) and a head circumference of 32.5 cm (+0.3 SD). She smiled socially and gained head control within 3 months of age, but then showed delayed psychomotor development, including the ability to bottom-shuffle at 1 year of age, to utter meaningful words at 1 year and 6 months of age, and to walk at 1 year and 11 months of age. Intellectual disabilities became evident thereafter, and she was diagnosed with nonspecific mental retardation. Her verbal skills remained at the level of single words throughout childhood. Although special support was necessary, she was able to attentively participate in activities of ordinary classes and run, dance, and sing with her classmates (Fig. 1A). However, tremors

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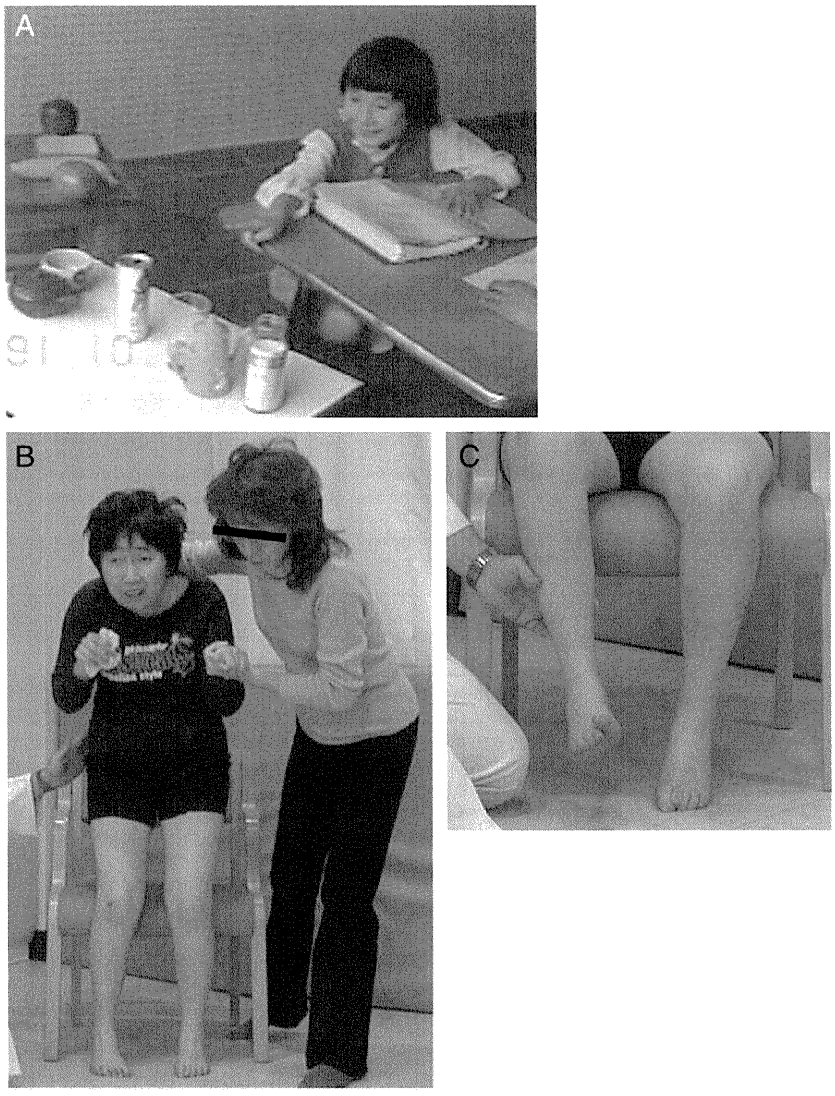

Fig. 1. The patient at ages of 8 (A) and 26 years (B, C). A: She was able to smile and point to a can with her index finger and say "juice." B: The patient showed occasional stereotyped hand behaviors, e.g., rubbing a small towel. She was not able to stand up or walk by herself. Kyphotic posture, flexion and adduction of the arms, and tapering of the lower extremities were noted. C: Spontaneous dorsiflexion of the toe (pallidal toe) on the right side.

in her right hand emerged at the age of 11 years, followed by stiffening of the right leg during walking and intermittent pronation, adduction, and flexion posturing of the right arm, which disabled the patient from dressing herself. In addition, her use of meaningful words decreased and she would merely look at someone and hold out her hand when she wanted aid by others. Her movement disorder markedly fluctuated; the patient could walk up to 100 m with assistance when she was in good condition, but could not walk at all when the symptoms were aggravated. This fluctuation showed an irregular periodicity varying from hours to days or months, without any specific factors triggering onset. Dysregulated circadian rhythm presented at 12 years of age; the patient either kept sleeping or used to be awake for up to several days at a time as well as occasionally became excited and screamed at midnight. Epileptiform discharges had been noted since 2 years of age, but epileptic seizures were first clinically diagnosed at 13 years of age. These seizures, which were accompanied with facial twitching and secondary generalizations, were kept under control with valproate sodium.

The patient was referred to our hospital at 26 years of age with suspicion of a neurodegenerative disorder. Her body height was 147 cm (−2.1 SD), body weight was 49.4 kg (−0.5 SD), and head circumference was 53 cm (−1.7 SD). She presented with thin lower legs, dorsiflexion of right first toe (Fig. 1C), and a kyphotic posture with mid-flexion of the upper extremities when sitting or standing with help (Fig. 1B). Stereotypy such as intermittent hand rubbing was rarely observed. Her muscle tonus was increased, and slight limitations in her ability to extend her elbows and ankles were noted. Pyramidal signs were negative at each extremity. Intension tremors appeared in the upper extremities when reaching for objects. The patient could speak a couple of words when encouraged by her mother, including "Good morning" and "Thank you," but otherwise did not verbally express herself. She occasionally mumbled to herself with phrases such as "Your fault" or "Good night, see you tomorrow," but these phrases were used without the purpose of communicating with others. Using the Enjoji developmental assessment scale, her overall psychomotor activities were assessed to be equivalent to the level of a 1-year 6-months-old child.

G-banded chromosome analysis showed a normal karyotype of the 46, XX. Vanillylmandelic acid, 5-hydroxyindoleacetic acid, 3-methoxy-4-hydroxyphenylglycol, neopterin, biopterin, and amino acids

levels were normal in the cerebrospinal fluid. Brain magnetic resonance imaging showed mild bilateral atrophy of the frontal lobe and cerebellar hemispheres (Fig. 2A). Single photon emission computed tomography revealed hypoperfusion at the bilateral frontal cortices and, to a more significant degree, over the left parietotemporal cortex and left striatum (Fig. 2B). Mutation analysis of the *MECP2* gene detected a heterozygous 5-bp deletion in exon 4 (c.1196_1200delCCACC), which caused a frameshift and a stop codon at position 1212 of the wildtype sequence. Truncation of the gene product (p.P399QfsX4) was expected (Fig. 3). This mutation was not present in the mother's DNA.

Treatment with L-DOPA and melatonin resulted in the transient amelioration of the disturbed sleep cycles but did not result in any significant effect on the movement disorder of this patient.

3. Discussion

MECP2 is thought to be important not only for the development of the central nervous system, but also for the maintenance of neuronal cell functions; overexpression of *IGFBP3* due to lack of *MECP2* has been shown to delay brain maturation [7]. The deterioration phase of RS may be related to the latter function of MECP2 in central nervous system. On the other hand, the initial regression period as late as 11 years of age and the fluctuating nature of the movement disorders in this patient are different from those previously observed in patients with *MECP2* mutations. To date, the effect of the type and location of *MECP2* mutations on the severity of RS have generally been elucidated: patients with missense mutations tend to manifest milder phenotypes than those with truncating mutations due to nonsense mutations or frameshift deletions, and those with mutations close to the 3′-terminal (late mutation) present with milder phenotypes (Fig. 3) [8,9]. Frameshift deletions/insertions in the last exon between nucleotides 1050 and 1200 are hot spots in *MECP2* mutants and are found in approximately 10% of RS patients [10]. We found 47 cases in the literature [4,10–19] with deletions in this region (Supplementary Table 1), which included 15 cases of preserved speech variant and additional six cases of atypical RS phenotype. Deletions downstream to the site as observed in the present patient were identified in five of the aforementioned cases, including one case of atypical RS and two cases of preserved speech variant. The same trend of C-terminal truncation could also be observed in the genotype–phenotype database (http://mecp2.chw.edu.au); 14 truncating mutations downstream to the site in the present case were registered and included six cases of classical RS, three of atypical RS, one of preserved speech variant, one of congenital variant, and three of X-linked/sporadic mental retardation (Supplementary Table 2). The relatively mild phenotypes of this group may be explained by the preservation of the MBD and TRD domains. However, even in this group and other cases of mild RS due to missense mutations, the initial regression almost exclusively appears at 3 years of age or younger [4]. Apart from rare adult cases of RS without any apparent deterioration periods [15], the present patient showed the latest onset of the regression stage. In fact, the "late childhood deterioration" group is even rarer than other atypical phenotypes of RS, and is not incorporated in the recently revised diagnostic criteria of variant RS [1]. Lack of regression by 5 years of age is assigned as questionable for RS diagnosis in these criteria. Only a few anecdotal cases with a regression period presenting at ages older than 5 years have been reported in the literature; however, the statuses of the *MECP2* mutations were not specified [3]. A case with a truncating mutation of c.1154_1167del44 (p.389X) where motor regression emerged during adolescence but intellectual deterioration was not evident has been reported [13].

Since c.1194_1195insT (p.P399fsX) causes classical RS (http://mecp2.chw.edu.au), the exact mechanism for the atypical presentation of this patient remains to be elucidated. Skewed X-inactivation has been proposed as an epigenetic factor in phenotypic variations that exist in RS cases with identical mutations [14], however, a correlation between phenotype and X-inactivation patterns has not been identified in larger case studies [8]. Random [20] or mutant allele-preserved [21] X-inactivation has been revealed in asymptomatic *MECP2* mutation carriers, which suggests that a mechanism other than X chromosome inactivation may be responsible for the modification of clinical phenotypes. Although the mild phenotype of our patient may be explained by skewed X-inactivation in her brain, the X-inactivation pattern of the peripheral tissue does not necessarily reflect the status of the brain [21].

The fluctuating nature of the right-sided dystonia is reminiscent of the symptoms of hereditary progressive dystonia with marked diurnal fluctuation (HPD, Segawa's disease), which is caused by a defect in GTP cyclohydrolase I that affects tyrosine hydroxylase activity by altering the metabolism of biopterin. Degenerative changes in the substantia nigra and decreases in presynaptic dopaminergic activities have been reported in RS patients [22]. However, cerebrospinal levels of biopterins and catecholamine metabolites were normal in the

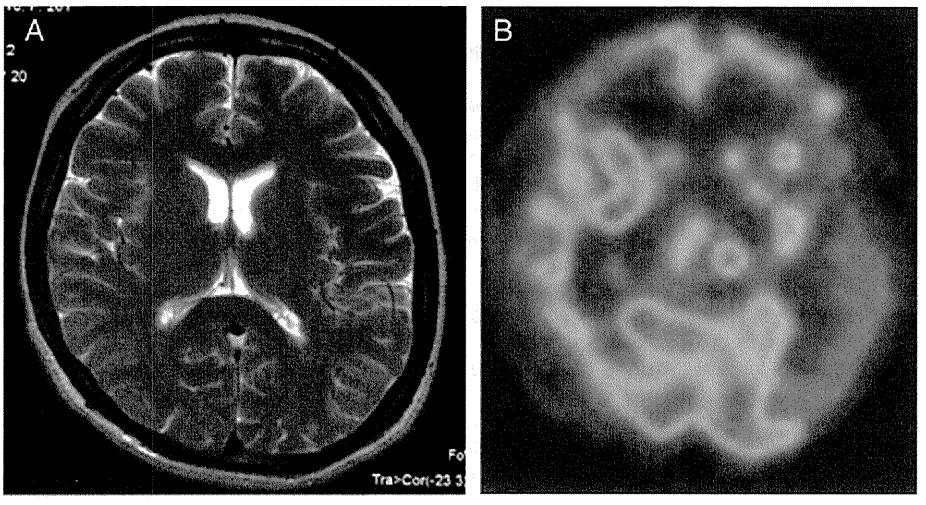

Fig. 2. Neuroimaging of the patient. A: T2-weighted magnetic resonance imaging. The frontal lobes show mild atrophy. B: ^{99m}Tc-ECD single photon emission computed tomography. Hypoperfusion over the left cerebral hemisphere and left striatum were noted. Diminished blood flow to the right frontal cortex was also noted.

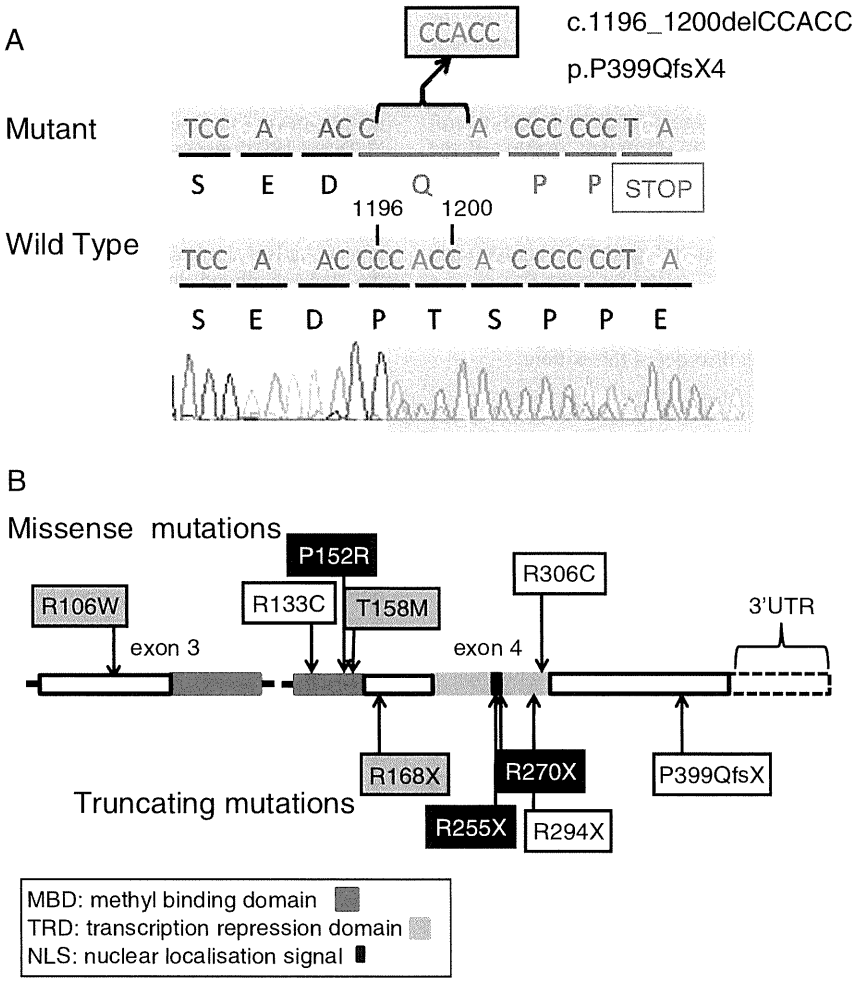

Fig. 3. Mutation analysis of the *MECP2* gene. A: Sequences around the mutation in the patient's *MECP2* gene. A 5-bp deletion of 1196_1200CCACC results in the expected alteration of P399Q and truncation of the protein product at the third codon downstream. B: Common mutations in the *MECP2* gene. The groups of 3′-terminal deletions at nucleotides 1050–1200 are hot spots for aberrations in this gene. Phenotypic severity is shown based on the findings of previous studies [8,9,12,14,16,17]. Black and white squares represent mutations that preferentially result in classic or atypical RS, respectively. Mutations in the gray squares can result in either of these phenotypes.

present patient, and L-DOPA did not have an effect on the fluctuating movement disorders; these findings are in contrast to HPD. On the other hand, the loss of postsynaptic striatal neurons is assumed to occur in the pathogenesis of dystonia and bradykinetic disorders that often appear in teenagers with RS [22]. Increases in glucose metabolism and glutamate receptors have been identified in the frontal lobe and basal ganglia of RS brains, which is in contrast to the decreased blood flow to these areas. This evidence supports the hypothesis that glutamate-induced neurotoxicity affects these areas [23]. It is interesting that increased glucose uptake in the cerebral hemispheres occurs predominantly on the left-side [24]. This may be related to the asymmetrical nature of dystonia and other neurological deficits that accompany the symptoms of RS in adult patients, whose symptoms predominantly present on the right-side [25]. Age-related onset and asymmetry of dystonia both occurred in the patient described in this report. Cerebral hypoperfusion contralateral to the asymmetrical dystonia of this patient may support the role of the cortex–basal ganglia glutamatergic system in the pathogenesis of movement disorders that is present in RS [26]. The presentation of a mild phenotype with dominating scoliosis has been interpreted as a manifestation of truncal dystonia and proposed as a characteristic of RS because of C-terminal truncations of MECP2 proteins [10]. However, apart from the case of another patient with late motor deterioration [13], we hardly found information on limb dystonia in these and other genetically proven cases with *MECP2* mutations (Supplementary Table 1). Genotype–phenotype correlation in this aspect of movement disorders in MECP2-related disorders is yet to be determined.

In conclusion, the cause of late regression and fluctuations in the motor symptoms of this patient remains unclear. However, it was confirmed that RS variants with these phenotypes result from *MECP2* mutations. This report could facilitate the identification of undiagnosed cases and encourages further exploration into the pathomechanisms of RS symptoms.

Supplementary materials related to this article can be found online at doi:10.1016/j.jns.2011.06.008.

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Neocortical Layer Formation of Human Developing Brains and Lissencephalies: Consideration of Layer-Specific Marker Expression

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To investigate layer-specific molecule expression in human developing neocortices, we performed immunohistochemistry of the layer-specific markers (TBR1, FOXP1, SATB2, OTX1, CUTL1, and CTIP2), using frontal neocortices of the dorsolateral precentral gyri of 16 normal controls, aged 19 gestational weeks to 1 year old, lissencephalies of 3 Miller–Dieker syndrome (MDS) cases, 2 X-linked lissencephaly with abnormal genitalia (XLAG) cases, and 4 Fukuyama-type congenital muscular dystrophy (FCMD) cases. In the fetal period, we observed SATB2+ cells in layers II–IV, CUTL1+ cells in layers II–V, FOXP1+ cells in layer V, OTX1+ cells in layers II or V, and CTIP2+ and TBR1+ cells in layers V and VI. SATB2+ and CUTL1+ cells appeared until 3 months of age, but the other markers disappeared after birth. Neocortices of MDS and XLAG infants revealed SATB2+, CUTL1+, FOXP1+, and TBR1+ cells diffusely located in the upper layers. In fetal FCMD neocortex, neurons labeled with the layer-specific markers located over the glia limitans. The present study provided new knowledge indicating that the expression pattern of these markers in the developing human neocortex was similar to those in mice. Various lissencephalies revealed abnormal layer formation by random migration.

Keywords: developing human neocortex, layer-specific marker, lissencephaly

Introduction

The experimental neurosciences have recently provided many new insights into the molecular mechanisms of mammalian cerebral formation. Past knowledge revealed that some molecules are regulated with a well-designed genetic algorithm during the developmental stages, with interrelated phenomena that include cell proliferation, fate determination and migration to the proper laminar, and final position in the cerebral cortex. Neocortical laminar formation is highly programmed by genetic control in the early embryonic period. At the decided time, projection neurons migrate into the cortical plate (CP) along the radial glial process from the subventricular germinal zone with an inside out pattern. At this neural migration stage, integration of reelin (RELN), Lis-1, doublecortin (DCX), and other molecules is required to form a complete neocortex (Guillemot et al. 2006; Mochida and Walsh 2004). Finally, mammalian brains commonly show a 6-layer neocortex, and each layer has a specific function with a synaptic connection. In each step, specific genes have important roles, and the molecular mechanism is well known in rodent brains (Arlotta et al. 2005; Alcamo et al. 2008). Satb2, a special AT-rich binding protein 2, generates callosal projection neurons in layers II–IV (Alcamo et al. 2008; Britanova et al. 2008). Ctip2, encoding a C2H2-type zinc finger protein, locates in layers V and VI and promotes corticospinal motor neuron projection (Arlotta et al. 2005; Britanova et al. 2008). Satb2 is a repressor of Ctip2 and makes not only the callosal projection but also the subcortical connections (Alcamo et al. 2008). Mouse Otx1, orthodenticle homeobox 1, is expressed in a number of cells in layers V and VI (Weimann et al. 1999). Tbr1, a member of the T-box homeobox gene family, expresses in preplate and layer VI in mouse fetal brain (Hevner et al. 2001) and layers I–III and layer VI in mouse adult brain (Bulfone et al. 1995). Tbr1 contributes to make corticocortical projection neurons (Hevner et al. 2001). Tbr1 expresses in the deep layer of the human fetus cortex (Sheen et al. 2006). A transcription factor Cutl1, drosophia homeobox CUT like 1, is expressed in pyramidal neurons of the upper layer (Nieto et al. 2004). Foxp1, a transcription factor of the winged-helix/forkhead family, expresses in layers III–V of mouse neocortex (Ferland et al. 2003) and layer V in human neocortex (Sheen et al. 2006). Foxp1 expresses in the deep layer of Miller–Dieker syndrome (MDS) neocortex (Sheen et al. 2006). However, many rodent studies show that the other layer-specific molecules also play very important roles in forming cortical lamination (Molyneaux et al. 2007) and that such gene disruption leads to profound cortical malformation (Mochida and Walsh 2004).

Lissencephaly, formed at the neuronal migration period, is classically recognized to be mainly of 2 types; smooth pacthygyria–agyria as type I lissencephaly and cobblestone lissencephaly as type II lissencephaly (Olson and Walsh 2002). Type I (classical) lissencephaly shows a thick 4-layer cortex and is typically known as MDS and double cortex syndrome. The causative genes of type I lissencephaly are known as RELN, Lis-1, DCX, and filamine. Interestingly, the gene products are associated with the microtubules and can alter the cytoskeleton size for cell movement (de Rouvroit and Goffinet 2001; Reiner and Sapir 2009) or its related molecules (Olson and Walsh 2002; Assadi et al. 2003). Typical type II (cobblestone) lissencephalies

of Muscle-eye-brain disease, Walker–Warburg syndrome (WWS), and Fukuyama-type congenital muscular dystrophy (FCMD), are caused by mutated genes encoding enzymes of alpha-dystroglycan glycosylation, such as POMGnT1, protein-O-mannosyltransferase (POMT) 1 and 2, and Fukutin, respectively (Mochida and Walsh 2004). The posttranslational glycosylated alpha-dystroglycan binds to extracellular matrix (Michele et al. 2002). Reduction of glycosylation leads to disruption of the glia limitans over which neurons migrate (Yamamoto et al. 2004).

Recently, it has been reported that X-linked lissencephaly with abnormal genitalia (XLAG), whose causative gene is *Aristaless*-related homeobox gene (*ARX*), is a new type of lissencephaly that shows a 3-layer neocortex (Dobyns et al. 1999; Kitamura et al. 2002; Bonneau et al. 2002; Okazaki et al. 2008). ARX has a homeodomain and decides the migration of interneurons in the ganglionic eminence. However, it is unknown why ARX dysfunction leads to abnormal radial neuronal migration in human XLAG, whereas *ARX*-null mice show reduced cortical proliferation but normal migration (Kitamura et al. 2002; Okazaki et al. 2008).

It is very important to reveal the molecular and morphological relationship between these malformed brains to understand human neocortical formation and pathophysiology, although little is known about the expression pattern of layer-specific markers in human developing brain (Hevner 2007). In the present study, we focus on layer formation and investigate the expression of layer-specific molecules in neocortices of human developing brains and lissencephalies.

Materials and Methods

Human Brain Tissues

All cerebral tissues used in the present study were approved for research usage by parents and Ethical Committees of the involved hospitals and institutes. For the developmental study, we used frontal cortices of the dorsolateral precentral gyri of 16 controls, showing no neuropathological findings (age 19 gestational weeks [GWs] to 1 year after birth) (Supplementary Material). In addition, we examined the same frontal cerebral hemispheres of lissencephaly, which were clinicopathologically diagnosed as MDS, XLAG, and FCMD (Supplementary Material). The postmortem interval (time from death to fixation) of all subjects was within 12 h (Supplementary Material). After removal, all brains were fixed in 10% buffered formalin or 4% paraformaldehyde for 2 weeks. Then, brains were dehydrated with 70–100% alcohol and embedded in paraffin. The serial sections were cut 6 μm thick for histological and immunohistological examination.

Histology and Immunohistochemistry

For investigation of brain architecture, the sections were stained with hematoxylin and eosin (HE) and Klüber-Barrera (KB) method. To investigate cortical layer formation, we performed immunohistochemistry using cortical layer-specific markers; polyclonal antibodies against TBR1 (dilution of 1:100; Abcam), FOXP1 (1:100; Abcam), and OTX1 (1:100; Abcam), as well as monoclonal antibodies against SATB2 (1:100; Bio Matrix Research Inc.), CUTL1 (1:100; Abnova), and CTIP2 (1:20; Abcam).

Our immunohistochemistry technique was previously described (Okazaki et al. 2008). Briefly, the serial sections were deparaffinized and rehydrated. For antigen retrieval, we performed an autoclave treatment (120 °C for 10 min in 10 mM citrate buffer, pH 6.0). Sections were incubated in primary antibodies at 4 °C for overnight, and then reacted with the secondary antibodies (Nichirei). We used amino ethyl carbazole (Nichirei) as a chromogen. For counterstaining, 0.2% methyl green was used. For double labeling, we used Alexafluor-488- and 568-conjugated secondary antibodies (Invitrogen Corporation) with 4′,6′-diamidino-2-phenylindole (DAPI). We observed the stained tissues with FLUOVIEW 500 fluorescent microscope (Olympus).

Results

Cortical Lamination of Normal Developing Brains

Generally, we confirmed cortical formation of all subjects with HE- and KB-staining. We observed the CP and intermediate zone around 20 GW (Fig. 1*A*). At this embryonic period, SATB2+ cells located in the upper region of CP (Fig. 1*B*). CUTL1+ cells were diffusely distributed in CP (Fig. 1*C*). FOXP1+ cells were restricted to the middle region of CP (Fig. 1*D*). OTX1+ cells and CTIP2+ cells are seen in the lower region of CP (Fig. 1*E,F*). The distribution of TBR1+ cells exhibited a 2-layer pattern of CP and SP (Fig. 1*G*).

At approximately 30 GW, the neocortex was divided into 6 layers (Fig. 2*A*). The distribution of SATB2+ cells was observed in layers II–V, predominantly in layers II and IV (Fig. 2*B*). CUTL1+ cells were diffusely seen in layers II–VI (Fig. 2*C*). FOXP1+ cells were in layer IV and the upper region of layer V (Fig. 2*D*). OTX1+ cells were concentrated in layers IV and V (Fig. 2*E*). CTIP2+ and TBR1+ cells were located in layers V and VI (Fig. 2*F,G*). The developmental expression pattern is shown in Supplementary Figure 1.

In the perinatal period, the expression pattern of the cortical layer-specific markers is very similar to that of around 30 GW (Fig. 3). In the late gestational period, SATB2 expressed in the superficial region of the neocortex and CUTL1, FOXP1, and CTIP2 gradually demonstrated in the deep region, while TBR1 was in the bottom. Interestingly, OTX1+ cells were only in layer V (Fig. 3*E*). After birth, SATB2+ and CUTL1+ cells appeared until 3 months of age, although the other markers had already disappeared (data not shown).

In order to investigate the relationships among these layer-specific markers, we performed double fluorescent staining of SATB2 and FOXP1, SATB2 and TBR1, CTIP2 and SATB2, SATB2 and OTX1, CTIP2 and FOXP1, and CTIP2 and TBR1 (Fig. 4, Supplementary Figure 1). FOXP1+ and SATB2+ merged (FOXP1+/SATB2+) cells were observed in the superficial CP of 23 GW but in the deep layer after 29 GW (Fig. 4*A*). Throughout the fetal period, FOXP1+/CTIP2+ cells might be in the deep layer (Fig. 4*E*), and many SATB2+/OTX1+ cells were in layers II and IV or V (Fig. 4*D*). However, SATB2+ cells did not express CTIP2+ (Fig. 4*C*). TBR1+ cells had no SATB2, but there were a few CTIP2 signals in layer VI (Fig. 4*B,F*). The double staining of layer-specific marker expression was shown in Supplementary Figure 1.

Layer-Specific Marker Expression of Various Lissencephalies

MDS brains were typical agyria and pachygyria with thick cortex and thin white matter. MDS showed a 4-layer neocortex as previously reported (Crome 1956): a molecular layer, an external cellular layer (layer I), a sparsely cellular layer (layer II), and an internal cellular layer (layer III) (Fig. 5*A*). In layers II, III, and IV, small neurons, which had immunoreactivities of SATB2, CUTL1, FOXP1, and TBR1, were observed diffusely but were few in number (Fig. 5*B–E*). Large pyramidal neurons in the upper layer II had TBR1 (Fig. 5*E*). The neocortex in XLAG exhibited a 3-layer pattern (Bonneau et al. 2002): a molecular layer (layer I), an intermediate layer with densely packed