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G. 知的所有権の取得状況

1. 特許取得
   なし

2. 実用新案登録
   なし

3. その他
   なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

レット症候群モデルマウスの無呼吸頻度の日内変動とモノアミン神経伝達

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研究要旨
MeCP2 が欠損している雄ノックアウトマウス（*Mecp2*$^{-/y}$）について，全身型プレチスモグラフを用いて無拘束下で呼吸を 24 時間連続測定し，得られたデータを野生型雄マウス（wild）と比較した。また延髄における小胞膜モノアミントランスポーター2（VMAT2）発現の免疫組織学的検討を行い，呼吸異常との関連性について調べた。その結果，*Mecp2*$^{-/y}$について，生後 7 週においてwild に比べ著明な無呼吸回数の増加がみられ、明期で有意に多かった。また，セロトニン・ノルアドレナリン再取り込み阻害薬であるミルナシプランの長期経口投与により無呼吸回数の有意な減少がみられ，*Mecp2*$^{-/y}$の寿命は約２０％延長した。*Mecp2*$^{-/y}$では wild に比べ生後８週において呼吸中枢を含む延髄での VMAT2 陽性 puncta 数の著しい減少がみられたことから，無呼吸回数の増加について，呼吸関連中枢におけるモノアミン作動性シナプスの異常が関与している可能性が示唆された。

A．研究目的

Mecp2$^{-/y}$では生後 5 週以降に無呼吸発作の頻度が著しく増加するとの報告があるが，呼吸動態の 24 時間変動に関する報告はまだなく，また脳内セロトニンやノルアドレナリン量と無呼吸との関連についても十分な知見が得られていない。そこで今回，MeCP2 の欠損が延髄の呼吸関連中枢におけるモノアミン神経伝達にどのような変化を惹起しているのかを明らかにすることを目的として研究を行った。

B．研究方法

①７週齢の *Mecp2*$^{-/y}$ならびに wild を一匹ずつ全身型プレチスモグラフ（PLY4211；Buxco Electronics, 大阪）のチャンバー内に入れ，明暗が 12:12 の条件下で呼吸波形を 24 時間連続で測定・記録したのち，1 秒以上の無呼吸の発生回数について解析を行った。また，セロトニン・ノルアドレナリン再取り込み阻害薬であるミルナシプランを飲料水中に溶解し，生後 3 週から継続して摂取させ，7 週齢で 24 時間の呼吸測定を行った。ミルナシプランの摂取量は約 4 mg/day となるように濃度調節した。

②*Mecp2*$^{-/y}$ならびに wild について８週齢で脳組織をとりだし，VMAT2 についての免疫組織染色を行った。呼吸を含めた自律神経機能の調節に関与している dorsal motor nucleus of the vagus（DMV），呼吸リズム形成を担っている nucleus of the solitary tract（NST）および ventral respiratory group（VRG）について，VMAT2 陽性 puncta 数を，画像処理ソフトウエア（ImagePro7.0）を用いて数えた。

（倫理面への配慮）
本研究は日本大学歯学部実験動物委員会の承認を得て実施し，実験動物の取扱いは同委員会の指針に従って行った。（承認番号 18-7　2006 歯 007-1, 007-2）

C．研究結果

Mecp2$^{-/y}$について、生後 7 週において wild に比べ著明な無呼吸回数の増加がみられ、明期で有意に多かった。またミルナシプランの経口投与により無呼吸回数の有意な減少がみられた。（*Mecp2*$^{-/y}$の寿命は約 20%延長した）

DMV、NTS、 VRG において、*Mecp2*$^{-/y}$ではいずれの部位についても VMAT2 陽性 puncta 数の著しい減少がみられた。

D．考察

Mecp2$^{-/y}$ の無呼吸回数が明期に多いという結果については，明期の光刺激が自律神経系に影響して呼吸を不安定化させている可能性が考えられ，今後検討が必要と思われる。ミルナシプランの経口投与により一日を通じての無呼吸回数の有意な減少がみられたことは，Rett 症候群患者にみられる無呼吸発作にセロトニンあるいはノルアドレナリン神経伝達の低下が関与するとの仮説を支持するが，今後その他の神経伝達物質の関与についても検討する必要がある。また組織学的検索により，延髄の呼吸関連中枢でのモノアミン作動性シナプスの減少に伴う神経伝達の低下を確認した。

E．結論

Mecp2 $^{-/y}$ では明暗条件下で明期において無呼吸の発生回数が増加することが明らかになり，ミルナシプランの長期経口投与により無呼吸回数の有意な減少がみられたことから，セロトニンあるいはノルアドレナリン神経伝達の向上が Rett 症候群患者の無呼吸の改善に結びつく可能性が示唆された。

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G．知的所有権の取得状況

1. 特許取得
   なし

2. 実用新案登録
   なし

3. その他
   なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
研究分担者研究報告書

非典型レット症候群の原因遺伝子*CDKL5*の分子機能及び遺伝子変異による病態機序の解析

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研究要旨
非典型レット症候群の原因遺伝子CDKL5の分子機能と遺伝子変異による病態機序の解明を目的として、酵母ツーハイブリッド法を用いたCDKL5相互作用蛋白の同定と、Cdkl5 ノックアウト（KO）マウスの作製及び表現型解析を行った。その結果、N末端側キナーゼ領域に対し19個、C末端側領域に対し2個の相互作用蛋白候補を同定した。またCdkl5 KOマウスにおいて、海馬神経細胞樹状突起スパインの異常、海馬スライス電気生理学的異常、不安の亢進等の情動異常などを同定した。

A．研究目的

Cyclin-dependent kinase-like 5（CDKL5）遺伝子は早期発症てんかんを伴う非典型レット症候群の原因遺伝子である。しかしその神経発達における分子機能、及び遺伝子変異による病態機序はほとんどわかっていない。そこで私はCDKL5遺伝子変異による病態解明を目指し、酵母ツーハイブリッドスクリーニングを用いた相互作用蛋白の同定と、CDKL5ノックアウト(KO)マウスの作製によるloss-of-function機構解析を行った。

B．研究方法

1. CDKL5相互作用蛋白の同定

Clontech Matchmaker GAL4 Two-Hybrid System 3を用い、(1) CDKL5 N末端側キナーゼ領域、(2) C末端側領域、をpGBKT7ベクターにクローニングしbaitベクターを作製し、マウス脳cDNA Library (Clontech)に対して相互作用スクリーニングを行った。

2. CDKL5 KOマウスの作製と解析

ES細胞相同組み換えにより、Cdkl5遺伝子のexon をloxPで挟んだCdkl5 floxマウス、及びfloxマウスとCAG-Creマウスとの交配により、Cdkl5 KOマウスを作製した。

C．研究結果

1. 酵母ツーハイブリッドスクリーニング

CDKL5 N末端側キナーゼ領域で19個、C末端側で2個の、微小管関連蛋白、細胞質モーター蛋白、シナプス関連蛋白、イオンチャネル、転写因子、などを含む相互作用蛋白候補を同定した。

2. Cdkl5 KOマウス

ジーンターゲティングによりCdkl5 floxマウス及びCdkl5 KOマウスを獲得した。Cdkl5 KOマウスの表現型解析の結果、海馬神経細胞樹状突起スパイン密度の異常、海馬スライスの電気生理学的異常、行動解析によって不安の亢進、うつ様行動の亢進、社会性の変化、情動異常、学習障害異常などを同定した。

D．考察

今回同定された相互作用蛋白候補は、複数のシナプス関連蛋白を含んでいた。CDKL5とこれら蛋白との相互作用がシナプス機能調節に関わる可能性が示唆された。

KOマウスの解析によって海馬神経細胞の形態・機能異常及び、情動・学習機能障害が認められ、ヒトの病態機序の解明に本マウスの詳細な解析が重要と考えられた。

E．結論

CDKL5相互作用蛋白候補21個が得られた。Cdkl5 KOマウスの作製・解析によって、海馬神経細胞の形態・電気生理学的異常、不安亢進等の情動・学習障害など異常が認められた。

F．研究発表

1. 論文発表

なし

2. 学会発表　（招待講演）

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G．知的所有権の取得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
研究分担者研究報告書

レット症候群の臨床、病態に関する研究 - 特に診断基準について

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瀬川昌也　瀬川小児神経学クリニック　院長
八森　啓　瀬川小児神経学クリニック　医師
木村一恵　瀬川小児神経学クリニック　医師
長尾ゆり　瀬川小児神経学クリニック　医師
林　雅晴　瀬川小児神経学クリニック　医師

研究要旨
我々はレット症候群の研究に長年関与してきておりそれらの研究結果に基づき本症が特異な神経発達障害であること、その病態にはモノアミン、カテコラミンの発達障害が重要な病態を成すことを述べてきているが、今回の研究ではそれらに基づき、可及的早期の正しい診断について述べ、かつ診断基準のfeasibilityを検討し、今後の診断、診療の方向性について検討した。

A．研究目的

レット症候群(RTT) は主として神経系を障害する特異な神経発達障害である。その臨床的特徴、病態に関する研究は1982年以来精力的になされてきているが未だ広くは理解されていないのが現状である。本研究ではそれらについての認識を広め、本症について可及的早期かつ正確な診断を成し、診療の方向を示していくことを目的とする。

B．研究方法

瀬川小児神経学クリニックにおける本症の最初の経験は1973年であり、1982年本症がレット症候群として再発見された以後多くの患者が日本各地から来院し、これまで約250名の患者が来院している。我々のこれまでの研究結果に基づいた臨床・病態研究を再提示し、自験例を対象とし、これまで述べられてきている診断基準について検討し、本症の病態に基づいた診療方針を示す。

C．研究結果

本症の臨床的特徴は乳児期早期より特異な症状が年齢依存性に出現することである。乳児期早期の症状が非常に軽微であるためRett先生、Hagberg先生共本症の発症は乳児期後期から幼児期早期にあり、乳児期早期の発達は正常であることがこれまでの診断基準の重要点として挙げられてきた。我々は詳細な発達歴、病歴の聴取より本症の発症は乳児期早期であることを述べ、終夜睡眠ポリグラフによる睡眠要素および睡眠・覚醒リズムの解析研究より本症の発症は胎生38週から生後4ヵ月であることを示した。また臨床的特徴を表1の如くまとめている。診断に関しては特徴的な症状が年齢依存性に出現することにより可能であることを述べてきた。

これまでの診断基準(1895, 1988, 2010) に我々の症例のデータを当てはめると、我々が臨床的にRett症候群と診断している症例の特徴をカバーしきれていないことが明らかである。しかし2010の診断基準には乳児期早期の発達は必ずしも正常とは云えないことが含まれており、前の診断基準より改良がみられる。

しかし今回我々の解析では本症の病因遺伝子であるMECP2 の変異をみた症例ではほぼこの診断基準をみたすが、変異をみなかった症例ではこの診断基準が当てはまらない症例が少なくなかった。

D．考察

本症は主として神経系をおかす特異な発達障害であり、我々はその臨床的解析、病態研究により本症の病態はモノアミン神経系、カテコラミン神経系の発達障害であることを示唆してきた。また、その原因遺伝子としてMECP2が解明されて以後、新たな方向の研究の発展がめざましい。

また、我々はこれまで本症の臨床的及び病態研究から本症の診断は特異な臨床症状が年齢依存性に出現することによりなされることを述べてきており、今回の研究でも再確認した。

我々(野村、瀬川)はこれまでの診断基準(1895, 1988, 2010)の作成にあたり、直接的に関与してきており、その問題点、改善すべき点について討論してきている。

今回我々はそれらの診断基準につきfeasibilityを検討したが2010年のものにおいて改善がみられる事が示唆された。しかし、MECP2の変異をみなかった例において必ずしもこの診断基準のすべてが当てはまらない例があった事は、本症の臨床、病態、病因に関する更なる研究が期待されるところであり、それが本症の診療について不可欠の事といえる。

E．結論

Rett症候群の臨床的研究、病態に関する研究を再考察し、診断基準の妥当性について検討した。その結果、新しい診断基準のfeasibilityには改善が見られたといえるが、臨床的な診断とは必ずしも合致しない例があり、これらの診断基準については一応の参考資料としていくことが真の臨床診断、それに基づいた治療の方向の提示、実践に大切であると云える。

F．研究発表

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2．学会発表

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G．知的所有権の取得状況

1．特許取得

なし

2．実用新案登録

なし

3. その他

なし

表1　Rett症候群における年齢依存性の症状出現

乳児期早期（初発症状）；	姿勢筋緊張低下、自閉傾向
乳児期後期；	ロコモーションの障害、頭囲停滞
早期小児期；	目的を持った手の運動機能の消失、 常同運動の出現 筋緊張亢進、ジストニー、側彎 奇異呼吸、てんかん、 精神遅滞
後期小児期-成人；	安定？

III. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kuki I, Kawawaki H, Okazaki S, Kimura S, Nakano T, Fukushima H, Inoue T, Tomiwa K, Itoh M.	Progressive Leukoencephalopathy with Intracranial Calcification, Congenital Deafness and Developmental Deterioration.	Am J Med Genet A	155	2832-2837	2011
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Yasui DH, Scoles HA, Horike S, Meguro-Horike M, Dunaway KW, Schroeder DI, Lasalle JM	15q11.2-13.3 chromatin analysis reveals epigenetic regulation of CHRNA7 with deficiencies in Rett and autism brain	Hum Mol Genet	20	4311-4323	2011
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Kuroki Y, Honda K, Kijima N, Wada T, Arai Y, Matsumoto N, Iwata K, Shirakawa T	In vivo morphometric analysis of inflammatory condylar changes in rat temporomandibular joint	Oral Dis	17	499-507	2011
Mikami Y, Ishii Y, Watanabe N, Shirakawa T, Suzuki S, Irie S, Isokawa K, Honda MJ	CD271/p75NTR inhibites the differentiation of mesenchymal stem cells into osteogenic, adipogenic, chondrogenic, and myogenic lineages.	Stem Cells Dev	20	901-913	2011
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IV. 研究成果の刊行物・別刷

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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

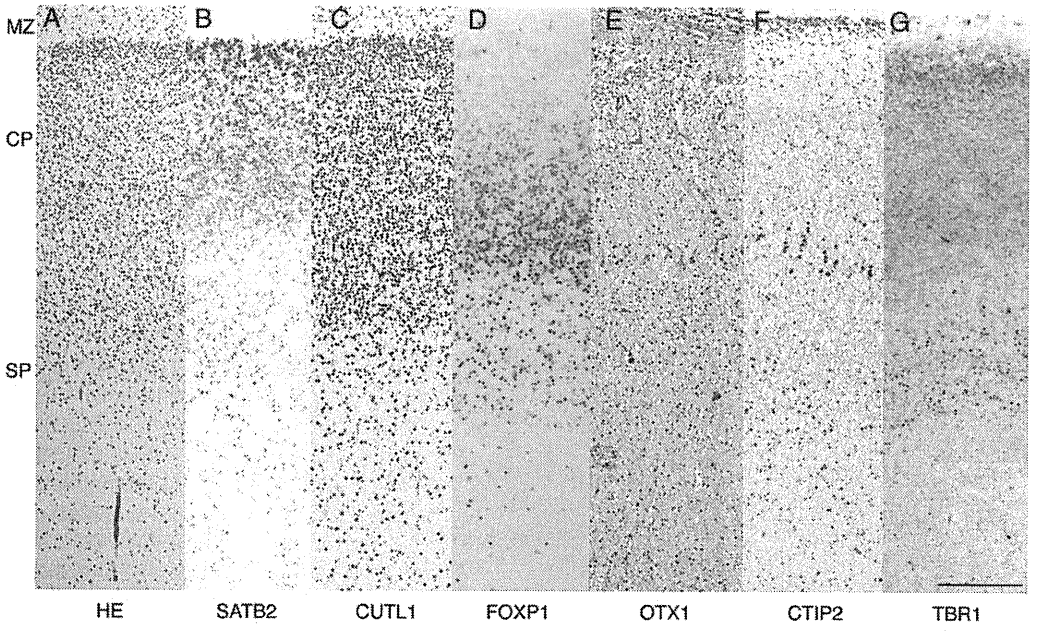

Figure 1. Layer-specific marker expression of the neocortex at 23 GWs. Around 20 GWs, the 3-layer pattern, that is, the marginal zone (MZ), CP, and subplate (SP), are seen (*A*). SATB2 expresses in the upper region of CP (*B*). CUTL1 diffusely expresses in the whole cortex and intermediate zone (*C*). FOXP1-positive cells locate in the middle region (*D*) and CTIP2-immunopositive cells (*F*) locate in the lower region of CP. OTX1 exhibits in CP and SP, predominantly lower region of CP (*E*). TBR1-immunopositive cells are in the lower region of CP and SP, as well as those fibers in CP (*G*). *A*, HE; *B–G*, SATB2, CUTL1, FOXP1, OTX1, CTIP2, and TBR1 immunohistochemistry, respectively. Scale bar: 100 μm.

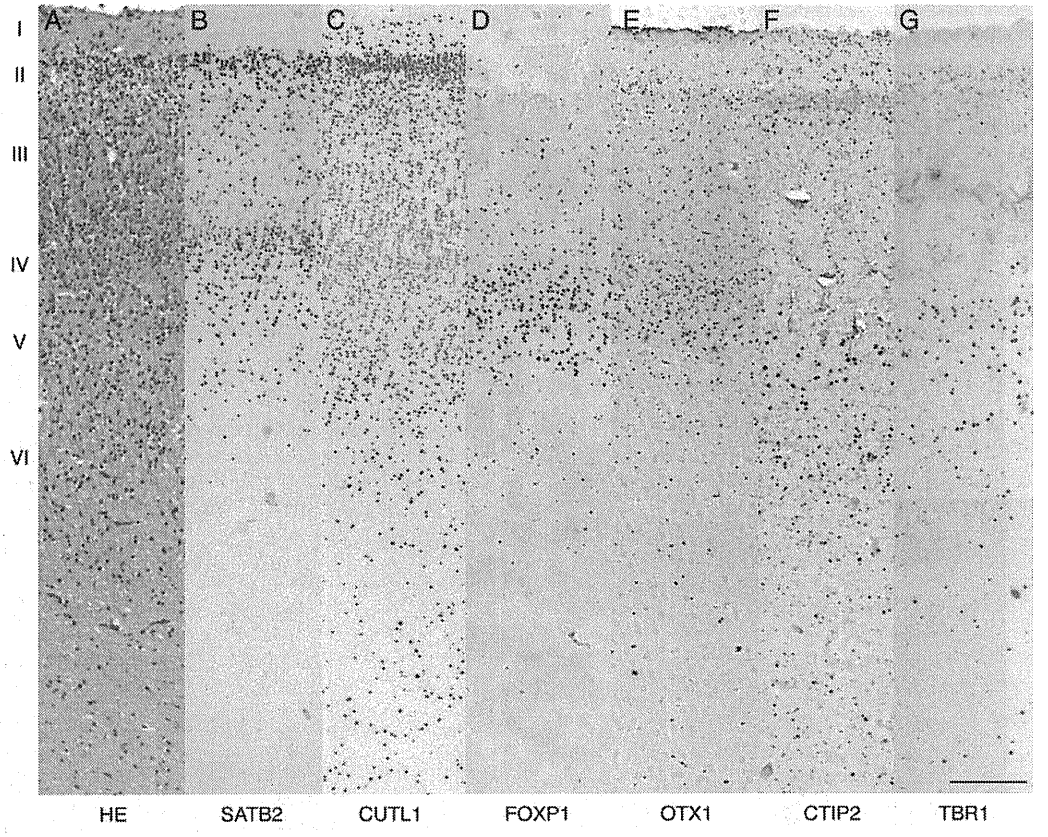

Figure 2. Layer-specific marker expression of the neocortex at 29 GWs. The 6-layer neocortex is shown (*A*). SATB2 expresses in layers II–V, especially layer II and upper region of layer IV (*B*). CUTL1 diffusely expresses in layers II–V and predominates in layer II (*C*). FOXP1 converges to layers VI and V (*D*). OTX expresses in upper layer and layers VI and V (*E*). CTIP2- and TBR1-immunopositive cells locate in layer V and layers V and VI (*F* and *G*). *A*, HE; *B-G*, SATB2, CUTL1, FOXP1, OTX1, CTIP2, and TBR1 immunohistochemistry, respectively. Scale bar: 100 μm.

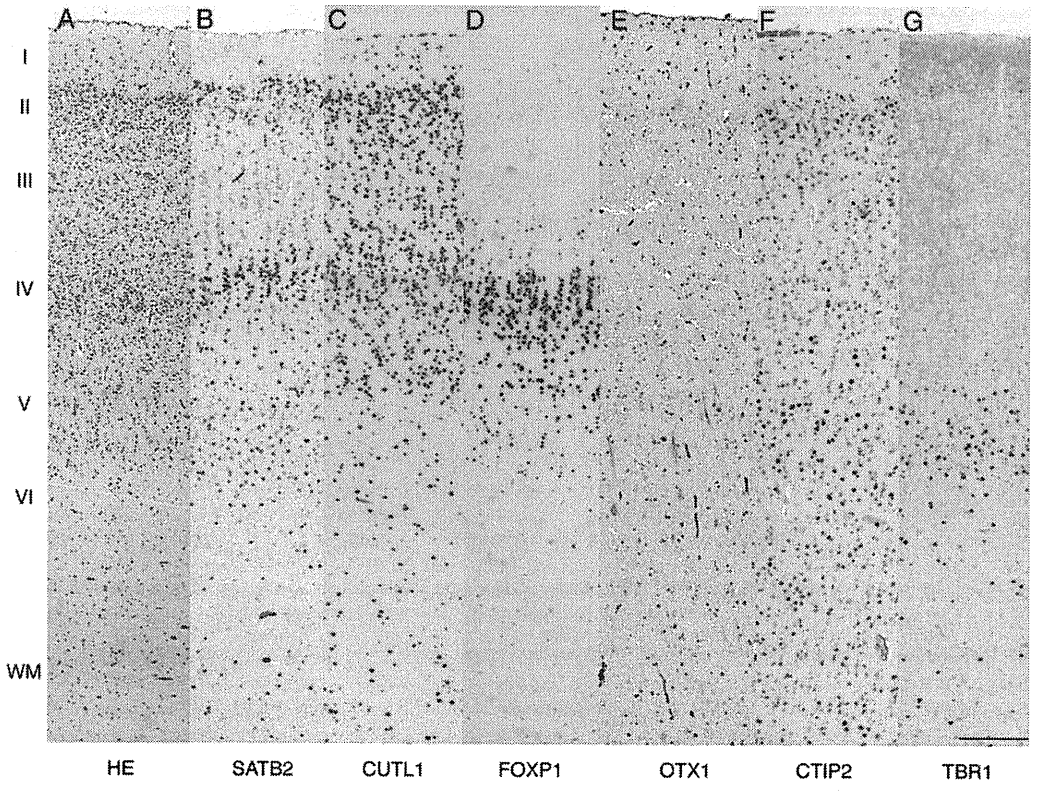

Figure 3. Layer-specific marker expression of the neocortex at 37 GWs. Expression of SATB2, CUTL1, FOXP1, OTX1, CTIP2, and TBR1 has a pattern similar to those at 29 GWs. OTX1 disappears in upper layer of neocortex (*E*). *A*, HE; *B–G*, SATB2, CUTL1, FOXP1, OTX1, CTIP2, and TBR1 immunohistochemistry, respectively. Scale bar: 100 μm.

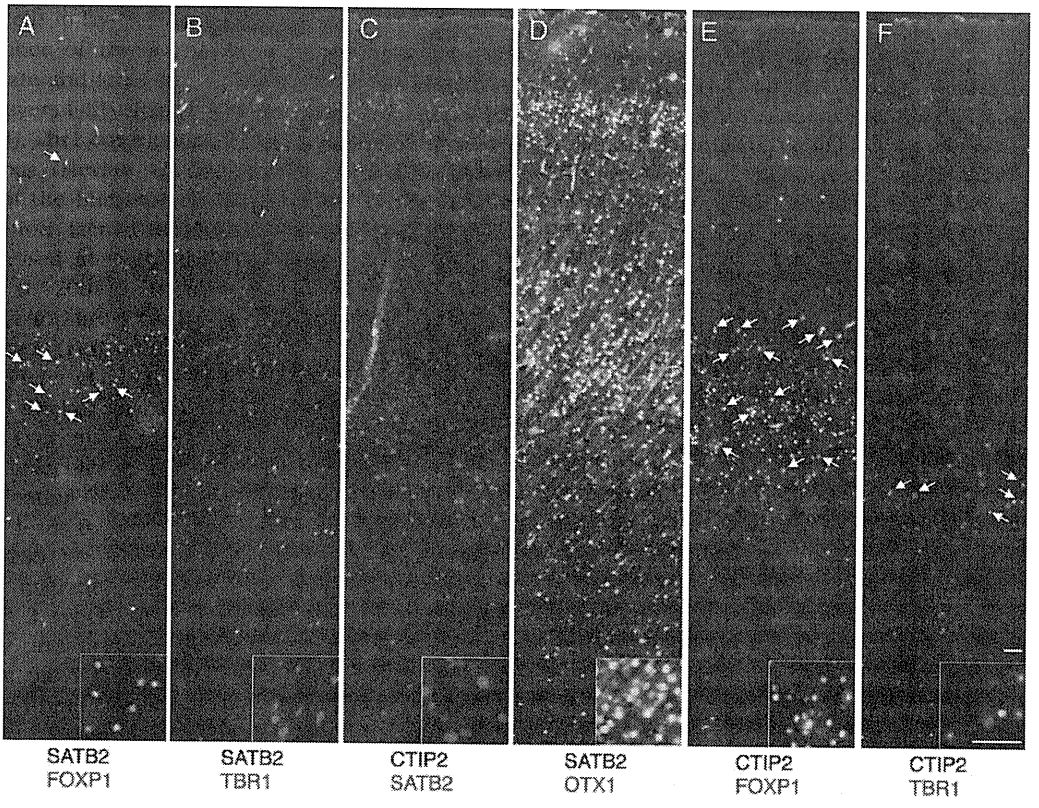

Figure 4. Immunofluorescence of layer-specific marker of neocortex at 29 GWs. FOXP1-immunopositive cells partially have SATB2 (merged color: arrows) in layers II–III and IV–V (*A*) and CTIP2 (merged color: arrows) in layers IV–VI (*E*). No double-positive cells for SATB2 and CTIP2 are scattered throughout all layers (*C*). No TBR1+ and SATB2+ cells are observed in layers V and VI (*B*), but a few TBR1+ and CTIP2+ cells are seen in layers V and VI (*F*). Many merged cells with SATB2 (red) and OTX1 (green) are diffusely demonstrated, predominantly in layers II and V (*D*). *A*, SATB2 (red) and FOXP1 (green) double fluorescence; *B*, SATB2 (red) and TBR1 (green); *C*, CTIP2 (red) and SATB2 (green); *D*, SATB2 (red) and OTX1 (green); *E*, CTIP2 (red) and FOXP1 (green); *F*, CTIP2 (red), and TBR1 (green). Scale bars: 20 μm.

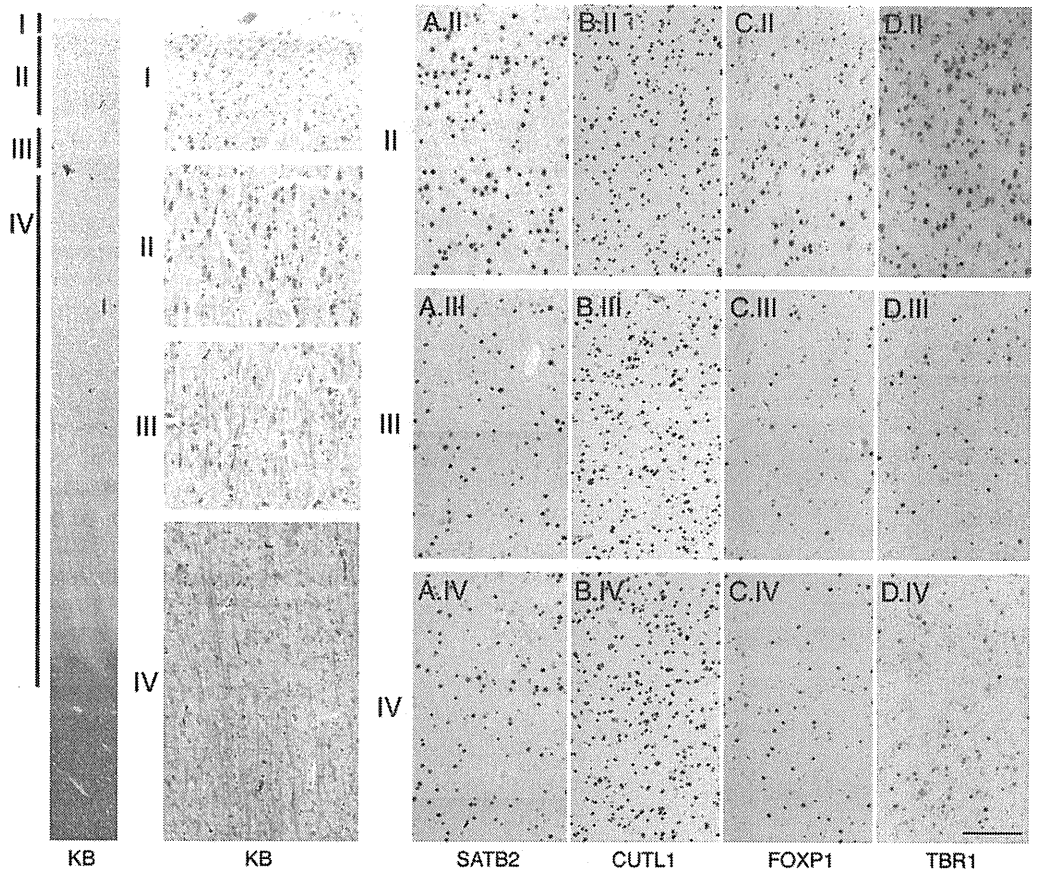

Figure 5. Layer-specific marker expression of the neocortex of 1-year-old patient with Miller–Dieker syndrome. Typical 4-layer pattern is shown. (*A*) SATB2, CUTL1, FOXP1, and TBR1 are diffusely expressed in layers II, III, and IV. Especially, TBR1-immunopositive cells locate in layer II (*E*). Enlargement of layer II shows *A.II*, *B.II*, *C.II*, and *D.II*. Enlargement of layer III shows *A.III*, *B.III*, *C.III*, and *D.III*. Enlargement of layer IV shows *A.IV*, *B.IV*, *C.IV*, and *D.IV*. Gross histology shows with KB staining. *A.II*, *A.III*, and *A.IV*, SATB2 in layers II, III, and IV; *B.II*, *B.III*, and *B.IV*, CUTL1; *C.II*, *C.III*, and *C.IV*, FOXP1; *D.II*, *D.III*, and *D.IV*, TBR1, respectively. Scale bar: 100 μm.

neurons (layer II), and a deep layer (layer III) (Fig. 6*A*). SATB2+ and CUTL1+ cells located in the intermediate layer and upper region of the deep layer (Fig. 6*B,C*). FOXP1+ cells and TBR1+ cells were also distributed in layer II and III (Fig. 6*D,E*). These labeled cells in the deep intermediate layer were large and dense but small and sparse in the upper region of the intermediate layer. Also, in the molecular layer, FOXP1+ and TBR1+ cells were few. No CTIP2+ and OTX1+ cells were observed in either malformed brain.

Usually, FCMD cerebral cortices show type II lissencephaly with cobblestone cortex. The cerebral cortices of FCMD fetus already revealed typical cobblestone lissencephaly (Fig. 7*A*). Neurons of the fetal neocortex migrated over the glia limitans. SATB2+, CULT1+, FOXP1+, CTIP2+, and TBR1+ cells were dense above the glia limitans and sparse below it (Fig. 7*B–D*), and TBR1+ cells were distributed predominantly below the glia limitans (Fig. 7*E*). However, no markers were detected in specimens from postnatal FCMD brains (data not shown).

The layer-specific marker expression pattern of 3 types of lissencephalies was summarized in Supplementary Figure 2.

Discussion

Very little is known about the molecular mechanism of human neocortex layer formation. Here, we presented new knowledge regarding the layer-specific marker expression in fetus development. Recent neuronal developmental studies have introduced some molecules as layer-specific markers. Among them, Satb2, Cutl1, Foxp1, Otx1, Ctip2, and Tbr1 are well-known transcriptional factors and highly conserved. The facts that SATB2 was relatively limited to layers II and IV of human fetus cortex and that Cutl1 was not known in human but was expressed in layers II–IV evidenced the same expression patterns of these molecules in rodent study (Nieto et al. 2004; Britanova et al. 2008). The migration pattern of callosal projection neurons may be the same as that in the mouse. FOXP1+ cells located in deep layers or layers IV–V before 30 GW and in layers IV–VI before birth. TBR1+ cells located in layers V–VI in the fetal period. FOXP1+ and TBR1+ cell localization in layers IV and V was similar to those in a previous human study (Sheen et al. 2006). However, TBR1+ cells were located beneath FOXP1+ cells but not colocalized. The restricted distribution of CTIP2+ cells in layer V may reflect the corticospinal projection formation, as indicated by mouse *ctip2* analysis (Arlotta et al. 2005). Interestingly, SATB2+ cells were located in the upper region of layer IV and FOXP1+ cells in the lower region of the same layer. This different localization indicates completely different neural functions between SATB2 and FOXP1, although the FOXP1 function in neocortex is unknown.

In mouse neocortex, Otx1+, Tbr1+, Ctip2+, Foxp1+, Cutl1+, and Satb2+ neurons are born around embryonic day 12.5, 10.0,

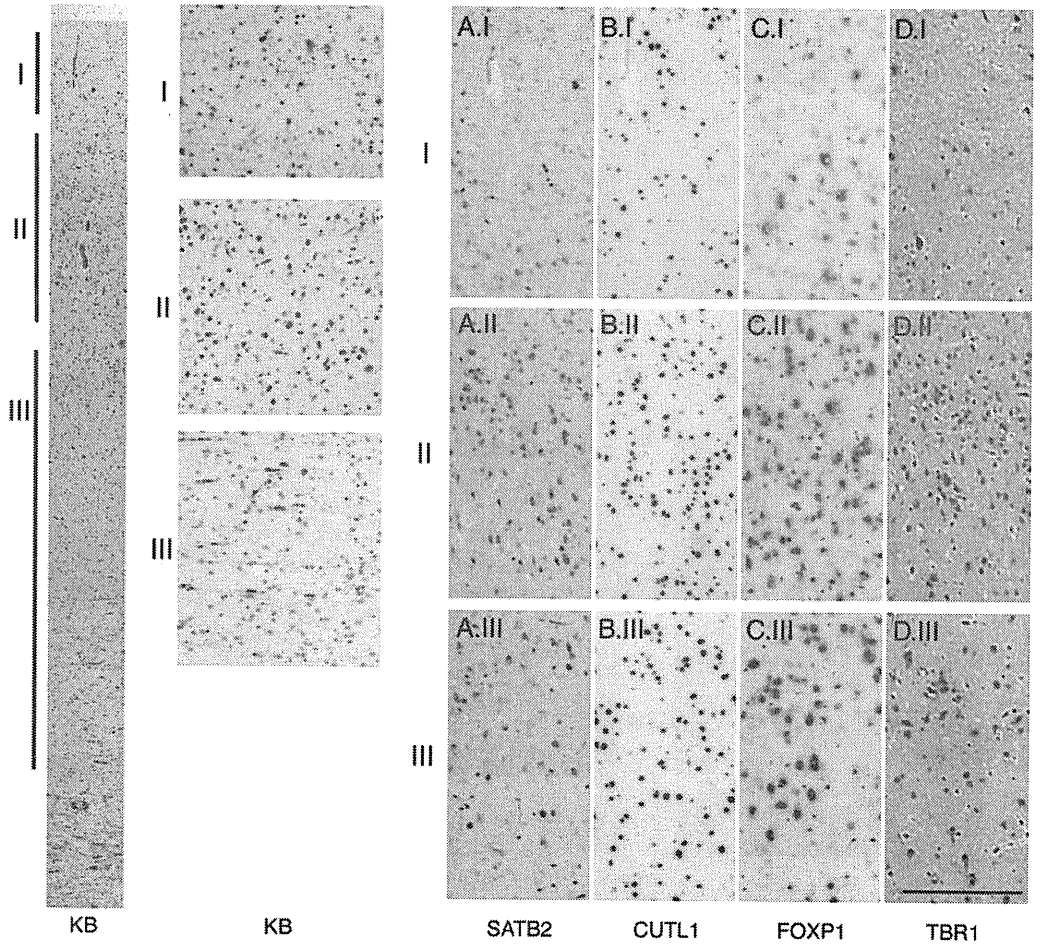

Figure 6. Layer-specific marker expression of the neocortex of 10-month-old boy with XLAG. Neocortex shows a thin 3-layer pattern. SATB2-, CUTL1-, FOXP1-, and TBR1-immunopositive cells locate diffusely (*A–D*). Gross histology shows with KB staining. *A.I*, *A.II*, and *A.III*, SATB2 in layers I, II, and III; *B.I*, *B.II*, and *B.III*, CUTL1; *C.I*, *C.II*, and *C.III*, FOXP1; *D.I*, *D.II*, and *D.III*, TBR1, respectively. Scale bar: 100 μm.

12.0, 14.5, 13.0, and 13.5, respectively (Simeone et al. 1993; Bulfone et al. 1995; Hevner et al. 2001; Ferland et al. 2003; Leid et al. 2004; Nieto et al. 2004; Arlotta et al. 2005; Britanova et al. 2005). These labeling neurons originate from progenitor cells residing in the ventricular zone (VZ) and the subventricular zone (SVZ) of early developing brain. Early progenitor cells in VZ produce deep layer neurons expressing Ctip2. On the contrary, late progenitor cells in SVZ form upper layers, expressing Cutl1 (Nieto et al. 2004). The previous data that Satb2-null mice show loss of Cutl1+cells in the superficial layers (Alcamo et al. 2008) suggest the profound molecular relationship of Satb2 and Cutl1. Satb2+ cells directly contribute to the formation of a callosal projection of the bilateral neocortical connection (Alcamo et al. 2008), while Ctip2+ cells contribute to the formation of a corticospinal projection forming a long pathway between the neocortex and anterior horn of the spinal cord (Arlotta et al. 2005). Interestingly, the expression patterns of SATB2 and CTIP2 in human neocortex mimicked those of rodent, and SATB2+ cells were also found in part of layer V. Although SATB2+ cells and CTIP2+ cells were in layer V, these double-marked cells were not observable. This may indicate these cells have different functions. From rodent study, 2 major projection neurons, callosal and subcortical, are formed by Satb2 and Ctip2 interaction (Leone et al. 2008), which may be at work in the human fetal neocortex. The finding of no double-labeled cells with CTIP2 and SATB2 in human neocortex is compatible with the rodent data (Leone et al. 2008). Otx1 in mouse brain also expresses in layer V and contributes to the formation of the corticospinal projection (Frantz et al. 1994; Weimann et al. 1999). CTIP2+/OTX1+ cells may be closely related to the forming of the corticospinal projection. Interestingly, we found many SATB2+/OTX1+ cells in layer V. OTX1 may play an essential role in the specification of both callosal and corticospinal projection neurons, although the detailed interaction between OTX1 and CTIP2 remains unknown. Moreover, FOXP1+ cells expressed SATB2 and CTIP2 in layer V. It is unknown whether a relationship exists between Foxp1 and Satb2 or Foxp1 and Ctip2, although Ctip2 is known to colocalize with Foxp1 in mouse striatum (Arlotta et al. 2008). FOXP1 may also contribute callosal and corticospinal projection neurons. FOXP1 disappeared earlier than OTX1 (Figs 2 and 3 and Supplementary Figure 1). FOXP1 could strongly control forming corticospinal projection. Tbr1+ cells derived from the earliest progenitor cells locate in layer VI (Hevner et al. 2003) and contribute to the development of corticothalamic projection neurons (Hevner et al. 2001, 2002; Guillemot et al. 2006; Leone et al. 2008). In our data, the TBR1+ cells that expressed CTIP2 in layer VI may form corticothalamic projections, as in rodent studies.

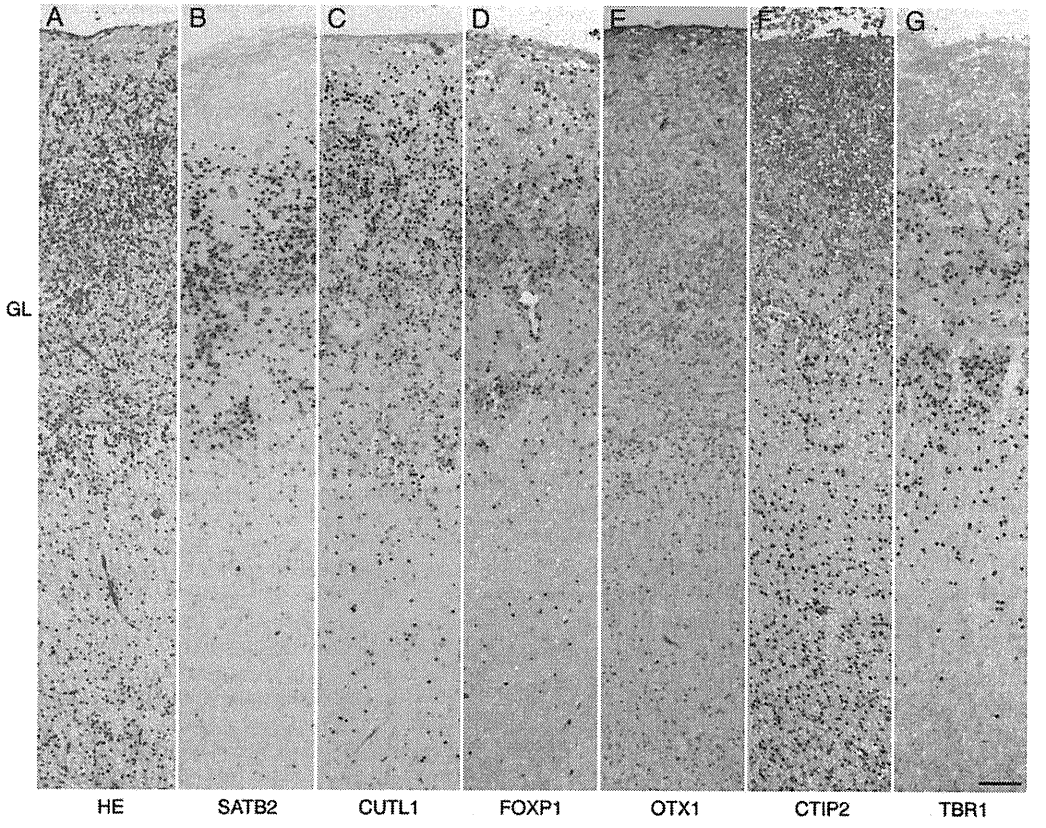

Figure 7. Layer-specific marker expression of the neocortex of 19-GW fetus with FCMD. Neocortex shows typical cobblestone lissencephaly feature. Many SATB2-, CUTL1-, FOXP1-, OTX1, CTIP2-, and TBR1-immunopositive cells migrate over the glia limitans (*B–G*), while some labeled cells locate under it. GL, glia limitans; *A*, HE; *B–G*, SATB2, CUTL1, FOXP1, OTX1, CTIP2, and TBR1 immunohistochemistry, respectively. Scale bar: 100 μm.

On the other hand, malformed neocortices revealed unique distributions of the layer-specific markers. In MDS, due to deletion of 17p13.3 with LIS1 gene, it has been thought that neurons of the superficial layer are neuronal components of the fundamental deep layers, and neurons of the deep layers consist of neuronal components of layers II–IV in the normal neocortex (Ferrer et al. 1987). Also, MDS neocortical lamination was found to have an inverted organization (Viot et al. 2004). However, recently the neocortex of 33 GW MDS has reportedly demonstrated FOXP1+ cell in the deep layers or TBR1+ cells in the first 3 layers (Sheen et al. 2006). MDS neocortical lamination was concluded to be preserved and noninverted. Our MDS findings supported noninverted lamination because of the diffuse expression pattern of all layer-specific markers. XLAG, caused by loss of function mutations of ARX gene concerned with differentiation and migration of γ-aminobutyric acidergic interneurons, shows a 3-layer lissencephalic neocortex (Kitamura et al. 2002; Bonneau et al. 2002; Cobos et al. 2005; Forman et al. 2005). Although ARX-null mice exhibit nearly normal layer formation of the cerebral cortex (Kitamura et al. 2002), the human XLAG neocortex was reported to consist of 3 layers with uniform pyramidal neurons (Bonneau et al. 2002; Okazaki et al. 2008). From our observation of layer-specific markers in layers II and III, XLAG might also be a random migration pattern. In human brain, ARX involves migration of not only interneurons but also projection neurons (Okazaki et al. 2008). XLAG neocortex may have an abnormal interneuron migration pattern, although in the present study this could not be demonstrated. Interestingly, our postnatal patients with MDS and XLAG revealed persistent expression of these layer-specific markers, which was not found in the normal neocortex. This suggests that MDS or XLAG neurons arrest in the premature or undifferentiated stage.

Further investigation is needed to determine why these layer-specific markers are expressed in postnatal brains, and the nature of their molecular function. Moreover, we investigated neocortices of typical type II lissencephaly, FCMD. Various-sized and/or disoriented neurons were widely scattered in the neocortex. In FCMD fetal brain, the layer-specific markers diffusely expressed over and under glia limitans (Fig. 7). Obviously, the FCMD fetal neocortex had completely lost its layer formation. The layer-formation pattern of WWS fetus presents the same result as ours (Hevner 2007). This type II lissencephaly, cobblestone lissencephaly, may commonly have this pathological construction. Postnatal FCMD demonstrated no expression of the layer-specific markers and was different from MDS and XLAG. Neuronal maturation of FCMD neocortex may be more advanced than other types of lissencephalies. This leads us to conclude that FCMD patients have a relatively low incidence of epilepsy and some cases are mild (Guerrini and Filippi 2005; Spalice et al. 2009).

Our study suggests that the laminar formation pattern of human and rodent neocortices is fundamentally the same. One of the characteristics of the human neocortex is its gyration, which is 1000-fold in the neocortical surface area between human and rodent (Bystron et al. 2006; Rakic 2009). It is thought that not only the number of neuronal progenitors but also the number of radial glial cells in human brain is much

larger than in the rodent. As a result, the human neocortex must fold and form gyrations. However, in case of abnormal expression of migration- or proliferation-related genes or environments such as trauma and infection, the number of neuronal progenitor cells, and radial glial cells may serve to reduce and influence the migration pattern.

We may conclude that the neocortex of lissencephalies is formed by a unique type of neuronal migration. The late-birth cells in MDS may migrate randomly but not the early-birth cells. In XLAG, SATB2+, and TBR1+ cells distribute in the relatively deep layers, but CUTL1+ and FOXP1+ cells may follow a random migration pattern. FCMD shows the most random pattern. We must seek to understand the mechanism behind these differences. The molecular mechanism of neuronal movement is well known. Lis-1 or Dcx is a modulator of radial migration and contributes to layer formation (Hirotsune et al. 1998; Meyer et al. 2002; Bai et al. 2003). In human layer formation, various projection neurons originate from VZ or SVZ and migrate radially depending on the time of cell birth. In interneuron development, Cutl1 and Cutl2 contribute to Reln expression and control the number of the interneuron subpopulation (Cubelos et al. 2008). However, little is known about interaction between the layer-specific markers (transcription factors) and neuron kinetic factors including Lis-1, Dcx and Reln. Further study is warranted to obtain more information in this regard.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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