The role of UNC5H4 in cortical development

（大脳皮質の発達における UNC5H4 の役割）

大阪大学大学院生命機能研究科

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Makoto Takemoto

Cellular and Molecular Neurobiology Laboratory (Yamamoto Lab.)
Graduate School of Frontier Biosciences, Osaka University
Suita, Osaka

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Summary

During development, neurons differentiate into specific cell types with distinct cell morphology, physiological properties and neural connections. One major question in developmental neuroscience is how cell type-specific differentiation is regulated. In general, it is known that intrinsic and extrinsic factors cooperate to regulate cell specification and differentiation. In the neocortex, neurons display different characteristics according to their laminar and area locations. Although much progress has been made in understanding the molecular basis for intrinsic regulations of laminar and area specification, it is still poorly understood how lamina- and area-specific molecules such as membrane-associated proteins contribute to cortical development through interacting with extrinsic cues.

In this thesis, I studied the molecular mechanisms underlying layer-specific cortical development, focusing on layer 4. For this purpose, I searched for the genes that are expressed in layer 4 of the developing rodent neocortex by constructing a subtraction cDNA library. As a result, *unc5h4*, a netrin receptor family member, was identified. *In situ* hybridization analysis revealed that *unc5h4* gene was expressed strongly in layer 4 of the primary sensory cortices, the target of sensory thalamic afferents.

To determine the ligand of UNC5H4, protein binding to UNC5H4 was examined. The result showed that netrin-4 as well as netrin-1 protein bound to UNC5H4-expressing HEK293T cells. Expression of *netrin-4* gene was observed in the neocortex with a higher level in layer 4 and sensory thalamic nuclei, suggesting that netrin-4 can affect cortical neurons which express *unc5h4*. To further study the role of UNC5H4 in cortical development, the effect of netrin-4 on *unc5h4*-expressing cortical neurons was investigated using dissociated cell culture. I found that cell death of *unc5h4*-expressing layer 4 cells was suppressed by the application of netrin-4 protein. In contrast, netrin-4 had no effect on deep layer cells even though *unc5h4* was overexpressed. These results suggest that UNC5H4 is predominantly expressed in layer 4 neurons in the primary sensory areas of the developing neocortex and may mediate the effect of netrin-4 on cortical cell survival in a lamina-specific manner.

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General Introduction

In the developing nervous system, neurons are born in the germinal zone, migrate to their appropriate positions and make neural connections, while some of them disappear due to programmed cell death (Sanes et al., 2006). These developmental processes lead to the formation of orderly brain structures, expressing complex functions. Neural progenitor cells acquire cell type-specificity during these processes. In many parts of the nervous system such as cerebellum, hippocampus, olfactory bulb, retina and tectum, cell type-specificity appears as layer-specificity. The mammalian neocortex also has laminar structure, which is mainly composed of six cell layers, and in addition, the layer structure and neuronal connectivity are different among areas (Ramon Y Cajal, 1911). About a century ago, Ramón Y Cajal had already found that cortical neurons have a variety in their morphology and neural connections according to their laminar and area locations (Ramon Y Cajal, 1911). Since then, a large number of studies have been accumulated, providing evidence that neurons display different cell morphology, physiological properties, neural connections and molecular expressions among layers and areas (Gilbert, 1983; O'Leary and Koester, 1993; Hevner et al., 2003). One of major questions in current developmental neuroscience is how such cell type-specific differentiation is regulated.

The molecular mechanisms that specify the identity of neural cells have begun to be elucidated in the dorsoventral patterning of the neural tube and motor neuron subtype specification (Jessell, 2000; Shirasaki and Pfaff, 2002). For instance, Sonic hedgehog, secreted by the notochord and floor plate, regulates the expression of a

combinatorial code of transcription factors that specifies the identity of motor neuron subtypes (Jessell, 2000; Shirasaki and Pfaff, 2002). Similarly, in the neocortex, it is generally hypothesized that specification and differentiation of neurons are controlled by coordinated action of intrinsic and extrinsic factors (Rakic, 1988; O'Leary, 1989; O'Leary and Nakagawa, 2002; Grove and Fukuchi-Shimogori, 2003; Guillemot et al., 2006; Molyneaux et al., 2007). As intrinsic mechanisms, transcription factors such as Emx2, Pax6, COUP-TFI and Sp8, which are expressed in distinct graded manners, may participate in area patterning during early corticogenesis (O'Leary and Sahara, 2008). A plausible mechanism in cortical neuron differentiation is that a combinatorial set of transcription factors confer laminar and area identity on cortical neuronal progenitors or postmitotic neurons, and the downstream molecules including intracellular, membrane-bound and secreted molecules regulate lamina- and area-specific cell differentiation and neuronal connectivity. Therefore, a key approach is to identify the molecules that are expressed with lamina- and area-specificity. Recently, much progress has been made in identifying the transcription factors involved in laminar and subtype specification including axonal connectivity. For instance, analyses of mutant mouse models have indicated that *Otx1*, *Fezl* and *Ctip2* may specify a subset of layer 5 subcortical and/or corticocortical projection neurons (Weimann et al., 1999; Arlotta et al., 2005; Chen et al., 2005; Molyneaux et al., 2005), while *Tbr1* and *Satb2* as well as *Cux2* may be involved in cell differentiation of a subset of layer 6 and upper layer neurons, respectively (Hevner et al., 2001; Cubelos et al., 2007; Britanova et al., 2008).

On the other hand, environmental factors also play a role in cortical cell differentiation. Molecules secreted from signaling centers, such as FGF8, FGF17, BMPs

and Wnts, have been reported to modulate the expression of some transcription factors such as *Emx2* and *COUP-TFI*, and also some area-specific marker genes (Fukuchi-Shimogori and Grove, 2001; Grove and Fukuchi-Shimogori, 2003; O'Leary and Nakagawa, 2002; O'Leary et al., 2007). In addition, a study demonstrated that brain-derived neurotrophic factor altered the laminar fate of cortical neurons by accelerating the completion of S-phase of a cell cycle (Fukumitsu et al., 2006). Another notable extrinsic factor is an afferent-derived factor. A transplantation experiment demonstrated that a piece of fetal rat occipital cortex, where the barrel is originally not formed, transplanted to the barrel field of newborn parietal cortex generated the barrels after thalamic innervations (Schlaggar and O'Leary, 1991). An organotipic coculture experiment also demonstrated that cell death of subplate cells in cortical explants was prevented by coculture with thalamic explants, whereas they died in isolated cortical explants (Price and Lotto, 1996). Another experiment using coculture of thalamic explants with cortical precursor cells further showed that the proliferation of cortical precursors was promoted by thalamic axons (Dehay et al., 2001). Thus, thalamic afferents may be also important extrinsic factors for cortical differentiation. Furthermore, an abnormal barrel cytoarchitecture in the primary somatosensory cortex was observed in several genetic mouse models, which cause an excess of serotonin in the brain (Cases et al., 1996; Persico et al., 2001), suggesting that serotonin derived from the brainstem may contribute to the barrel formation in layer 4 of the neocortex as an extrinsic factor.

After laminar and area fate determination, several membrane-associated protein genes such as *cadherins*, a G-protein-coupled receptor like molecule *rCNL3* and

an Ig-containing cell adhesion molecule *MDGA1*, are expressed in a layer- or area-specific fashion (Suzuki et al., 1997; Chenn et al., 2001; Takeuchi and O'Leary, 2006). Moreover, MDGA1 is required for the migration of layer 2/3 neurons to their appropriate position (Takeuchi and O'Leary, 2006). Thus, these molecules may play an important role in the subsequent cell development including cell migration, morphological and physiological cell maturation, the formation and maintenance of neural connections and cell survival, by interacting with environmental cues. However, it is still poorly understood how extrinsic factor-related molecules, especially expressed as a result of cell fate determination, contribute to the subsequent layer- and area-specific cell differentiation during cortical development.

Our group has been focusing on layer 4 of the neocortex because of thalamocortical connectivity. The thalamocortical projection exhibits typical laminar specificity (Jones, 1981; Gilbert, 1983) and has also been well characterized during development (Lund and Mustari, 1977; Ghosh and Shatz, 1992; Agmon et al., 1993; Kageyama and Robertson, 1993; Catalano et al., 1996; Molnár et al., 1998). Our previous investigations have suggested that thalamocortical axon branching is induced by membrane-associated molecules in layer 4, whereas termination of axonal growth is regulated by growth-inhibitory molecules in layers 2/3 and 4 (Yamamoto, 2002). These findings suggest that the factors regulating thalamocortical axon targeting and differentiation of target layer cells are expressed specifically in the upper layers.

In this study, to elucidate the molecular mechanisms underlying layer-specific cortical cell differentiation and neural circuit formation, in chapter I, I searched for genes that are expressed in upper layers of the developing rodent neocortex and

identified that *unc5h4* gene was predominantly expressed in layer 4 and the primary sensory areas, and in chapter II, I studied the ligand of UNC5H4 and their role in cortical development.

References for general introduction

Agmon A, Yang LT, O'Dowd DK, Jones EG (1993) Organized growth of thalamocortical axons from the deep tier of terminations into layer IV of developing mouse barrel cortex. J Neurosci 13:5365-5382.

Arlotta P, Molyneaux BJ, Chen J, Inoue J, Kominami R, Macklis JD (2005) Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. Neuron 45:207-221.

Britanova O, de Juan Romero C, Cheung A, Kwan KY, Schwark M, Gyorgy A, Vogel T, Akopov S, Mitkovski M, Agoston D, Sestan N, Molnar Z, Tarabykin V (2008) Satb2 is a postmitotic determinant for upper-layer neuron specification in the neocortex. Neuron 57:378-392.

Cases O, Vitalis T, Seif I, De Maeyer E, Sotelo C, Gaspar P (1996) Lack of barrels in the somatosensory cortex of monoamine oxidase A-deficient mice: role of a serotonin excess during the critical period. Neuron 16:297-307.

Catalano S, Robertson RT, Killackey H (1996) Individual axon morphology and thalamocortical topography in developing rat somatosensory cortex. J Comp Neurol 366, 36-53.

Chen B, Schaevitz LR, McConnell SK (2005) Fezl regulates the differentiation and axon targeting of layer 5 subcortical projection neurons in cerebral cortex. Proc Natl Acad Sci U S A 102:17184-17189.

Chenn A, Levin ME, McConnell SK (2001) Temporally and spatially regulated expression of a candidate G-protein-coupled receptor during cerebral cortical development. J Neurobiol 46:167-177.

Cubelos B, Sebastian-Serrano A, Kim S, Moreno-Ortiz C, Redondo JM, Walsh CA, Nieto M (2007) Cux-2 Controls the Proliferation of Neuronal Intermediate Precursors of the Cortical Subventricular Zone. Cereb Cortex.

Dehay C, Savatier P, Cortay V, Kennedy H (2001) Cell-cycle kinetics of neocortical precursors are influenced by embryonic thalamic axons. J Neurosci 21:201-214.

Fukuchi-Shimogori T, Grove EA (2001) Neocortex patterning by the secreted signaling molecule FGF8. Science 294:1071-1074.

Fukumitsu H, Ohtsuka M, Murai R, Nakamura H, Itoh K, Furukawa S (2006) Brain-derived neurotrophic factor participates in determination of neuronal laminar fate in the developing mouse cerebral cortex. J Neurosci 26:13218-13230.

Ghosh A, Shatz CJ (1992) Pathfinding and target selection by developing geniculocortical axons. J Neurosci 12:39-55.

Gilbert CD (1983) Microcircuitry of the visual cortex. Annu Rev Neurosci 6:217-247.

Grove EA, Fukuchi-Shimogori T (2003) Generating the cerebral cortical area map. Annu Rev Neurosci 26:355-380.

Guillemot F, Molnar Z, Tarabykin V, Stoykova A (2006) Molecular mechanisms of cortical differentiation. Eur J Neurosci 23:857-868.

Hevner RF, Daza RA, Rubenstein JL, Stunnenberg H, Olavarria JF, Englund C (2003) Beyond laminar fate: toward a molecular classification of cortical projection/pyramidal neurons. Dev Neurosci 25:139-151.

Hevner RF, Shi L, Justice N, Hsueh Y, Sheng M, Smiga S, Bulfone A, Goffinet AM, Campagnoni AT, Rubenstein JL (2001) Tbr1 regulates differentiation of the preplate and layer 6. Neuron 29:353-366.

Jessell TM (2000) Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet 1:20-29.

Jones EG (1981) Anatomy of cerebral cortex: Columnar input-output organization. In: The Organization of the Cerebral Cortex (Schmitt FO,Worden FG, Adelman G, Dennis SG, ed.) pp.199-235. Cambridge, Massachusetts, London, England: The MIT press.

Kageyama GH, Robertson RT (1993) Development of geniculocortical projections to visual cortex in rat. J Comp Neurol 335:123-148.

Lund RD, Mustari MJ (1977) Development of geniculocortical pathway in rats. J Comp Neurol 173:289-306.

Molnár Z, Adams R, Blakemore C (1998) Mechanisms underlying the early establishment of thalamocortical connections in the rat. J Neurosci 18:5723-5745.

Molyneaux BJ, Arlotta P, Menezes JR, Macklis JD (2007) Neuronal subtype specification in the cerebral cortex. Nat Rev Neurosci 8:427-437.

Molyneaux BJ, Arlotta P, Hirata T, Hibi M, Macklis JD (2005) Fezl is required for the birth and specification of corticospinal motor neurons. Neuron 47:817-831.

O'Leary DD (1989) Do cortical areas emerge from a protocortex? Trends Neurosci 12:400-406.

O'Leary DD, Koester SE (1993) Development of projection neuron types, axon pathways, and patterned connections of the mammalian cortex. Neuron 10:991-1006.

O'Leary DD, Nakagawa Y (2002) Patterning centers, regulatory genes and extrinsic mechanisms controlling arealization of the neocortex. Curr Opin Neurobiol 12:14-25.

O'Leary DD, Sahara S (2008) Genetic regulation of arealization of the neocortex. Curr Opin Neurobiol.

O'Leary DD, Chou SJ, Sahara S (2007) Area patterning of the mammalian cortex. Neuron 56:252-269.

Persico AM, Mengual E, Moessner R, Hall FS, Revay RS, Sora I, Arellano J, DeFelipe J, Gimenez-Amaya JM, Conciatori M, Marino R, Baldi A, Cabib S, Pascucci T, Uhl GR, Murphy DL, Lesch KP, Keller F (2001) Barrel pattern formation requires serotonin uptake by thalamocortical afferents, and not vesicular monoamine release. J Neurosci 21:6862-6873.

Price DJ, Lotto RB (1996) Influences of the thalamus on the survival of subplate and cortical plate cells in cultured embryonic mouse brain. J Neurosci 16:3247-3255.

Rakic P (1988) Specification of cerebral cortical areas. Science 241:170-176.

Ramon Y Cajal S (1911) Histology of the Nervous System of Man and Vertebrates (translated in 1995). New York: Oxford University Press.

Sanes DH, Reh TA, Harris WA (2006) Development of the nervous system, Second Edition: Elsevier Academic Press.

Schlaggar BL, O'Leary DD (1991) Potential of visual cortex to develop an array of functional units unique to somatosensory cortex. Science 252:1556-1560.

Shirasaki R, Pfaff SL (2002) Transcriptional codes and the control of neuronal identity. Annu Rev Neurosci 25:251-281.

Suzuki SC, Inoue T, Kimura Y, Tanaka T, Takeichi M (1997) Neuronal circuits are subdivided by differential expression of type-II classic cadherins in postnatal mouse brains. Mol Cell Neurosci 9:433-447.

Takeuchi A, O'Leary DD (2006) Radial migration of superficial layer cortical neurons controlled by novel Ig cell adhesion molecule MDGA1. J Neurosci 26:4460-4464.

Weimann JM, Zhang YA, Levin ME, Devine WP, Brulet P, McConnell SK (1999) Cortical neurons require Otx1 for the refinement of exuberant axonal projections to subcortical targets. Neuron 24:819-831.

Yamamoto N (2002) Cellular and molecular basis for the formation of lamina-specific

thalamocortical projections. Neurosci Res 42:167-173.

Chapter I

Identification of a layer 4-specific gene and analysis of its expression in the developing neocortex

Summary

The neoocortex is fundamentally composed of six cell layers, and the laminar properties are different among areas. During development, neurons differentiate into a variety of cell types based on their laminar and area specification. An intriguing question is how layer- and area-specific development of cortical neurons is regulated. To explore the molecular basis, I searched for genes that are expressed in a laminar specific fashion. Especially I focused on layer 4 since it is related to the thalamocortical connectivity, and therefore constructed a subtraction cDNA library that was enriched for genes expressed in layer 4 of the rat neocortex at postnatal day 7, by when laminar organization and fundamental cortical circuitries have been established. As a result of differential screening and *in situ* hybridization analysis, one of cDNA fragments obtained from the library was expressed specifically in layer 4 of developing rat neocortex, although it showed no homology to any known sequence. To identify the gene of this fragment, it was extended by the 5' race method and found to be homologous to human *unc5h4* gene. Detailed analysis of the *unc5h4* expression further revealed that it was expressed in layer 4 with the predominance of the primary somatosensory and visual areas at around first postnatal week. Moreover, *unc5h4* was preferentially expressed in the target neurons for thalamic afferents in the barrel field. In addition, the area-specificity of the expression pattern in the mouse neocortex emerged at postnatal day 3. These results suggest that *unc5h4* is predominantly expressed in developing layer 4 neurons in primary sensory areas of the neocortex and may play a role in layer- and area-specific development of cortical cells and circuitries.

Introduction

The neocortex is fundamentally composed of six cell layers, which are distinguishable by cellular morphology and the extrinsic and intrinsic connections they make, and such laminar properties are different among areas (Ramon Y Cajal, 1911; Krieg, 1946; McConnell, 1989). An intriguing question is how cortical neurons differentiate into a particular laminar and area type, and are connected with a specific population of subcortical and cortical neurons. A plausible mechanism is that a set of transcriptional factors expressed in a given layer or area determine cell fate, and expression of downstream molecules including ligand and receptor molecules, are required for cell type-specific differentiation and axonal guidance of cortical afferents and efferents. Therefore, a key approach is to identify the molecules that are expressed with laminar and area specificity.

In this study, I attempted to identify lamina-specific genes, focusing on thalamocortical connectivity. The thalamocortical projection exhibits typical laminar specificity (Jones, 1981; Gilbert, 1983) and has also been well characterized during development (Lund and Mustari, 1977; Ghosh and Shatz, 1992; Agmon et al., 1993; Kageyama and Robertson, 1993; Catalano et al., 1996; Molnár et al., 1998). To date, *in vitro* studies using organotypic cocultures of the cortex with the thalamus have demonstrated that there is a target recognition mechanism by which thalamocortical axons recognize layer 4, their target (Yamamoto et al., 1989, 1992; Molnár and Blakemore, 1991, 1999; Bolz et al., 1992). Our previous investigations have further suggested that thalamocortical axon branching is induced by membrane-associated

molecules in layer 4, whereas termination of axonal growth is regulated by growth-inhibitory molecules in layers 2/3 and 4 (Yamamoto et al., 1997, 2000a, 2000b). These findings suggest that the factors regulating thalamocortical axon targeting and differentiation of target layer cells are expressed specifically in the upper layers. Based on this hypothesis, I searched for the molecules expressed specifically in these layers, in particular, layer 4, by constructing a subtraction cDNA library.

Materials and Methods

Animals

Sprague Dawley (SD) rats (Nihon Animals, Osaka, Japan) were used for the construction of a subtraction cDNA library and *in situ* hybridization analysis for screening. For the detailed gene expression analysis and immunohistochemistry for serotonin transporter (5-HTT), C57/BL6 mice and Institute of Cancer Research (ICR) mice were used. The day of vaginal plug detection is designated E0, and the day of birth is postnatal day 0 (P0).

Construction of a subtraction cDNA library

The whole brain was removed from P7 rats. Coronal slices (250 μm thickness) were cut with a microtome in ice-cold Hanks' solution. Layer 4 strips, roughly 500 μm in length, were dissected from the somatosensory cortex with a small scissors (Fig. I-1). The cortical barrel structures, which were visible under a trans-illuminating microscope, were used as a landmark for layer 4 (Fig. I-1). Virtually the same size of layer 5 strip

was dissected beneath layer 4 (Fig. I-1). Three to four pieces for each layer were collected, from which total RNAs were extracted (RNeasy Mini Kit, Qiagen, Tokyo, Japan). Approximately, 10-20 ng/μl of total RNA (30 μl) was obtained. Layer 4 and layer 5 cDNAs were synthesized from approximately 50 ng of these RNAs with reverse transcriptase (SMART PCR cDNA Synthesis Kit, Clontech, Tokyo, Japan). These cDNAs were amplified with a primer (AAGCAGTGGTAACAACGCAGAGT), and subjected to *RsaI* digestion for the following hybridization. The layer 4 DNA fragments were hybridized with an excess amount of the layer 5 DNA fragments (PCR Select cDNA Subtraction Kit, Clontech, Tokyo, Japan). Thereafter, unhybridized DNA fragments were further amplified, cloned into pGEM-T vectors (Promega, Tokyo, Japan), and stored as a subtraction cDNA library (layer 4 minus layer 5). Likewise, a reverse subtraction library (layer 5 minus layer 4) was produced.

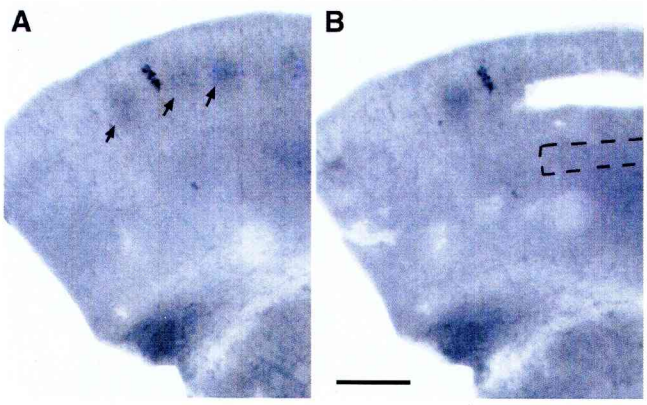


Fig. I-1. Dissection of layer 4 and 5 strips from P7 rat somatosensory cortex. (A) Somatosensory cortical slice under transilluminating light before dissection. Arrows indicate the barrels. (B) The same cortical slice after dissection of layer 4 strip. The area surrounding the dashed lines represents dissected layer 5 strip. Bar indicates 0.5 mm.

Differential screening and sequence analysis

To eliminate false-positive clones a differential screening was performed prior to *in situ* hybridization. After transformation of the subtraction cDNA library, colonies were picked randomly and grown in a 96-well plate. Each insert was amplified by PCR from the bacterial culture solution. Amplified DNAs were denatured with 0.6 M NaOH and duplicated onto two nylon membranes. Two kinds of DNA probes (layer 4 and layer 5 probes) were produced with Digoxigenin (DIG)-labeled nucleotides (Roche, Tokyo, Japan) by amplifying inserts from the two subtraction cDNA libraries (layer 4 minus layer 5 and layer 5 minus layer 4). The duplicate membranes were subjected to hybridization with each probe and detected with chemilluminescence reaction. The clones showing more than 2-fold intensity with layer 4 probes were selected and subjected to *in situ* hybridization.

The selected clones were applied to sequence analysis with the plasmid-specific sequences. In some cases, to determine the additional sequence of genes of the selected clones, extended DNA fragments were obtained by 5'race method (Smart race kit, Clontech, Tokyo, Japan). To analyze gene homology to the sequence of obtained DNA fragments, DDBJ/GenBank/EMBL DNA databases were utilized.

Isolation of the rat unc5h4 cDNA

To synthesize the *unc5h4* riboprobe for *in situ* hybridization, the cDNA encoding a part of *unc5h4* coding region was obtained as follows: Total RNA was prepared from a SD rat cortex at E18, and the first-strand cDNA was produced using the SuperScript II reverstranscriptase (Invitrogen). The cDNA encoding rat *unc5h4* (1449 bps) was

isolated by RT-PCR using the following primer pair: 5'-TTGTGAGGGAATGTCAGTG-3' and 5'-TCCTGGCATGCAGTGAATG-3'. Although the sequence of rat *unc5h4* gene was not still in public, the sequence of the fragment obtained by RT-PCR was 95.8% homologous to the 1163-2623 bp portion of mouse *unc5h4* (Accession# NM_153135). This fragment was then cloned into the pGEM-T vector (Promega).

In situ hybridization

DIG-labeled RNA probes were used for hybridization. The DNA fragments obtained from the subtraction library and another *unc5h4* fragment obtained by RT-PCR (see above) were used as templates for RNA probes. To produce linearized templates for the synthesis of riboprobes, inserts in pGEM-T vectors were amplified by PCR using oligonucleotides that contain T7 and SP6 promoter sequences. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega), and *in vitro* transcription was carried out (DIG-RNA Synthesis Kit, Roche, Tokyo). Finally, these probes were purified with gel filtration columns (mini Quick Spin RNA Columns, Roche, Tokyo) and kept at –80 degrees.

Animals were decapitated after anesthesia to obtain whole brains from postnatal animals (P0, 3, 7, 14 for rats; P0, 2, 3, 6 for mice). E18 rat brains were taken from fetuses under deep anesthesia. The brains were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) for 1 hour at room temperature and then 2-3 hours at 4 ℃. After overnight incubation with a sequential step of 15% and 30% sucrose in PBS, the brains were frozen and then cut into 10 or 20 μm sections (coronal or sagittal sections) with a cryostat. 40 μm-thick tangential sections were prepared as

described below. The serial coronal sections were prepared at approximately 250 μm intervals.

Sections were refixed in 4% PFA in 0.1 M PB, washed with distilled water and 0.1 M triethanolamine, then acetylated in 0.25% acetic acid in 0.1 M TAE, followed by a final wash in PBS. Prehybridization was carried out for 1 hour in hybridization buffer (50% formamide, 5% SDS, 5×SSPE, 0.2 mg/ml tRNA), followed by hybridization for 12 hours at 60 ℃ in hybridization buffer containing 1 μg/ml DIG-labeled RNA probe. After three washes in 50% formamide and 2×SSC at 60 ℃, these sections were subjected to blocking (blocking regent, Roche, Tokyo, Japan) for 1-2 hours at room temperature, and then incubated overnight at 4 ℃ with alkaline phosphatase-conjugated anti-DIG antibody (1:2000, Roche, Tokyo, Japan). After washing five times at room temperature, the color reaction was carried out at room temperature or 4 ℃ in BM Purple (Roche, Tokyo, Japan). The reaction was terminated by immersing the sections in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) overnight at 4 ℃, and then the sections were fixed with 4% PFA in 0.1 M PB for 15 minutes. Then sections were treated in 70%, 80%, 90% and 100% ethanol, and xylene, and then embedded.

For Nissl staining, adjacent or proximal sections were used. These sections were immersed in 0.5% cresyl violet for several minutes and then subjected to the ethanol series and embedding.

Immunohistochemistry for 5-HTT

5-HTT immunohistochemistry as a marker of the thalamocortical projections was performed mainly as described elsewhere (Rebsam et al., 2002). P7 ICR mouse brains

were fixed with 4% PFA in 0.125 M PB, followed by cryoprotection with a sequential step of 15% and 30% sucrose in PBS. To obtain tangential sections, cortical hemispheres were separated from a P7 mouse brain, flattened between two glass slides separated by 1 mm-thick spacers, and fixed for 3 hours as described above. Then, 40 μm-thick frozen sections were prepared using a cryostat. Sections were washed with PBS- (0.12 M PB, 9% saline) and incubated with PBS+ (PBS containing 0.2% gelatin and 0.25% Triton X-100) for 30 min, followed by incubation with a rabbit polyclonal anti-5-HTT antibody (1:5000, Calbiochem) overnight at room temperature. After washing with PBS+, sections were incubated with a biotinylated goat anti-rabbit antibody (1:200; Vector Laboratories) for 2 hours at room temperature, followed by a streptavidin-biotin-peroxidase complex (1:400; Amersham Biosciences) for 1.5-2 hours at room temperature, and then detected by a solution containing 0.02% diaminobenzidine, 0.003% H_2O_2 in 0.05 M Tris-HCl, pH 7.6.

Results

Construction of a subtracted cDNA library and isolation of a clone expressed in layer 4 of the neocortex

To identify the genes that are expressed in layer 4 or layer 2/3-4, I constructed a subtraction cDNA library in which cDNAs derived from layer 4 strips of P7 rat somatosensory cortex was enriched by subtracting cDNAs from layer 5 strips (see Materials and Methods). Approximately one thousand clones from the subtraction library were subjected to the differential screening in the first-round screening. As a

result, I obtained 130 positive clones, which showed stronger signals to the layer 4 probe than the layer 5 probe. Then, *in situ* hybridization was performed with each of the 49 positive clones which were selected randomly, to examine laminar specificity on P7 rat brain, in which cortical laminar configuration is established. Although most of the clones tested showed virtually no or very faint signals, four clones (#571, #585, #746 and #846) exhibited specific expression in layer 4 or layer 2/3-4 (Fig. I-2).

Among them, #571 exhibited a highly restricted expression in layer 4 (Fig. I-2A, E, I-3). Moreover, its expression was higher in the caudal rather than the rostral regions in the neocortex (Fig. I-3B). Another characteristic of this gene was its prominent expression in the piriform cortex, the amygdala and hippocampus, especially in CA3 and dentate gyrus (Fig. I-3). Moderate expression was also found in the hypothalamus (Fig. I-3B). In the thalamus, the expression was observed in the ventral part of the lateral geniculate nucleus (Fig. I-3B). In other brain regions, it was expressed in midbrain, mitral cell layers of the olfactory bulb and the Purkinje cell layer of the cerebellum (Fig. I-3B).

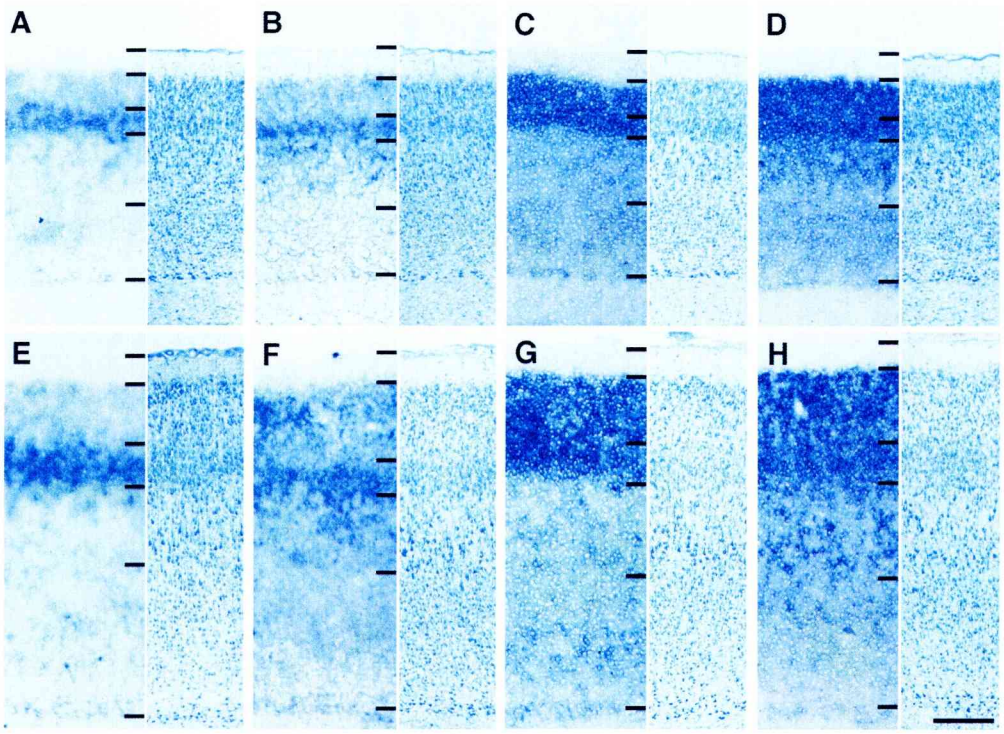


Fig. I-2. Four clones which are expressed in layer 4 or in layer 2/3-4.
In situ hybridization shows laminar expression patterns in the visual (A-D) and somatosensory (E-H) cortices of P7 rat. DIG-labeled probes were used. In each panel, *in situ* hybridization is shown to the left, and Nissl staining in adjacent sections is to the right. #571 (A and E) and #585 (B and F) genes are more specifically expressed in layer 4, while #746 (C and G) and #846 (D and H) genes are expressed in layer 2/3-4. Bar represents 0.2 mm.

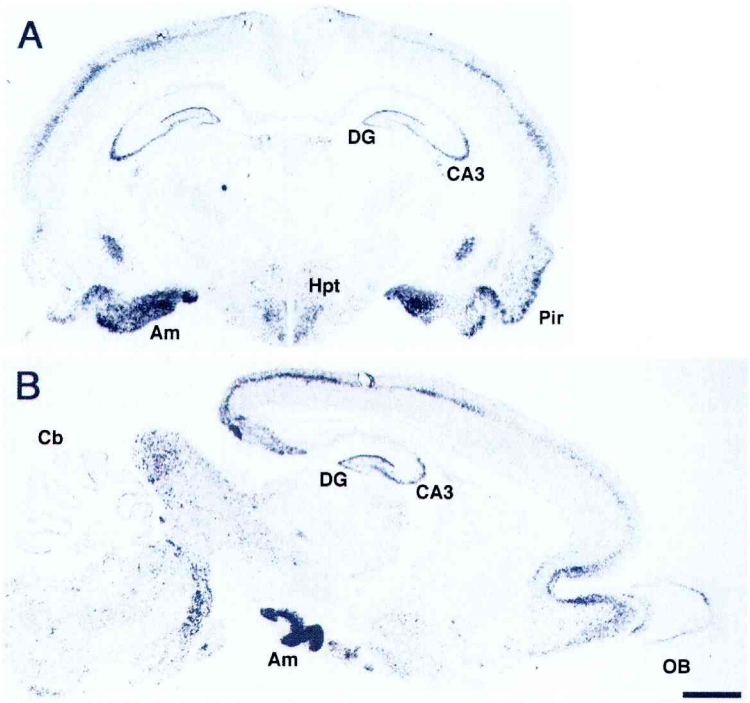


Fig. I-3. The gene expression of #571 in the P7 rat brain. Panels A and B show the gene expression pattern of #571 in the coronal and sagittal brain sections, respectively. The signals are observed in the neocortex, hippocampus, olfactory bulb, midbrain, limbic system and hypothalamus. Am, amygdala; Cb, cerebellum; DG, dentate gyrus; Hpt, hypothalamus; OB, olfactory bulb; Pir, piriform cortex. Bar indicates 1 mm.

Identification of the layer 4-specific gene

To determine the gene of the DNA fragment #571, sequence analysis was performed. However, it showed no homology to any known gene, though there were ESTs (expression sequence tags) that exactly matched these clones. However, an extended product of #571 from the 5' race method was homologus to an unreported human cDNA, which was obtained from a human brain cDNA library (Nagase, 1997, 1999). The transcript contained an open reading frame (2847 bp) and 3' UTR of more than 4000 bp. The deduced amino acid sequence (948 aa) revealed its features of a transmembrane protein, including a signal peptide sequence, two immunoglobulin and thrombospondin domains (Fig. I-4). Its cytoplasmic region consists of ZU5 and death domains, which are

common to UNC-5-like netrin receptors (Leonardo et al., 1997). We hereafter designate this novel member of the *unc-5* family as *unc5h4*. The nucleotide sequence of human *unc5h4/KIAA1777* was deposited into DDBJ/GenBank/EMBL DNA databases (Accession# AB055056). *KIAA1777* is an alias for this new gene in the human brain cDNA database.

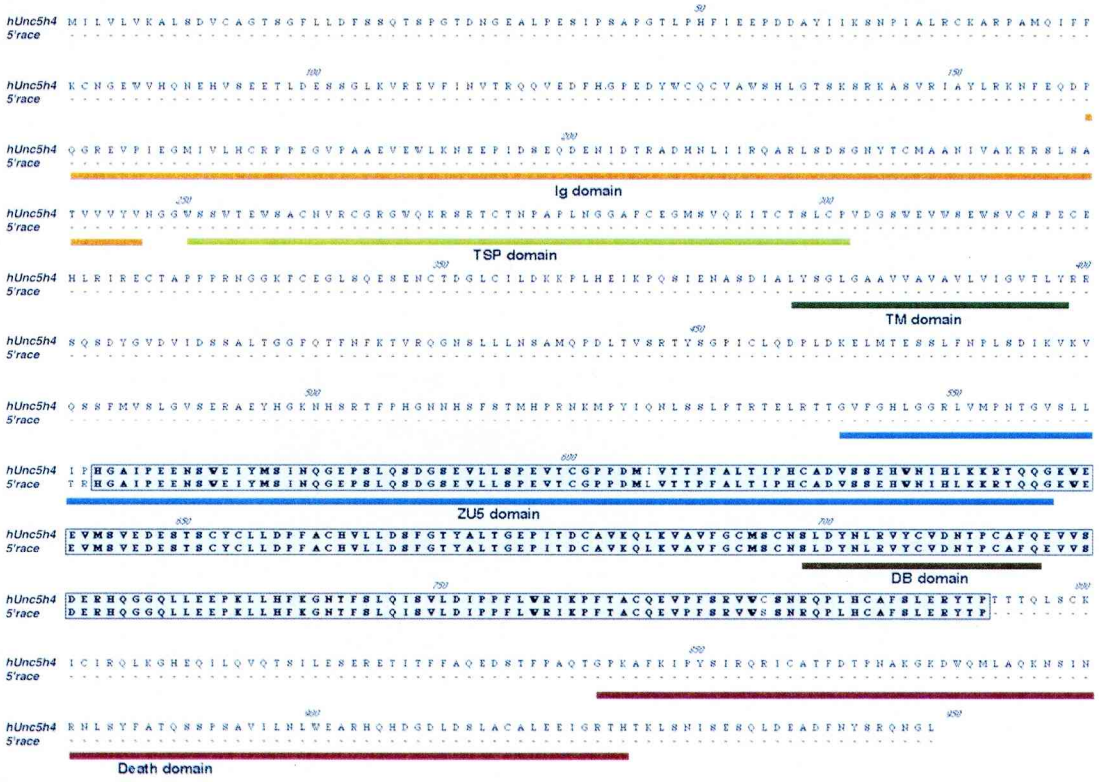


Fig. I-4. Amino acid sequence of human UNC5H4/KIAA1777 and extended product of #571 by 5' race method. The matching sequences are indicated by gray boxes. Ig, immunoglobulin; TSP, thrombospondin; TM, transmembrane; DB, DCC-binding.

Development of laminar expression of *unc5h4*

To gain an insight into how *unc5h4* gene is associated with laminar property, cellular differentiation and afferent invasion, their expression patterns were studied in the

developing somatosensory cortex (Fig. I-5). The expression of *unc5h4* was observed in the subventricular zone (SVZ) and lower part of the intermediate zone (IZ) at E16 (data not shown) and E18. At P0, the weak expression appeared just beneath the marginal zone, and then in slightly lower layers at P3. At P7, the message was largely restricted to layer 4. Thus, the expression pattern of this gene during development was closely related to the laminar locations of the cells destined to layer 4, but the signal was rather weakened at P14.

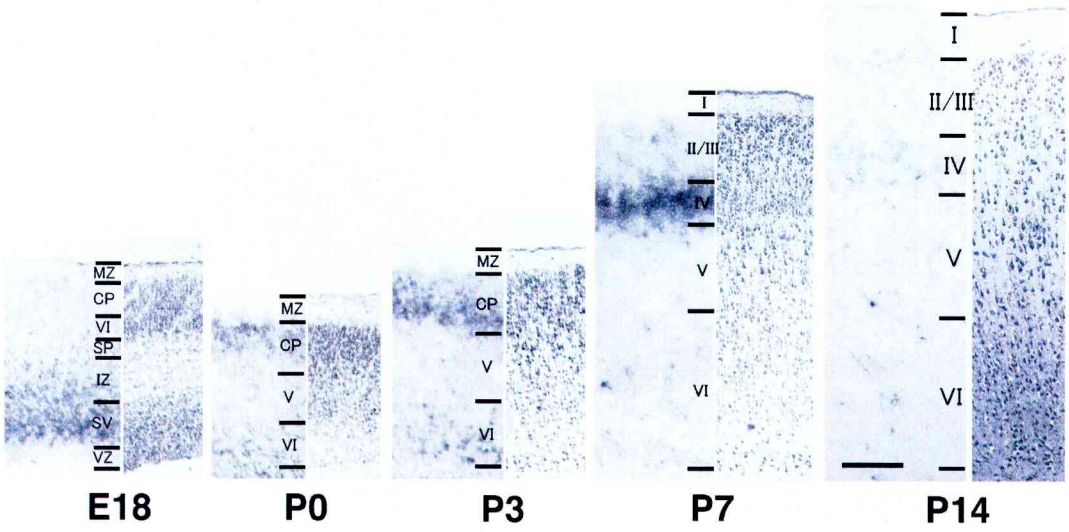


Fig. I-5. Developmental changes of laminar expression of *unc5h4* gene. *Unc5h4* gene expressions in the parietal region (above the hippocampus) of rat neocortices are shown. *In situ* signals are shown to the left of each stage, and Nissl staining is shown to the right. The laminar pattern of *unc5h4* signals is highly similar to the migratory pattern of layer 4 neurons. MZ, marginal zone; CP, cell-dense cortical plate; SP, subplate; SV, subventricular zone; VZ, ventricular zone; I, II/III, IV, V, VI, cortical layers. Bar represents 0.2 mm.

Predominant expression of *unc5h4* gene in the primary sensory areas of the neocortex

The screening based on the *in situ* hybridization analysis demonstrated that the gene expression of *unc5h4* was largely restricted to layer 4 of the developing rat neocortex. To

study the detailed gene expression of *unc5h4*, *in situ* hybridization analysis was then performed in serial coronal sections of the mouse neocortex at P6, when laminar configuration is established. In parietal sections, the *unc5h4* signal within layer 4 was much higher in the lateral part of the neocortex (Fig. I-6A). In the occipital cortex, however, the predominant region was shifted to the dorsal part (Fig. I-6A). The expression pattern also showed clear boundaries (Fig. I-6B, C). To determine predominant *unc5h4*-expressing cortical areas, the pattern was compared with adjacent Nissl-stained sections (Fig. I-6C, D) in reference to the architectonic map of the mouse neocortex (Caviness, 1975) (Fig. I-6F). For example, the expression boundary observed in the parietal sections was determined to be the border between the primary (area 3) and the secondary (area 40) somatosensory areas based on cell density in layer 4 (Fig. I-6C, D). Reconstruction of the serial sections revealed mainly two regions where predominant signals of *unc5h4* were observed (Fig. I-6E). These regions were found to correspond to the primary somatosensory area (area 3) and the primary visual area (area 17) (Fig. I-6E, F). In addition, the comparably higher expression was observed in the primary auditory area (data not shown). The expression was also strong in the insular cortex, the piriform cortex and the amygdale of the mouse brain (Fig. 1-6A, data not shown).

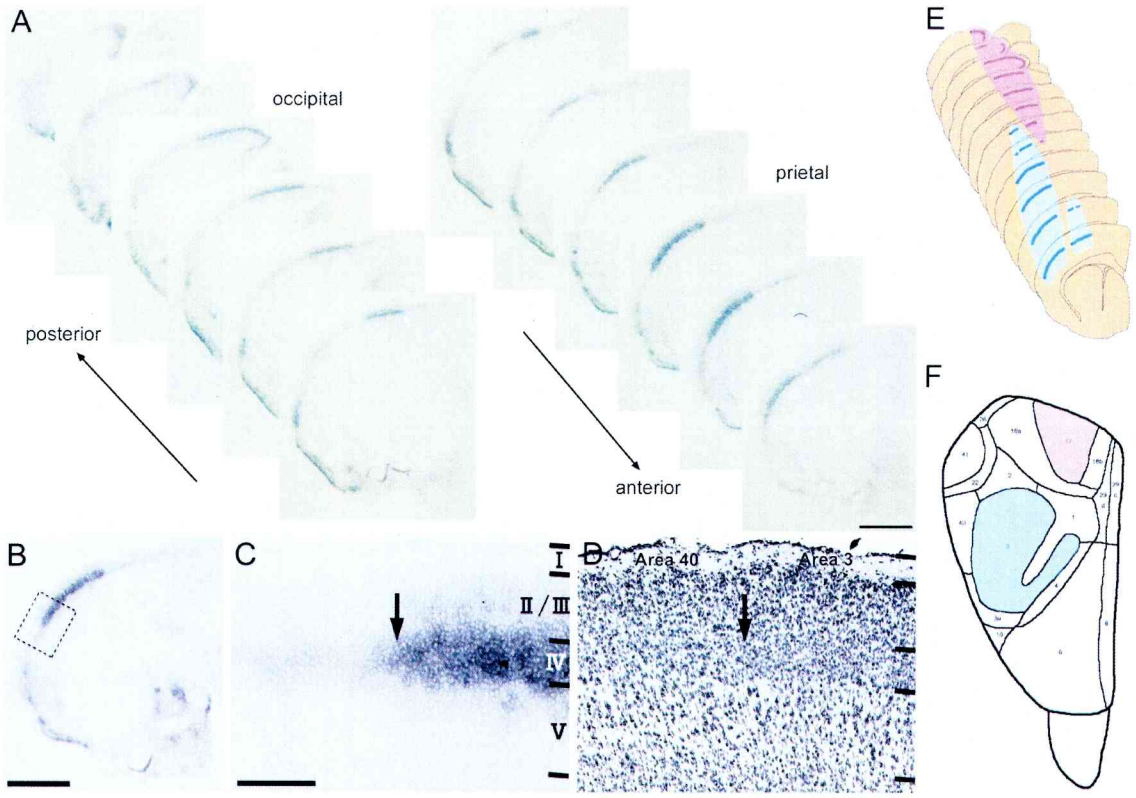


Fig. I-6. Area-specific gene expression of *unc5h4*. (A) *In situ* hybridization of *unc5h4* in serial coronal sections of the P6 mouse brain. In the parietal cortex, *unc5h4* expression is higher in the lateral part (right, white arrows). In the occipital cortex, the strong expression is seen in the dorsal part (left, white arrows). (B, C) *In situ* signals show clear boundaries (arrow in C). C represents a higher magnification view of the box in B. (D) Nissl staining on an adjacent section shows area boundary recognized by cell density in layer 4 (arrow). Ⅰ, Ⅱ/Ⅲ, Ⅳ, Ⅴ, cortical layers. (E) Reconstruction of the serial sections (A) revealed two predominant regions (blue and red). These two regions correspond to the primary somatosensory area (blue, area 3), and the primary visual area (red, area 17) in reference to the architectonic map of the mouse neocortex (F, adapted from Caviness, 1975). Numbers in F indicate Brodmann area. Scale bars: 1 mm (A, B), 0.2 mm (C, D).

Unc5h4 expression in the target cells for thalamocortical afferents

To compare the *unc5h4* expression with thalamocortical innervations, thalamocortical projections were visualized by immunostaining with an anti-serotonin-transporter (5-HTT) antibody, since 5-HTT is transiently expressed in thalamocortical axon fibers until around the first postnatal week (Rebsam et al., 2002). 5-HTT immunopositive

regions were largely overlapped with *unc5h4* gene expression areas in layer 4 on the coronal sections, although *unc5h4* was also moderately expressed in the upper layers in some regions (Fig. I-7A, B). I also found strong signals of *unc5h4* in the barrel field in the primary somatosensory area (Fig. I-7A, B). To determine the detailed location of *unc5h4*-expressing cells in the barrel field, *in situ* hybridization was performed with a tangential section of the flattened cortex. I found that *unc5h4* was preferentially expressed in the barrel walls, but was hardly expressed in the barrel hollows and in the septal regions (Fig. I-7C, D). Since the barrel walls are primarily comprised of the neurons that receive thalamic inputs (Woolsey and Van der Loos, 1970; Killackey and Leshin, 1975), these results suggest that *unc5h4* may be preferentially expressed by the target neurons for thalamocortical axons.

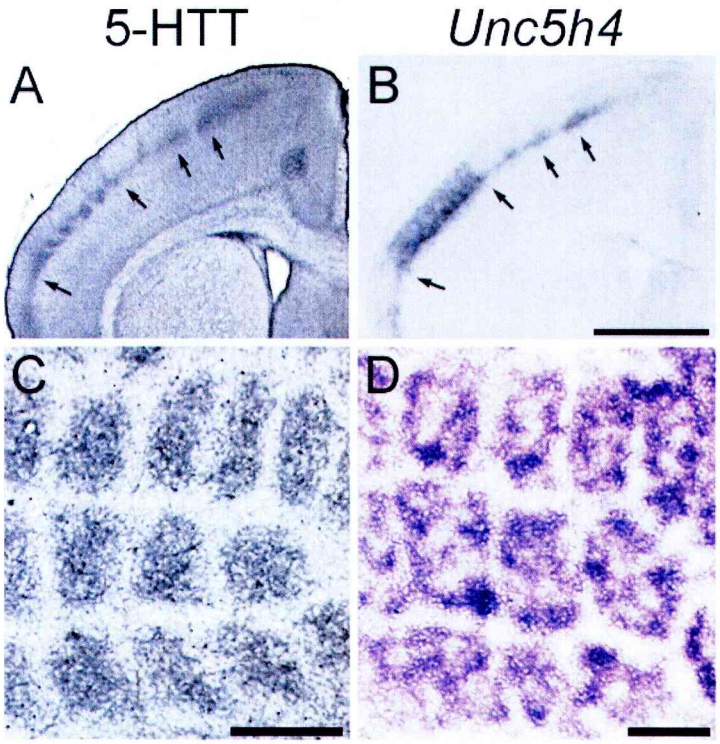


Fig. I-7. Comparison between gene expression pattern of *unc5h4* and thalamocortical projections. (A, B) 5-HTT immunopositive areas (arrows in A) on a coronal section at P7

are largely overlapped with *unc5h4* gene expression pattern (arrows in B) of a proximal section in the same brain. (C) 5-HTT immunohistochemistry on a tangential section of P7 flattened cortex clearly shows thalamic axon terminals in the barrel field of the primary somatosensory area. (D) Intense signals of *unc5h4* are recognized in the barrel walls and in a part of barrel hollows, but the signals are hardly seen in the septal regions. Scale bars: 1 mm (A, B), 0.2 mm (C, D).

Emergence of area-specificity of *unc5h4* expression

To address when the area-specific expression in the neocortex emerges, I analyzed the developmental expression during perinatal stages. On an E18 rat sagittal section, predominant expression of *unc5h4* was observed uniformly along the anterior-posterior axis in the SVZ and the lower IZ of the neocortex (Fig. I-8A). At birth, decreased expression was observed in the SVZ and a part of the white matter (Fig. I-8B), and weak expression emerged in the upper part of the cortical plate at P2 (Fig. I-8C). At P3, however, the expression was clearly recognized in upper layers with a discontinuous pattern (Fig. I-8D). Especially, within presumptive layer 4 at P3, the discontinuous signal was observed with a high level in the parietal and occipital regions but a lower level in the frontal region (Fig. I-8D). The obvious area-specific expression within layer 4 was observed by P6 (Fig. I-6). Thus, the area-specific expression pattern began to emerge at the early postnatal stage.

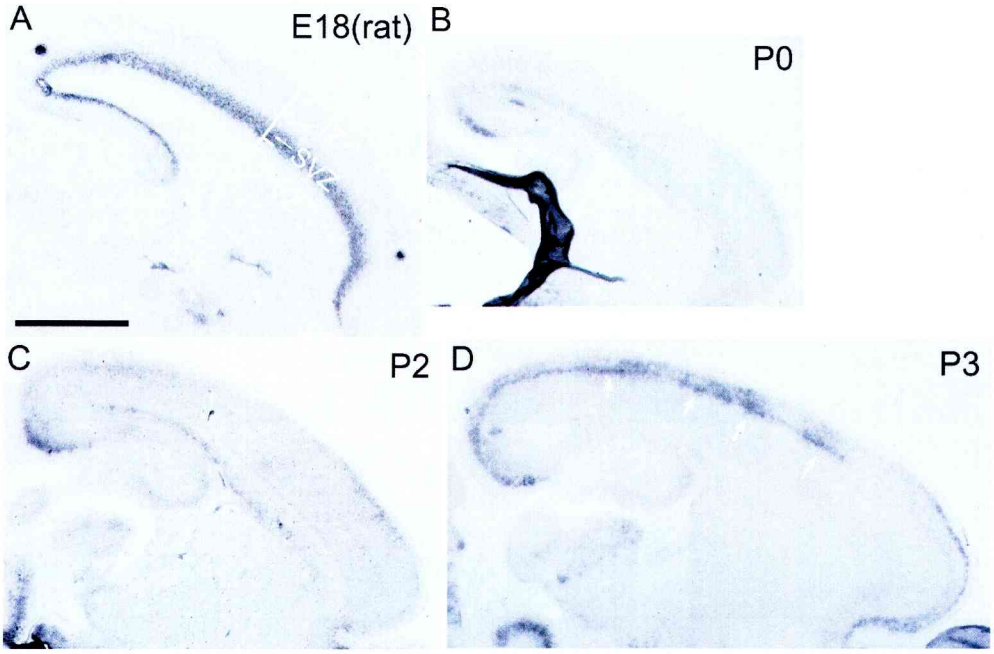


Fig. I-8. Developmental changes of area expression of *unc5h4* gene. *In situ* hybridization for *unc5h4* on the sagittal sections. (A) The signals are detected uniformly along the anterior-posterior axis in the SVZ and lower part of the IZ in the E18 rat section. (B, C) Weak signals are observed in the white matter at P0 (B), and also in the upper part of the cortex at P2 (C) in mice. (D) At P3, clear discontinuous expression pattern begins to emerge around layer 4 in the parietal and occipital regions (arrows). CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Bar represents 1 mm.

Discussion

I constructed a subtraction cDNA library where layer 4-derived cDNA fragments were enriched. I obtained four genes from the library that were expressed in the upper layers in P7 rat cortex when laminar configuration is established. Of those, one gene was predominantly expressed in layer 4, and it was identified as *unc5h4*, a member of netrin receptor genes. Thus, the present study using systematic screening of the subtraction cDNA library clearly demonstrates that certain types of genes including a receptor molecule, which might be involved in cellular differentiation and neural circuit

formation, are expressed in the upper layers of the developing neocortex. Furthermore, *unc5h4* was also predominantly expressed in the primary sensory areas. These observations suggest that *unc5h4* may contribute to layer- and area-specific cortical development. They may also provide some clues to the relationship between the development of layer 4 neurons and thalamocortical connections.

The spatiotemporal gene expression of *unc5h4* during development

Unc5h4, a new member of *unc-5* family, was expressed most specifically in layer 4 of P7 rat neocortex. After the sequence of human *unc5h4/KIAA1777* was registered in GenBank/DDBJ database, its mouse homologue was reported (Engelkamp, 2002). However, the present study is the first to demonstrate its molecular characteristics and expression pattern in the brain. In terms of the laminar specificity, the expression profile during development showed a migrating behavior of *unc5h4* expression. Indeed, in rats, layer 4 cells are born at E16-17 in the ventricular zone and migrate to the subventricular and intermediate zones at E18. At P0, they move to the most superficial part of the CP, and gradually settle in layer 4 by P6 (Berry and Rogers, 1965; Lund and Mustari, 1977). *Unc5h4* expression closely resembles this migration pattern, suggesting that *unc5h4* is expressed during development by the cells destined to form layer 4 of the neocortex. In addition, the embryonic expression pattern of *unc5h4* is also compatible with recent studies suggesting that intermediate neuronal precursor cells which are destined for upper layer neurons might accumulate within the SVZ during embryonic stages. Indeed, in addition to *unc5h4*, *Svet1* and *Cux2*, which are expressed by neurons destined for upper layers, continue to be expressed in the SVZ over some embryonic

stages (Tarabykin et al., 2001; Zimmer et al., 2004; Sasaki et al., 2008).

Furthermore, the screening by *in situ* hybridization also revealed a discontinuous expression of *unc5h4* within layer 4, prompting us to examine its area-specificity. As a result of the detailed expression analysis, I found that *unc5h4* was predominantly expressed in the primary sensory areas at around first postnatal week. At a late embryonic stage, however, *unc5h4* was expressed uniformly along the anterior-posterior axis in the SVZ and lower part of the IZ. Taken together, these findings indicate that the area distribution of *unc5h4*-expressing cells is changed developmentally while the laminar specificity may not be changed. Nevertheless, further studies are needed to address the question of whether the expression of *unc5h4* becomes downregulated in some regions in late embryonic and early postnatal stages, or *unc5h4*-expressing SVZ cells themselves disappear specifically in the regions except for the primary sensory areas.

Molecular properties of *unc5h4*

It has been shown that UNC-5 family members are involved in axon guidance and cell migration as netrin-1 receptors (Ackerman et al., 1997; Hong et al., 1999). In addition, these members have been shown to suppress neuronal apoptosis, upon receiving a ligand signal (Hofmann and Tschopp, 1995; Llambi et al., 2001). Although the molecular interaction with UNC5H4 has not yet been well understood, it is possible that the ligand may influence cell survival of layer 4 neurons expressing *unc5h4*. The survival effect might further influence neuronal connectivity. Another possibility is that UNC5H4 acts as a ligand molecule. The extracellular domains containing

immunoglobulin and thrombospondin domains might directly influence ingrowing thalamic axons. Identification of the molecules that interact with UNC5H4 would be necessary to elucidate its role to a great extent.

Implications of the presence of upper layer-specific genes

To date, it has been shown that putative regulatory genes, *ROR-β*, also referred to as *RZR-β*, (Becker-Andre et al., 1994; Park et al., 1997; Schaeren-Wiemers et al, 1997) and *COUP-TFI* (Liu et al., 2000) are expressed in layer 4 of the neocortex, although the functions of these genes in layer 4 are not clear. Thalamocortical projections are disrupted in *COUP-TFI* mutants, but it is likely that the phenotype is attributable to the lack of subplate neurons rather than its direct influences on thalamocortical axon targeting in layer 4 (Zhou et al., 1999).

It is also important to reveal extracellular molecules such as cell surface or extracellular matrix molecules, in order to understand the molecular basis of cellular interactions and circuit formation (Yamamoto, 2002). In this sense, *unc5h4* is the gene that encodes a cell surface protein and is expressed rather specifically in cortical cells destined to settle in layer 4 during development. Although how *unc5h4* contributes to cellular differentiation and connectivity in the neocortex is unknown, its molecular characteristics raise the possibility that it is involved in the interactions between cortical cells, or between layer 4 cells and thalamocortical fibers. Recent studies have shown that *ephrin-A5*, an Eph ligand, affects thalamocortical axon behavior by its expression in layer 4 (Mann et al., 2002), but this is unlikely to be the case with the entire sensory thalamocortical projections (Donoghue and Rakic, 1999,

Mackarehtschian et al., 1999; Vanderhaeghen et al., 2000; Yabuta et al., 2000). In contrast, localization of *unc5h4* in layer 4 is found throughout the sensory cortices.

Cadherin-6 and *rCNL3* are known cell surface molecules that are expressed in the upper layers of the developing cortex (Suzuki et al., 1997, Chenn et al., 2001). At early postnatal stages, *SemaK1*, a GPI-anchored semaphorin, is also expressed in the upper layers (Xu et al., 1998), while layer 4 is devoid of the expression of phosphacan, a proteoglycan (Maeda and Noda, 1996), though their expression patterns during development are not well characterized. It would be worthwhile to examine whether these molecules affect thalamic axonal growth, as previous investigations have indicated that thalamocortical axon termination is governed by molecules distributed in layers 2/3-4. (Yamamoto et al., 2000a, b; Noctor et al., 2001; Yamamoto, 2002). A similar laminar transition has been reported in the expression of *Sema3A/SemaD*, a potential molecule, which might be responsible for the formation of thalamocortical projections (Skaliora et al, 1998).

Several transcription factors have been shown to be expressed in a lamina-specific fashion: *Otx1* (Frantz et al., 1995) and *Id2* (Bulfone et al., 1995) in layer 5 and *Tbr-1* (Bulfone et al., 1995; Hevner et al., 2001) in layer 6. As for layer 4, *COUP-TFI* and *ROR-β* are expressed in layer 4, as described above. To determine the relation between these regulatory genes and their down stream molecules would also be informative to understand the molecular basis of cortical cell differentiation and neural circuit formation.

References

Ackerman SL, Kozak LP, Przyborski SA, Rund LA, Boyer BB, Knowles BB (1997) The mouse rostral cerebellar malformation gene encodes an UNC-5-like protein. Nature 386: 838-842.

Agmon A, Yang LT, O'Dowd DK, Jones EG (1993) Organized growth of thalamocortical axons from the deep tier of terminations into layer IV of developing mouse barrel cortex. J Neurosci 13: 5365-5382.

Becker-Andre M, Wiesenberg I, Schaeren-Wiemers N, Andre E, Missbach M, Saurat JH, Carlberg C (1994) Pineal gland hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. J Biol Chem 269:28531-28534.

Berry M, Rogers AW (1965) The migration of neuroblasts in the developing cerebral cortex. J Anat 99: 691-709.

Bulfone A, Smiga SM, Shimamura K, Peterson A, Puelles L, Rubenstein JL (1995) T-brain-1: a homolog of Brachyury whose expression defines molecularly distinct domains within the cerebral cortex. Neuron 15:63-78.

Bolz J, Novak N, Staiger V (1992) Formation of specific afferent connections in organotypic slice cultures from rat visual cortex with lateral geniculate nucleus. J Neurosci 12:3054-3070.

Catalano S, Robertson RT, Killackey H (1996) Individual axon morphology and thalamocortical topography in developing rat somatosensory cortex. J Comp Neurol 366, 36-53.

Caviness VS, Jr. (1975) Architectonic map of neocortex of the normal mouse. J Comp Neurol 164:247-263.

Chenn A, Levin ME, McConnell SK (2001) Temporally and spatially regulated expression of a candidate G-protein-coupled receptor during cerebral cortical development. J Neurobiol. 46:167-177. Erratum in: J Neurobiol 47:244.

Donoghue MJ, Rakic P (1999) Molecular evidence for the early specification of presumptive functional domains in the embryonic primate cerebral cortex. J Neurosci 19:5967-79.

Engelkamp (2002) Cloning of three mouse Unc5 genes and their expression patterns at mid-gestation. Mech Dev. 118:191-197.

Frantz GD, Weimann JM, Levin ME, McConnell SK (1995) Otx1 and Otx2 define layers and regions in developing cerebral cortex and cerebellum. J Neurosci 14:5725-5740.

Ghosh A, Shatz CJ (1992) Pathfinding and target selection by developing geniculocortical axons. J Neurosci 12:39-55.

Gilbert CD (1983) Microcircuitry of the visual cortex. Ann Rev Neurosci 6:217-247.

Hevner RF, Shi L, Justice N, Hsueh Y, Sheng M, Smiga S, Bulfone A, Goffinet AM, Campagnoni AT, Rubenstein JL (2001) Tbr1 regulates differentiation of the preplate and layer 6. Neuron 29:353-366.

Hofmann K, Tchopp J (1995) The death domain motif found in Fas (Apo-1) and TNF receptor is present in proteins involved in apoptosis and axonal guidance. FEBS Lett 371:321-323.

Hong K, Hinck L, Nishiyama M, Poo MM, Tessier-Lavigne M, Stein E (1999) A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. Cell 97:927-941.

Jones EG (1981) Anatomy of cerebral cortex: Columnar input-output organization. In: The Organization of the Cerebral Cortex (Schmitt FO,Worden FG, Adelman G, Dennis SG, ed.) pp.199-235. Cambridge, Massachusetts, London, England: The MIT press.

Kageyama GH, Robertson RT (1993) Development of geniculocortical projections to visual cortex in rat. J Comp Neurol 335:123-148.

Killackey HP, Leshin S (1975) The organization of specific thalamocortical projections to the posteromedial barrel subfield of the rat somatic sensory cortex. Brain Res 86:469-472.

Krieg WJ (1946) Connections of the cerebral cortex. I. The albino rat. B. Structure of the cortical areas. J Comp Neurol 84:277-323.

Leonardo ED, Hinck L, Masu M, Keino-Masu K, Ackerman SL, Tessier-Lavigne M (1997) Vertebrate homologues of C. elegans UNC-5 are candidate netrin receptors. Nature 386: 833-838.

Liu Q, Dwyer ND, O'Leary DD (2000) Differential expression of COUP-TFI, CHL1, and two novel genes in developing neocortex identified by differential display PCR. J Neurosci. 20:7682-7690.

Llambi F, Causeret F, Bloch-Gallego E, Mehlen P (2001) Netrin-1 acts as a survival factor via its receptors UNC5H and DCC. EMBO J 20:2715-2722.

Lund RD, Mustari MJ (1977) Development of geniculocortical pathway in rats. J Comp Neurol 173:289-306.

Mackarehtschian K, Lau CK, Caras I, McConnell SK (1999) Regional differences in the developing cerebral cortex revealed by ephrin-A5 expression. Cereb Cortex 9:601-10.

Maeda N, Noda M (1996) 6B4 proteoglycan/phosphacan is a repulsive substratum but promotes morphological differentiation of cortical neurons. Development 122:647-658.

Mann F, Peuckert C, Dehner F, Zhou R, Bolz J (2002) Ephrins regulate the formation of terminal axonal arbors during the development of thalamocortical projections. Development 129:3945-3955.

McConnell SK (1989) The determination of neuronal fate in the cerebral cortex. Trends Neurosci. 12:342-349.

Molnár Z, Adams R, Blakemore C (1998) Mechanisms underlying the early establishment of thalamocortical connections in the rat. J Neurosci 18:5723-5745.

Molnár Z, Blakemore C (1991) Lack of regional specificity for connections formed between thalamus and cortex in coculture. Nature 351:475-477.

Molnár Z, Blakemore C (1999) Development of signals influencing the growth and termination of thalamocortical axons in organotypic culture. Exp Neurol 156:363-393.

Nagase T, Ishikawa K, Nakajima D, Ohira M, Seki N, Miyajima N, Tanaka A, Kotani H, Nomura N, Ohara O. (1997) Prediction of the coding sequences of unidentified human genes. VII. The complete sequences of 100 new cDNA clones from brain which can code for large proteins in vitro. DNA Res 4:141-150.

Nagase T, Ishikawa K, Suyama M, Kikuno R, Hirosawa M, Miyajima N, Tanaka A, Kotani H, Nomura N, Ohara O (1999) Prediction of the coding sequences of unidentified human genes. XIII. The complete sequence of 100 new cDNA clones from brain which code for large proteins in vitro. DNA Res 6:63-70.

Noctor SC, Palmer SL, McLaughlin DF, Juliano SL (2001) Disruption of layers 3 and 4 during development results in altered thalamocortical projections in ferret somatosensory cortex. J Neurosci 21:3184-3195.

Park HT, Kim YJ, Yoon S, Kim JB, Kim JJ (1997) Distributional characteristics of the mRNA for retinoid Z receptor beta (RZR beta), a putative nuclear melatonin receptor, in the rat brain and spinal cord. Brain Res 747:332-337.

Ramon Y Cajal S (1911) Histology of the Nervous System of Man and Vertebrates (translated in 1995). New York: Oxford University Press.

Rebsam A, Seif I, Gaspar P (2002) Refinement of thalamocortical arbors and emergence of barrel domains in the primary somatosensory cortex: a study of normal and monoamine oxidase a knock-out mice. J Neurosci 22:8541-8552.

Sasaki S, Tabata H, Tachikawa K, Nakajima K (2008) The cortical subventricular zone-specific molecule Svet1 is part of the nuclear RNA coded by the putative Netrin receptor gene Unc5d and is expressed in multipolar migrating cells. Mol Cell Neurosci.

Schaeren-Wiemers N, Andre E, Kapfhammer JP, Becker-Andre M (1997) The expression pattern of the orphan nuclear receptor RORbeta in the developing and adult rat nervous system suggests a role in the processing of sensory information and in circadian rhythm. Eur J Neurosci 9:2687-2701.

Skaliora I, Singer W, Betz H, Püschel AW. (1998) Differential patterns of semaphorin expression in the developing rat brain. Eur J Neurosci 10:1215-1229.

Suzuki SC, Inoue T, Kimura Y, Tanaka T, Takeichi M (1997) Neuronal circuits are subdivided by differential expression of type-II classic cadherins in postnatal mouse brains. Mol Cell Neurosci 9:433-447.

Tarabykin V, Stoykova A, Usman N, Gruss P (2001) Cortical upper layer neurons derive from the subventricular zone as indicated by Svet1 gene expression. Development 128:1983-1993.

Vanderhaeghen P, Lu Q, Prakash N, Frisén J, Walsh CA, Frostig RD, Flanagan JG (2000) A mapping label required for normal scale of body representation in the cortex. Nature Neurosci 3:358-365.

Woolsey TA, Van der Loos H (1970) The structural organization of layer IV in the somatosensory region (SI) of mouse cerebral cortex. The description of a cortical field composed of discrete cytoarchitectonic units. Brain Res 17:205-242.

Xu X, Ng S, Wu ZL, Nguyen D, Homburger S, Seidel-Dugan C, Ebens A, Luo Y (1998) Human semaphorin K1 is glycosylphosphatidylinositol-linked and defines a new subfamily of viral-related semaphorins. J Biol Chem 273:22428-22434.

Yabuta NH, Butler AK, Callaway EM (2000) Laminar Specificity of Local Circuits in Barrel Cortex of Ephrin-A5 Knockout Mice. J Neurosci 20:RC88:1-4.

Yamamoto N, Kurotani T, Toyama K (1989). Neural connections between the lateral geniculate nucleus and visual cortex in vitro. Science 245:192-194.

Yamamoto N, Yamada K, Kurotani T, Toyama K (1992) Laminar specificity of extrinsic cortical connections studied in coculture preparations. Neuron 9:217-228.

Yamamoto N, Higashi S, Toyama K (1997) Stop and Branch behaviors of geniculocortical axons: A time-lapse study in organotypic cocultures. J Neurosci 17:3653-3663.

Yamamoto N, Inui K, Matsuyama Y, Harada A., Hanamura K, Murakami F, Ruthazer ES, Rutishauser U and Seki T (2000a) Inhibitory mechanism by polysialic acid for lamina-specific branch formation of thalamocortical axons. J Neurosci 20:9145-9151.

Yamamoto N, Matsuyama Y, Harada A, Inui K, Murakami F, Hanamura K (2000b) Characterization of factors regulating lamina-specific growth of thalamocortical axon. J Neurobiol 42:56-68.

Yamamoto N (2002) Cellular and Molecular basis for the formation of lamina-specific thalamocortical projections. Neurosci Res 42:167-173.

Zhou C, Qiu Y, Pereira FA, Crair MC, Tsai SY, Tsai MJ (1999) The nuclear orphan receptor COUP-TFI is required for differentiation of subplate neurons and guidance of thalamocortical axons. Neuron 24:847-859.

Zimmer C, Tiveron MC, Bodmer R, Cremer H (2004) Dynamics of Cux2 expression suggests that an early pool of SVZ precursors is fated to become upper cortical layer neurons. Cereb Cortex 14:1408-1420.

Chapter II

Analysis of the ligand of UNC5H4 and their role in cortical development

Summary

UNC5H4 is a member of UNC-5 homologue receptors that are known to regulate cell survival and axon guidance depending on netrin-1, but the role of UNC5H4 and its ligands in cortical development are still unknown. In this study, I examined the binding of putative ligands to UNC5H4 using a cell surface binding experiment and demonstrated that netrin-4 as well as netrin-1 protein bound to *unc5h4*-transfected HEK293T cells. Moreover, the gene expression analysis revealed that *netrin-4* but not *netrin-1* was predominantly expressed in layer 4 of the sensory cortex and sensory thalamic nuclei, suggesting that netrin-4 is one of the ligands of UNC5H4 and could affect on *unc5h4*-expressing layer 4 neurons in the neocortex. To examine the role of UNC5H4 on cortical neuron development, *unc5h4* was transfected into cortical cells and the effect of netrin-4 on *unc5h4*-expressing cortical neurons was investigated using dissociated cell culture. The result demonstrated that cell death of *unc5h4*-expressing layer 4 cells was significantly suppressed by exogenous application of netrin-4 protein. In contrast, netrin-4 had no effect on deep layer cells even though *unc5h4* was ectopically expressed in the cells. Taken together, these results suggest that UNC5H4 is predominantly expressed by layer 4 neurons in the primary sensory areas of the developing neocortex and may mediate the effect of netrin-4 on cortical cell survival in a lamina-specific manner.

Introduction

During development, cortical neurons differentiate into a variety of cell types, migrate and make neural connections, while some of them undergo cell death, leading to the arrangement of cortical cytoarchitecture and precise neural circuits. The identification of molecules involved in such developmental events is a key approach to elucidate the mechanisms. In chapter I, I have characterized that *unc5h4* gene is predominantly expressed in layer 4 in the primary sensory areas of perinatal rodent neocortex. The result suggests that *unc5h4* may play a role in layer- and area-specific cortical development. *Unc5h4* was cloned as a novel member of vertebrate homologues of *Caenorhabditis elegans unc-5* gene (Engelkamp, 2002). UNC-5 homologues, comprising UNC5H1, UNC5H2, UNC5H3 and UNC5H4 (also named UNC5A, UNC5B, UNC5C and UNC5D), are type I transmembrane proteins composed of an extracellular sequence with two Ig-like domains and two thrombospondin type I domains, and an intracellular sequence with a ZU5 domain, a DB (DCC-binding) domain and a death domain (Leonardo et al., 1997; Engelkamp, 2002). UNC-5 homologues are known as netrin receptors based on the cell surface binding experiments demonstrating that UNC5H1-3 bound to netrin-1 (Leonardo et al., 1997) and netrin-3 (Wang et al., 1999), and that UNC5H1 bound to netrin-4 (Qin et al., 2007). Several *in vitro* studies have proposed that these receptors may regulate apoptotic cell death as a dependence receptor on netrin-1: they induce apoptosis in the absence of netrin-1 through the caspase-3 cleavage of a particular cytoplasmic domain, whereas they transduce a positive signal in the presence of netrin-1, leading to cell survival and differentiation (Mehlen and

Mazelin, 2003; Porter and Dhakshinamoorthy, 2004). *Unc5h1*-deficient mice also showed decreased apoptosis and increased number of neurons *in vivo* (Williams et al., 2006). Moreover, it has been suggested that they may play a role in neuronal cell migration and axon guidance (Ackerman et al., 1997; Hong et al., 1999; Finger et al., 2002; Watanabe et al., 2006). The overall sequence homologies of *unc5h4* to *unc5h1*, *unc5h2* and *unc5h3* are 44, 48.7 and 47.6%, respectively, and *unc5h4* is also expressed in developing limb and mammary gland (Engelkamp, 2002). However, it is unclear whether UNC5H4 functions in a netrin-dependent manner, and the role of UNC5H4 and its ligand molecules in cortical development is poorly understood.

In this study, I investigated the binding of the possible ligand netrin-1 and netrin-4, and also analyzed the gene expression of *netrin-1* and *netrin-4* in the early postnatal mouse brain. I further examined the role of UNC5H4 and the ligand molecule in cortical development using dissociated cell culture.

Materials and Methods

Cell surface binding assay

Cell surface binding experiments were performed as described previously (Wang et al., 1999) with slight modifications. Before transfection, human embryonic kidney 293T (HEK293T) cells were plated on a 0.1 mg/ml poly-L-ornithine coated dish. Transfection of expression constructs pcDNA3-UNC5H4-HA (encoding HA-tagged mouse UNC5H4 of which intracellular domain is truncated, gift from K. Nakajima, Keio University, Tokyo) and pEGFP-N1 (Clontech) into the cells was performed using Lipofectamine 2000

(Invitrogen). Forty-eight hours after transfection, the cells were washed with Dulbecco's PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, followed by incubation with 2 μg/ml recombinant polyhistidine-tagged mouse netrin-1 protein (R&D systems) or recombinant polyhistidine-tagged mouse netrin-4 protein (R&D systems) in PBS supplemented with 1% normal goat serum, 2 μg/ml heparin sodium, and 0.05% sodium azide at 37 ℃ for 1 hour. After washing three times with PBS, the cells were fixed with 4% PFA in PBS at room temperature for 15 min. After blocking with buffer G (Dulbecco's PBS- containing 5% normal goat serum, 0.1% Triton X-100, and 0.05% sodium azide), HA-tagged UNC5H4 overexpressed in HEK293T cells and bound His-tagged netrin proteins were stained with mouse anti-HA monoclonal antibody (1:600, Roche) and rabbit anti-His-tag polyclonal antibody (1:500, MBL), respectively. Overexpressed UNC5H4 and bound netrins were then visualized with secondary antibodies Alexa Fluor 488-conjugated goat anti mouse IgG (1:500, Molecular probes) and Cy3-conjugated donkey anti-rabbit IgG (1:600, Chemicon), respectively.

In situ hybridization

In situ hybridization using DIG-labeled antisense riboprobes was carried out as described in chapter I. For synthesis of riboprobes, total RNA was prepared from a SD rat cortex at E18, or a C57/BL6 mouse cortex at E16, and cDNAs were produced using the SuperScript II reverstranscriptase (Invitrogen). The cDNAs encoding rat *netrin-1* (1188 bps; the 388-1575 bp portion of NM_053731), mouse *netrin-3* (881 bps; the 2150-3030 bp portion of NM_010947), and mouse *netrin-4* (1945 bps; the 215-2159 bp portion of NM_021320) were isolated by RT-PCR using the following primer pairs:

5'-CACAACGTTACGCTCACTC-3' and 5'-TGTGCCCTGCTTGTACAC-3' for *netrin-1*, 5'-CAAGCTGGGCGTGGACTG-3' and 5'-AAAGAGGTGGGGACCCTAG-3' for *netrin-3*, 5'-CTGCCGCTTCATCCCACC-3' and 5'-GTGCTTAAGACCTTCAGTGC-3' for *netrin-4*. These fragments were then cloned into the pGEM-T or pGEM-T easy vectors (Promega).

In utero electroporation

To express full-length UNC5H4 protein together with the enhanced green fluorescent protein (EGFP) in cortical neurons (see below), the expression plasmid pCAG-UNC5H4-Myc-IRES-EGFP was constructed as follows. The full-length human *unc5h4* cDNA fragment, the signal sequence of which (encoding 14 amino acid MILVLVKALSDVCA) was replaced with the human IgG1 signal sequence (encoding MDWTWRILFLVAAATGAHS) to enhance its expression, was obtained from pCAG-human UNC5H4-6x His (gift from A. Tamada, RIKEN, Wako), and subcloned into pCAG-Myc vector containing the c-Myc epitope sequence (also gift from A. Tamada). The cDNA encoding UNC5H4-Myc was excised from pCAG-UNC5H4-Myc and then subcloned into pIRES2-EGFP vector (Clontech) which contains the internal ribosome entry site (IRES) and the EGFP coding region. Subsequently, the cDNA encoding UNC5H4-Myc-IRES-EGFP was further inserted downstream of the CAG promoter of a plasmid vector. The control vector pCAG-IRES-EGFP was made by deletion of the *unc5h4* sequence from pCAG-UNC5H4-Myc-IRES-EGFP. The protein expression of c-Myc-tagged UNC5H4 in COS7 cells transfected with pCAG-UNC5H4-Myc-IRES-EGFP was confirmed by western blotting and immunocytochemistry using an antibody against c-Myc (data not shown).

The electroporation was performed as described previously (Tabata and Nakajima, 2001) with slight modifications. Pregnant ICR mice were purchased from Japan Clea (Tokyo, Japan) or Japan SLC (Shizuoka, Japan). The expression plasmids were purified using the PureLink HiPure Plasmid Maxiprep Kit (Invitrogen) and dissolved in Dulbecco's PBS- at a concentration of 2-3 μg/μl, followed by addition of Fast Green solution at the final concentration of approximately 0.01% (w/v) to visualize the plasmid solution. For plasmid injection, a micropipette was prepared from a glass capillary tube 1.2 mm in diameter (Narishige, Tokyo, Japan) pulled by using a micropipette puller (PP830; Narishige, Tokyo, Japan) and the pulled tip was broken at an external diameter of 30-40 μm. At E13.5 or E12.5, pregnant mice were anesthetized with pentobarbital sodium (Nembutal; Dainippon Sumitomo Pharma, Osaka, Japan), injected intraperitoneally with Dulbecco's PBS- at 60-65 μg per gram of body weight. Then, approximately 1-2 μl of plasmid solution was injected into the lateral ventricle of an embryo in the uterus with the glass micropipette. Subsequently, the embryo was placed between tweezers-type electrodes, which have platinum disc electrodes of 5 mm in diameter at the tip (CUY650P5; Nepa Gene, Chiba, Japan), and electric pulses (five times at 30 V with 50 ms duration and 95 ms intervals) were then charged with an electroporator (CUY-20; BEX, Tokyo, Japan). The uteri were placed back into the abdominal cavity to allow the embryos to continue normal development until their brains were applied to culture experiments (see below). Either of three plasmids, pCAG-UNC5H4-Myc-IRES-EGFP, pCAG-IRES-EGFP or pCAGGS-DsRed was electroporated into individual littermates of a pregnant mouse.

Dissociated cell culture and analysis of cell survival

After electroporation, cortical explants containing transfected regions were dissected at E16.5 under a laser microscope (MZFLIII; Leica), and dissociated in Dulbecco's PBS-containing 0.125% trypsin and 5 μM EDTA. Subsequently, the dissociated cells were seeded in 0.1 mg/ml poly-L-ornithine coated plates at a density of 1 or 2 × 10^4 /cm^2 and cultured in Dulbecco's Modified Eagle Medium with Nutrient Mixture F-12 (D-MEM/F-12) containing 0.2 × B-27 supplement (Gibco) and 1% fetal bovine serum (FBS) (Hyclone). The concentration of B-27 supplement and FBS was adjusted so that approximately 60% of cultured cortical cells would undergo cell death in 2 days *in vitro* (DIV) (see below). For the application of netrin-4 or netrin-1 proteins, the dissociated cells were seeded in the culture media containing recombinant polyhistidine-tagged mouse netrin-4 or netrin-1 proteins (R&D systems) at the final concentration of 400 ng/ml. The cells were cultured at 37 ℃ in 5% CO_2 until they were analyzed.

Since considerable weak fluorescence of EGFP in cultured cells transfected with the pCAG-UNC5H4-Myc-IRES-EGFP or pCAG-IRES-EGFP plasmids made it difficult to observe the cells, EGFP fluorescence was enhanced by GFP immunocytochemistry. After cultured for 2 DIV, the cells were fixed in 4% PFA in PBS (0.1 M PB, pH 7.4, 0.15 M NaCl) for 15 min at room temperature, and then washed three times with Dulbecco's PBS-. After blocking with buffer G (see above), the cells were stained with a rat anti-GFP monoclonal antibody (1:2000, Nacalai tesque) or were double-stained with an anti-GFP antibody and a rabbit anti-cleaved caspase-3 polyclonal antibody (1:500, Cell Signaling Technology), and an Alexa Fluor 488-conjugated goat anti-rat IgG (1:500, Molecular probes) and a Cy3-conjugated donkey anti-rabbit IgG (1:400, Chemicon) for

secondary antibodies. The cells were finally counterstained with DAPI.

The viability of the cultured cells was quantified as follows. Photographs were taken from six to twelve different fields, which were randomly selected from one or four culture dishes per an experimental condition, using a microscope with appropriate fluorescent filters. The cells were identified by both clear cell bodies in a bright field and DAPI-stained nuclei. The cells with glia-like structure apparently distinguishable from that of neurons were excluded from the analysis. Dying cells were recognized by the pyknosis (nuclear condensation or fragmentation) detected by DAPI staining, while viable cells were identified by their healthy nuclei. The number of EGFP-labeled and unlabeled cells as well as pyknotic and viable ones were counted and then the percentage of pyknotic cells in labeled or unlabeled cells was calculated in each photograph. The data was obtained from two individual experiments for cells electroporated at E13.5 and one experiment for cells electroporated at E12.5, and then the average of the percentage was calculated in each experimental condition. Groups were compared using a Student's *t*-test.

Results

Binding of netrin proteins to *unc5h4*-expressing cells

As candidate ligands that interact with UNC5H4, the binding of netrins to UNC5H4 was examined using a cell surface binding assay. When the recombinant netrin-4 protein was applied to HEK293T cells transfected with an *unc5h4* construct, netrin-4 signals were observed on the surface of *unc5h4*-transfected cells (Fig. II-1A-A'). In

contrast, netrin-4 proteins showed no significant binding to EGFP-expressing cells above the background (Fig. II-1B-B'). Similarly, the signal of bound netrin-1 protein was observed on *unc5h4*-transfected cells (Fig II-1 C, C'), which is consistent with the protein binding between UNC5H4 and netrin-1 that was previously demonstrated by a biochemical assay (Wilson et al., 2006).

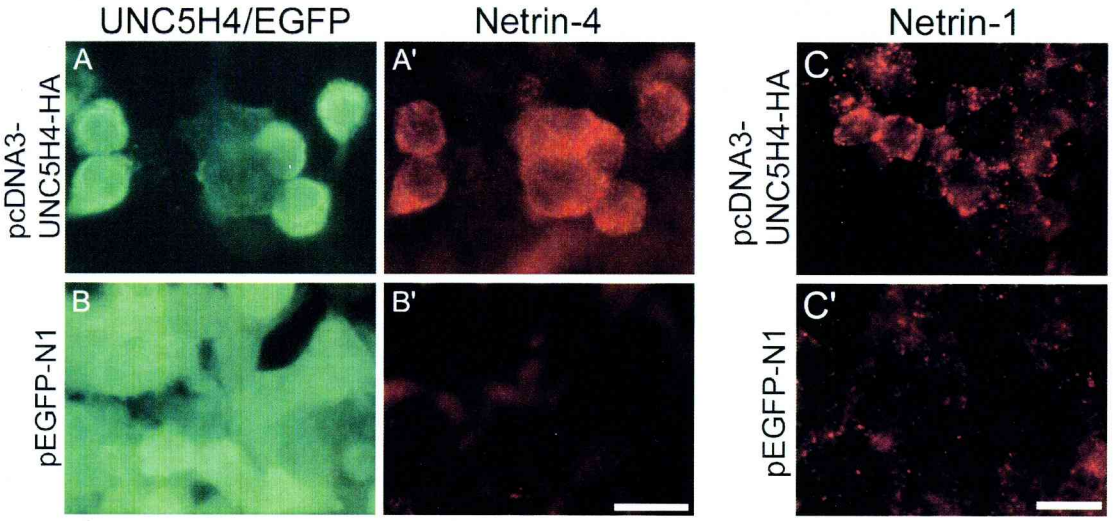


Fig. II-1. Binding of netrin protein to *unc5h4*-transfected cells. (A) The protein expression of UNC5H4-HA in HEK293T cells is detected with an anti-HA antibody. (A') Binding of recombinant His-tagged netrin-4 to cells transfected with an *unc5h4* plasmid is detected with an anti-His-tag antibody. (B, B') In the control experiment, netrin-4 does not bind to cells transfected with an EGFP plasmid. (C, C') Bound recombinant His-tagged netrin-1 protein is detected by immunostaining with an anti-His-tag antibody. Netrin-1 binds to UNC5H4-expressing cells (C) but not to EGFP-expressing cells (C'). Scale bars: 20 μm.

Netrin gene expressions in the developing neocortex and thalamus

To further determine the ligand interacting with UNC5H4 expressed by layer 4 cells, the gene expression of *netrin-1* and *netrin-4* was examined using P5 mouse brain sections. *Netrin-1* was markedly expressed in the caudate putamen, but was hardly observed in the neocortex and the thalamus (Fig. II-2A, E). In contrast, *netrin-4* was

preferentially expressed in the somatosensory and visual cortex (Fig. II-2B, F). These regions were partially overlapped with the *unc5h4* expression areas (Fig. II-2C, G). In the sensory cortex, *netrin-4* was predominantly expressed in layer 4 (Fig. II-2I). In addition, *netrin-4* was also expressed in the sensory thalamic nuclei including the dorsal lateral geniculate nucleus and the ventrobasal nucleus (Fig. II-2F), where neurons extend their axons mainly to the primary sensory areas of the neocortex. Taken together, these results suggest that netrin-4 but not netrin-1 may be one of the ligands for UNC5H4 in layer 4.

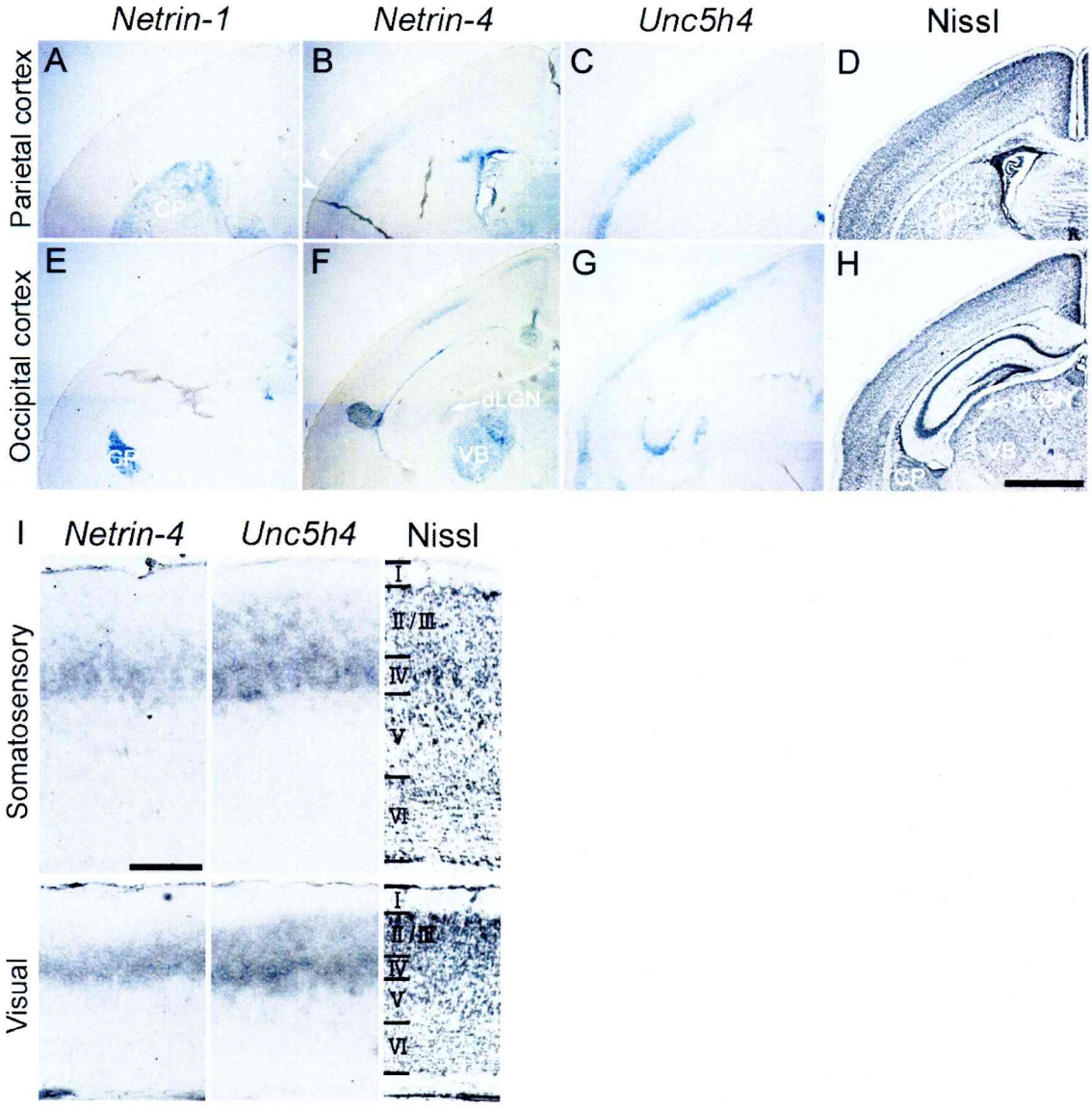


Fig. II-2. *Netrin* gene expressions in the cortex and the thalamus. *In situ* hybridization for *netrin-1, netrin-4* and *unc5h4* on coronal sections of the P6 mouse brain including either somatosensory cortices (A-D) or visual cortices (E-H). (A, E) *Netrin-1* is hardly expressed in the neocortex and the thalamus, while it is highly expressed in the CP. (B, F) *Netrin-4* is expressed preferentially in the sensory cortex (arrowheads) and in the thalamic nuclei including the dLGN and VB. (C, G) The cortical expression pattern of *netrin-4* is partially overlapped with that of *unc5h4*. (D, H) Nissl-stained sections proximal to the sections in A-C (for D) and in E-G (for H). (I) *Netrin-4* is predominantly expressed in layer 4 of the sensory cortices similarly to *unc5h4*, although *unc5h4* is also weakly expressed in layer 2/3. dLGN, dorsal lateral geniculate nucleus; VB, ventrobasal nucleus; CP, caudate putamen. Ⅰ, Ⅱ/Ⅲ, Ⅳ, Ⅴ, cortical layers. Scale bars: 1 mm (A-H), 20 μm (I).

The effect of netrin-4 on the survival of *unc5h4*-expressing cortical cells *in vitro*

To study the role of UNC5H4 in cortical neurons, I analyzed the effect of netrin-4 on layer 4 neurons using dissociated cell culture, since *unc5h4* gene is endogenously expressed in layer 4 (see chapter I). To distinguish layer 4 cells from the other layer cells which largely do not express *unc5h4*, layer 4 cells were labeled with EGFP or DsRed by *in utero* electroporation into E13.5 mouse embryos (Fig. II-3A, see Materials and Methods), because a previous study using the *in utero* electroporation technique demonstrated that most of cells transfected with a reporter gene in the lateral ventricle of E13.5 mouse embryos are destined to settle in layer 4 of the neocortex (Ajioka and Nakajima, 2005). To examine the effect of netrin-4 on cortical cell development, cortical cells containing labeled cells were cultured with or without exogenous application of netrin-4 prtoein. After two days in culture, many cultured cells showed pyknosis with condensed or fragmented nuclei that could be recognized by DAPI staining (Fig. II-3B'-D'). Immunostaining for cleaved caspase-3 demonstrated that almost all pyknotic cells (~98%, n = 80 for control-labeled cells, n = 121 for *unc5h4*-transfected labeled cells, see below) were also immnopositive for cleaved caspase-3, suggesting that cell death of labeled cells is due to apoptosis (Fig. II-3B"-D"). Interestingly, I found that the number of dying labeled cells was slightly decreased when the cells were cultured in the presence of netrin-4. On the other hand, I never found the influence of netrin-4 application on neurite growth or guidance in this culture condition (data not shown).

To evaluate the influence of netrin application on cell survival, the percentage of pyknotic cells in untreated culture and in netrin-treated culture was calculated and compared between them. In the absence of netrin proteins, 63.2 $\pm$ 4.7% of labeled

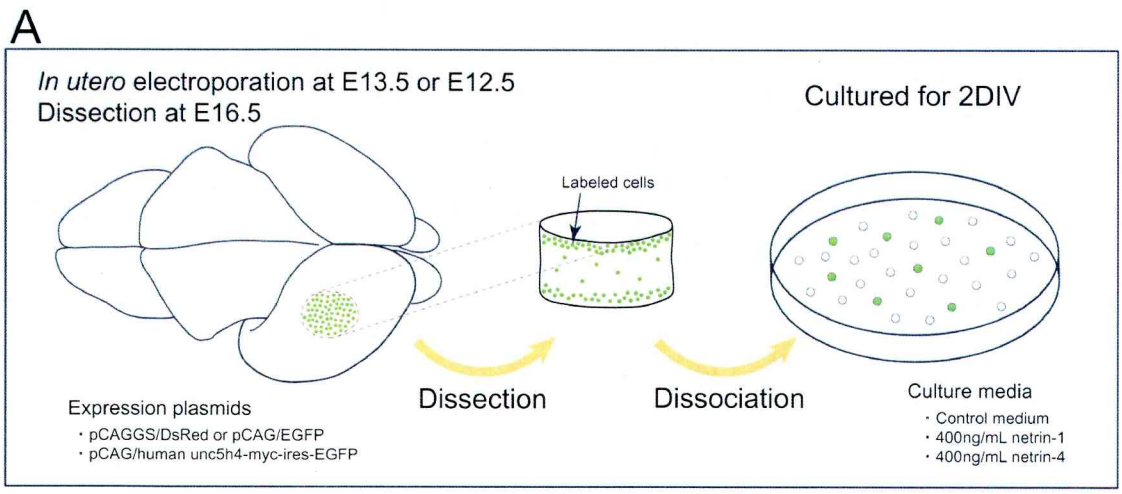
presumptive layer 4 cells showed pyknosis. In the presence of netrin-4, however, the percentage of pyknotic labeled cells was slightly but significantly decreased (47.7 ± 3.1%, $P < 0.02$). In contrast, in the presence of netrin-1, no significant difference was observed (59.9 ± 5.0%) compared to that in the absence of netrins (mean ± SEM, n = 16 for each group) (Fig. II-3E).

To examine the effect of netrin-4 on the other layer cells, cell death of unlabeled cells were then analyzed. In this case, however, no marked reduction of the percentage of pyknotic cells was observed in the presence of netrin-4 as well as netrin-1 (58.5 ± 1.8% for no netrins, 64.5 ± 2.0% for netrin-1, 53.8 ± 1.9% for netrin-4, mean ± SEM, n = 22 for each group) (Fig. II-3F). These results indicate that netrin-4 acts as a survival factor for layer 4 cells but not for the other layer cells.

In this experiment, it was not sure to what extent the number of labeled layer 4 cells practically expressed UNC5H4. In addition, I observed only a slight reduction of the percentage of pyknotic labeled cells by exogenous application of netrin-4 protein. For these reasons, to confirm whether UNC5H4 mediates the effect of netrin-4, presumptive layer 4 cells were then electroporated with an expression plasmid encoding UNC5H4-IRES-EGFP instead of the control plasmid, and analyzed in the same culture assay. The percentage of pyknotic *unc5h4*-transfeted EGFP-labeled cells was markedly decreased in the presence of netrin-4 protein (51.1 ± 3.6% for no netrins, 30.3 ± 3.6% for netrin-4, $P < 0.001$) (Fig. II-3G). In addition, the influence of netrin-4 application was significantly greater for *unc5h4*-transfeted labeled cells than for control-labeled cells (47.7 ± 3.1% for control-labeled, 30.3 ± 3.6% for *unc5h4*-transfected, $P < 0.001$) (Fig. II-3G). In contrast, the percentage of pyknotic labeled cells in the presence of

netrin-1 (52.6 ± 3.9%) was almost equivalent to that in the absence of netrins (mean ± SEM, n = 16 for each group) (Fig. II-3G). These results suggest that UNC5H4 may mediate the cell survival effect of netrin-4.

To further test whether UNC5H4 is sufficient to mediate the cell survival effect of netrin-4 in the other layer neurons which originally do not express *unc5h4*, the *unc5h4* plasmid was introduced into deeper layer cells by *in utero* electroporation at E12.5, and cell survival was examined in the same way. However, I found no effect of netrin-4 on cell survival of labeled cells regardless of ectopic *unc5h4* expression (54.9 ± 4.7% for no netrins, 50.7 ± 3.9% for netrin-4, mean ± SEM, n = 10), while the percentage of pyknotic unlabeled cells containing layer 4 cells was slightly decreased in the presence of netrin-4 (56.5 ± 1.5% for no netrins, 52.0 ± 1.5% for netrin-4, mean ± SEM, n = 20, $P < 0.02$) (Fig. II-3H). Taken together, these results suggest that UNC5H4 can function only in layer 4 neurons, but is not sufficient to mediate the effect of netrin-4 in the other layer neurons, which normally do not express *unc5h4*.



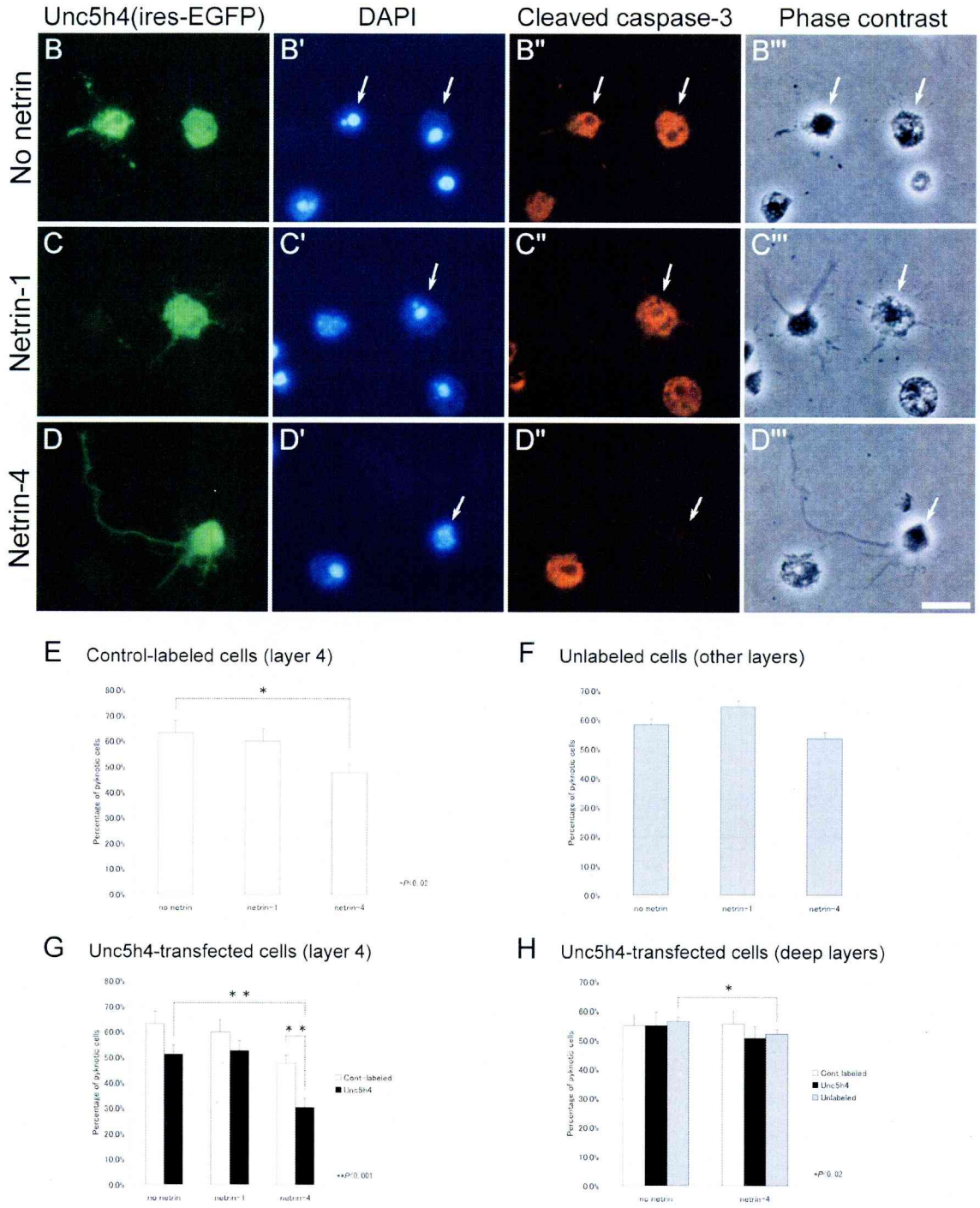


Fig. II-3. The effect of netrin-4 application on the survival of *unc5h4*-expressing cortical cells in dissociated cell culture. (A) Schematic representations of the experimental design for cortical cell culture. Many labeled cells showed pyknosis (arrows in B' and C') both in the absence of netrins (B, B') and in the presence of 400 ng/mL nterin-1 protein (C, C'). (D, D') Decreased numbers of labeled cells showed pyknosis in the presence of 400 ng/mL netrin-4 protein. (B"-D") Pyknotic cells were immunopositive for cleaved caspase-3, indicating their apoptotic cell death. (B"'-D"') Phase contrast images of B-D. (E-H) Quantification of the percentage of pyknotic cells. (E) The application of netrin-4 but not netrin-1 protein slightly reduced the percentage of pyknotic layer 4 cells labeled

with EGFP or DsRed (* $P < 0.02$). (F) Neither netrin-1 nor netrin-4 significantly reduced the percentage of pyknotic unlabeled (the other layer) cells. (G) Netrin-4 but not netrin-1 markedly suppressed pyknosis of *unc5h4*-transfected layer 4 cells (black bars, ** $P < 0.001$, versus no netrins). The transfection of *unc5h4* significantly enhanced the influence of netrin-4 application (** $P < 0.001$, versus control-labeled cells). (H) Netrin-4 had no significant effect on cell survival of control-labeled and *unc5h4*-transfected deep layer cells, whereas it slightly reduced the percentage of unlabeled (layer 4-containing) pyknotic cells (* $P < 0.02$). Scale bar represents 20 μm. Error bars represent ± SEM.

Discussion

My results demonstrated that layer 4-specific gene *unc5h4* was also strongly expressed in the primary somatosensory and visual areas in the early postnatal stages. I also found that netrin-4 as well as netrin-1 protein bound *unc5h4*-transfected cells and that *netrin-4* but not *netrin-1* gene was predominantly expressed in layer 4 of the sensory cortex and sensory thalamic nuclei. Furthermore, the percentage of pyknotic cells for *unc5h4*-expressing cortical layer 4 cells, but not for deep layer cells, was reduced by netrin-4 application *in vitro*. These results suggest that UNC5H4 is predominantly expressed by layer 4 neurons in the primary sensory areas of the developing neocortex and may mediate the effect of netrin-4 on cortical cell survival in a lamina-specific manner.

Netrins as possible ligands of UNC5H4

The spatiotemporal *unc5h4* gene expression in the neocortex together with the fact that UNC-5 homologues are known as receptor molecules suggest that UNC5H4 expressed by layer 4 cells may interact with environmental cues during cortical development. So, I attempted to determine the ligands of UNC5H4. My present study demonstrated that

netrin-4 as well as netrin-1 protein bound to *unc5h4*-transfected HEK293T cells. Similarly, previous studies reported that netrin-1 as well as netrin-3 protein bound to cell lines transfected with *unc5h1*, *unc5h2*, or *unc5h3* (Leonardo et al., 1997; Wang et al., 1999). In addition, alkaline phosphatase tagged netrin-4 fusion protein bound to *unc5h1*-transfected cells (Qin et al., 2007). However, an immunoprecipitation analysis could not detect the binding of netrin-4 to UNC5H4-Fc fusion protein, while netrin-1 bound to UNC5H4-Fc (Wilson et al., 2006). Thus, my results raise the possibility that netrin-4 can interact with UNC5H4 in a physiological situation.

Furthermore, *in situ* hybridization analysis revealed that *netrin-4* but not *netrin-1* was expressed in layer 4 of the sensory cortex and sensory thalamic nuclei. These results raise a hypothesis that netrin-4 is supplied to *unc5h4*-expressing layer 4 neurons by cortical cells or possibly by thalamic afferents, thereby functionally interacting with UNC5H4. Although I have not tested the binding of netrin-3 to UNC5H4, the gene expression pattern of *netrin-3* in the neocortex and thalamus was likely to be close to that of *netrin-4* (data not shown), raising the possibility that netrin-3 might also interact with UNC5H4 in layer 4 of the neocortex.

The function of UNC5H4 in cortical cell survival and its laminar specificity

In this study, I found the survival effect of netrin-4 on *unc5h4*-expressing cortical cells *in vitro*. However, I never observed the positive induction of apoptosis in *unc5h4*-transfected cells even in the absence of netrin proteins. These observations indicate that UNC5H4 unoccupied by netrins may not induce but permit cell death, whereas once it is occupied by netrin-4, it may mediate the effect of netrin-4 on cell

survival. Recent studies using cell lines demonstrated that apoptosis was induced in the cells expressed with each of UNC5H1-3 receptors in the absence of netrin-1, but the cell death was suppressed in the presence of netrin-1 (Llambi et al., 2001; Tanikawa et al., 2003; Thiebault et al., 2003; Williams et al., 2003; Llambi et al., 2005). In support of this observation, UNC5H1-3 proteins have a caspase-3 cleavage site (Llambi et al., 2001) and a death domain (Engelkamp, 2002) in their cytoplasmic region, both of which are conserved within UNC-5 homologues including UNC5H4. On the other hand, loss of *netrin-1* never affected apoptosis in the spinal cord, although loss of *unc5h1* decreased apoptosis in the same region (Williams et al., 2006). Thus, it remains controversial whether UNC-5 homologues regulate apoptosis as dependence receptors.

It is also noted that the effect of UNC-5 homologue receptors on cell survival or death depends on cell types where they are expressed, owing to coexistence of a specific mediator molecule that interacts with a cytoplasmic domain of the receptors and is responsible for signal transduction (Williams et al., 2003; Llambi et al., 2005; Tang et al., 2008). In agreement with this view, the present study demonstrated that UNC5H4 could mediate the survival effect of netrin-4 in layer 4 cells but was not sufficient to function in deep layer cells. Thus, it is conceivable that the function of UNC5H4 might also be dependent on its specific mediator molecules, although they have not yet been identified. The result may further reflect that such molecules are expressed with laminar specificity, as well as UNC5H4. It remains to be elucidated whether UNC5H4 could function in an area-specific fashion within layer 4.

Putative process of netrin supply to *unc5h4*-expressing cortical neurons

I observed the gene expression of *netrin-4* predominantly in layer 4 of the sensory cortex, raising the possibility that netrin-4 produced in cortical cells promote cell survival by interacting with UNC5H4 in an autocrine or a paracrine manner. This idea is compatible with the hypothesis that tumor suppressor p53 induces the transcription of both *netrin-1* and *unc5h2* to activate a survival pathway, which acts as a negative-feedback loop for the regulation of p53-dependent apoptosis (Arakawa, 2004). Further studies to reveal what regulates the expression of *netrins*, as well as *unc5h4*, in cortical cells would help us understand the role of netrin/UNC5H4-mediated cell survival in cortical development.

I also observed that *netrin-4* was predominantly expressed in the sensory thalamic nuclei which project to layer 4 of the primary sensory areas. This result raises another possibility that netrin-4 is released from thalamic axon terminals and influences on *unc5h4*-expressing cortical cells. Interestingly, the timing of the emergence of the area-specific expression of *unc5h4* was likely to be concomitant with that when thalamocortical axons begin to develop their terminal arbors in layer 4 of the neocortex (Agmon et al., 1993). Indeed, at P0 and P2, when thalamocortical axons have reached the neocortex but hardly made their terminal arbors, *unc5h4* was weakly expressed in the neocortex. However, at P3, when thalamocortical axons begin to develop their terminal arbors, discontinuous pattern of *unc5h4* expression clearly emerged in presumptive layer 4. The spatiotemporal correlation between *unc5h4* gene expression and thalamocortical projections implies that thalamic inputs might be important for the distribution of *unc5h4*-expressing cells in the neocortex. The

expression in the barrel field is also consistent with this view (see Fig. I-7). Indeed, several studies support the importance of thalamic axons in cortical cell development. For example, *in vitro* experiments have suggested that the thalamus provides trophic support for subplate cells which are transient target for thalamocortical axons (Price and Lotto, 1996). Moreover, layer 4 was absent due to excessive cell death in *COUP-TFI* mutant mice, in which thalamocorical axons failed to reach the neocortex (Zhou et al., 1999). Therefore, *unc5h4*-expressing layer 4 neurons could be affected by netrin-4 derived from thalamic axons. However, additional studies are needed to confirm whether netrin-4 protein is transported to the axon terminals. Furthermore, it is also conceivable that thalamic inputs might promote the production of netrin-4 in cortical cells. Although I cannot exclude the possibility that area-specificity of *unc5h4* expression in the neocortex is regulated by cues except for thalamic afferents, the observations in this study may provide clues to the relationship between the thalamic inputs and cortical development.

References

Ackerman SL, Kozak LP, Przyborski SA, Rund LA, Boyer BB, Knowles BB (1997) The mouse rostral cerebellar malformation gene encodes an UNC-5-like protein. Nature 386:838-842.

Agmon A, Yang LT, O'Dowd DK, Jones EG (1993) Organized growth of thalamocortical axons from the deep tier of terminations into layer IV of developing mouse barrel cortex. J Neurosci 13:5365-5382.

Ajioka I, Nakajima K (2005) Birth-date-dependent segregation of the mouse cerebral cortical neurons in reaggregation cultures. Eur J Neurosci 22:331-342.

Arakawa H (2004) Netrin-1 and its receptors in tumorigenesis. Nat Rev Cancer 4:978-987.

Engelkamp D (2002) Cloning of three mouse Unc5 genes and their expression patterns at mid-gestation. Mech Dev 118:191-197.

Finger JH, Bronson RT, Harris B, Johnson K, Przyborski SA, Ackerman SL (2002) The netrin 1 receptors Unc5h3 and Dcc are necessary at multiple choice points for the guidance of corticospinal tract axons. J Neurosci 22:10346-10356.

Hong K, Hinck L, Nishiyama M, Poo MM, Tessier-Lavigne M, Stein E (1999) A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. Cell 97:927-941.

Leonardo ED, Hinck L, Masu M, Keino-Masu K, Ackerman SL, Tessier-Lavigne M (1997) Vertebrate homologues of C. elegans UNC-5 are candidate netrin receptors. Nature 386:833-838.

Llambi F, Causeret F, Bloch-Gallego E, Mehlen P (2001) Netrin-1 acts as a survival factor via its receptors UNC5H and DCC. Embo J 20:2715-2722.

Llambi F, Lourenco FC, Gozuacik D, Guix C, Pays L, Del Rio G, Kimchi A, Mehlen P (2005) The dependence receptor UNC5H2 mediates apoptosis through DAP-kinase. Embo J 24:1192-1201.

Mehlen P, Mazelin L (2003) The dependence receptors DCC and UNC5H as a link between neuronal guidance and survival. Biol Cell 95:425-436.

Porter AG, Dhakshinamoorthy S (2004) Apoptosis initiated by dependence receptors: a new paradigm for cell death? Bioessays 26:656-664.

Price DJ, Lotto RB (1996) Influences of the thalamus on the survival of subplate and cortical plate cells in cultured embryonic mouse brain. J Neurosci 16:3247-3255.

Qin S, Yu L, Gao Y, Zhou R, Zhang C (2007) Characterization of the receptors for axon guidance factor netrin-4 and identification of the binding domains. Mol Cell Neurosci 34:243-250.

Tabata H, Nakajima K (2001) Efficient in utero gene transfer system to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex. Neuroscience 103:865-872.

Tang X, Jang SW, Okada M, Chan CB, Feng Y, Liu Y, Luo SW, Hong Y, Rama N, Xiong WC, Mehlen P, Ye K (2008) Netrin-1 mediates neuronal survival through PIKE-L interaction with the dependence receptor UNC5B. Nat Cell Biol 10:698-706.

Tanikawa C, Matsuda K, Fukuda S, Nakamura Y, Arakawa H (2003) p53RDL1 regulates p53-dependent apoptosis. Nat Cell Biol 5:216-223.

Thiebault K, Mazelin L, Pays L, Llambi F, Joly MO, Scoazec JY, Saurin JC, Romeo G,

Mehlen P (2003) The netrin-1 receptors UNC5H are putative tumor suppressors controlling cell death commitment. Proc Natl Acad Sci U S A 100:4173-4178.

Wang H, Copeland NG, Gilbert DJ, Jenkins NA, Tessier-Lavigne M (1999) Netrin-3, a mouse homolog of human NTN2L, is highly expressed in sensory ganglia and shows differential binding to netrin receptors. J Neurosci 19:4938-4947.

Watanabe K, Tamamaki N, Furuta T, Ackerman SL, Ikenaka K, Ono K (2006) Dorsally derived netrin 1 provides an inhibitory cue and elaborates the 'waiting period' for primary sensory axons in the developing spinal cord. Development 133:1379-1387.

Williams ME, Strickland P, Watanabe K, Hinck L (2003) UNC5H1 induces apoptosis via its juxtamembrane region through an interaction with NRAGE. J Biol Chem 278:17483-17490.

Williams ME, Lu X, McKenna WL, Washington R, Boyette A, Strickland P, Dillon A, Kaprielian Z, Tessier-Lavigne M, Hinck L (2006) UNC5A promotes neuronal apoptosis during spinal cord development independent of netrin-1. Nat Neurosci 9:996-998.

Wilson BD, Ii M, Park KW, Suli A, Sorensen LK, Larrieu-Lahargue F, Urness LD, Suh W, Asai J, Kock GA, Thorne T, Silver M, Thomas KR, Chien CB, Losordo DW, Li DY (2006) Netrins promote developmental and therapeutic angiogenesis. Science 313:640-644.

Zhou C, Qiu Y, Pereira FA, Crair MC, Tsai SY, Tsai MJ (1999) The nuclear orphan receptor COUP-TFI is required for differentiation of subplate neurons and guidance of thalamocortical axons. Neuron 24:847-859.

General Discussion

During development, neurons differentiate into specific cell types. After cell type-specification, neurons come to express cell type-specific molecules, including intracellular and membrane-associated molecules, which may play a role in regulation of intracellular environment, communications with surrounding cells or response to the extracellular environment. Extracellular communications lead to the arrangement of cytoarchitecture and neural circuits as well as the maintenance and maturation of cellular conditions. An intriguing question is how cell type-specific differentiation is regulated.

In the neocortex, two models were hypothesized two decades ago, with respect to the mechanism of cortical development especially focusing on area map formation (Rakic, 1988; O'Leary, 1989). In the protomap model (Rakic, 1988), the cortical primordium is patterned as cells are dividing in the VZ. Intrinsic area differences are specified by molecular determinants, and as neurons migrate radially out of the VZ, they transfer the protomap to the CP. An alternative model is the protocortex model (O'Leary, 1989), in which the cortical primordium is essentially homogeneous as it is generated and is patterned into area later by cues derived from axons growing from the thalamus. Today it has been proposed that intrinsic and extrinsic factors cooperate to generate the mature cortical area map (Grove and Fukuchi-Shimogori, 2003).

Since cortical areas are defined by the difference of layer cytoarchitecture between areas (Caviness, 1975), the exploration of the mechanisms of laminar differentiation will lead to understanding that of area differentiation. Recently, much

progress has been made in identifying transcription factors as intrinsic factors for laminar specification (Molyneaux et al., 2007). Such transcription factors may regulate the expression of several membrane-bound and extracellular molecules in a lamina-specific fashion. During development, such kind of molecules may play an important role in determination of cell locations, the formation of lamina-specific neural circuits or other aspects of layer-specific developmental events through interacting with environmental factors. Although several lamina-specific genes encoding membrane-associated molecules have been identified so far, it is poorly understood how they contribute to the layer-specific development by interacting with extrinsic cues, and also lamina-specific molecules themselves have not been still identified enough to classify overall laminar characteristics of cortical neurons.

In chapter I, I searched for genes expressed specifically in the upper layers, and identified four genes from a subtraction cDNA library including a netrin receptor gene *unc5h4*. *Unc5h4* was expressed specifically in layer 4 during perinatal stages when laminar organization and circuit formation are taking place, suggesting that this receptor might act on such developmental events by receiving extrinsic cues. Furthermore, *unc5h4* was predominantly expressed by layer 4 cells in the primary sensory areas, the target cells of thalamocortical axons, suggesting that it may also contribute to area-specific differentiation, possibly as a result of interaction with extrinsic factors derived from thalamic axons. It remains to be clear whether the transcription of *unc5h4* gene is regulated by only an intrinsic genetic program or additionally by extrinsic factors. However, the finding of layer- and area-specific genes in the developing neocortex, including *unc5h4*, indicates that a larger number of

molecules may participate in the layer- and area-specific developmental events including neural circuit formations, some of which may act through interacting with extrinsic cues.

In chapter II, I studied the ligands of UNC5H4 and the role of UNC5H4 in cortical development. As candidate ligands of UNC5H4, netrin-1 and netrin-4 proteins were analyzed by a cell surface binding assay and revealed their binding to *unc5h4*-expressing cells. Furthermore, the expression analysis for the *netrin* genes showed that *netrin-4* but not *netrin-1* were expressed in the neocortex and sensory thalamic nuclei. Taken together, these results indicate that netrin-4 is one of practical ligands of UNC5H4 in the neocortex. The expression pattern of *netrin-4* gene leads to two possible ways of the influence of netrin-4 protein on *unc5h4*-expressing cortical neurons. One possibility is that netrin-4 is supplied by surrounding cortical cells or by *unc5h4*-expressing cells themselves. In this case, the expression of *netrin-4* may be controlled by an intrinsic program or induced by thalamic inputs. Another possibility is that netrin-4 is supplied by thalamic neurons through their axons. The latter possibility further leads to the idea that some molecules might be supplied to the target neurons through thalamocortical axons, thereby affecting layer- and area-specific cortical cell differentiation. In this sense, it will be valuable to identify thalamus-derived molecules in the future study for understanding the molecular mechanisms of thalamic afferent-induced cortical differentiation. The gene profiles in cortical cells influenced by thalamic inputs will also help us understand what kind of differentiation is induced by axonal inputs. These studies will provide supporting evidence to the protocortex hypothesis. In the present study, I observed the cell survival effect of netrin-4 on

unc5h4-expressing cortical neurons *in vitro*. Neuronal cell survival and death might be one of the important processes in the development of the nervous system including the neocortex (Oppenheim, 1991).

In conclusion, the results of my present work indicate that UNC5H4 is predominantly expressed by layer 4 neurons in the sensory cortex during perinatal stages and may play a role in cell survival by interating with netrin-4 in a layer-specific manner. These results also imply that layer 4 neuorns may mature or maintain themselves by interacting with extrinsic factors derived from cortical cells and/or thalamic axons, mediated by membrane-associated molecules such as UNC5H4.

References for general discussion

Caviness VS, Jr. (1975) Architectonic map of neocortex of the normal mouse. J Comp Neurol 164:247-263.

Grove EA, Fukuchi-Shimogori T (2003) Generating the cerebral cortical area map. Annu Rev Neurosci 26:355-380.

Molyneaux BJ, Arlotta P, Menezes JR, Macklis JD (2007) Neuronal subtype specification in the cerebral cortex. Nat Rev Neurosci 8:427-437.

O'Leary DD (1989) Do cortical areas emerge from a protocortex? Trends Neurosci 12:400-406.

Oppenheim RW (1991) Cell death during development of the nervous system. Annu Rev Neurosci 14:453-501.

Rakic P (1988) Specification of cerebral cortical areas. Science 241:170-176.

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Papers and conference presentations related to this thesis

(Papers)

Zhong Y, Takemoto M, Fukuda T, Hattori Y, Murakami F, Nakajima D, Nakayama M, Yamamoto N (2004) Identification of the genes that are expressed in the upper layers of the neocortex. Cereb Cortex 14:1144-1152.

Yoneshima H, Yamasaki S, Voelker CC, Molnar Z, Christophe E, Audinat E, Takemoto M, Nishiwaki M, Tsuji S, Fujita I, Yamamoto N (2006) Er81 is expressed in a subpopulation of layer 5 neurons in rodent and primate neocortices. Neuroscience 137:401-412.

Takemoto M, Hattori Y, Yamamoto N.
UNC5D is expressed in layer 4 of the primary sensory areas of the neocortex and mediates the effect of netrin-4 on cortical cell survival *in vitro*. (in preparation)

(Conference presentations)

A STUDY OF LAYER 4-SPECIFIC GENES IN THE RAT NEOCORTEX
Makoto Takemoto, Tsuyoshi Fukuda, Fujio Murakami, Nobuhiko Yamamoto.
Neuroscience Research Suppl. 23 (1999) S148.

Unc5h4 and netrin-4 regulate survival and neurite outgrowth of mouse neocortical layer IV neurons in culture
Makoto Takemoto, Yuki Hattori, Nobuhiko Yamamoto.
Neuroscience Research Suppl. 58S (2007) S199.

Unc5h4 is expressed in layer IV of the primary sensory areas of the neocortex and regulates neuronal cell survival by interacting with netrin-4 *in vitro*
竹本 誠
神経組織の成長・再生・移植研究会　第23回学術集会　2008年5月17日

LAMINA- AND AREA-SPECIFIC EXPRESSION OF *UNC5H4* AND ITS ROLE IN CORTICAL DEVELOPMENT
Makoto Takemoto, Yuki Hattori, Nobuhiko Yamamoto.
Cortical Development Meeting, 22-25 May 2008, Chania, Crete, Greece.

Identification of the Genes that are Expressed in the Upper Layers of the Neocortex

Yun Zhong[1,3], Makoto Takemoto[1], Tsuyoshi Fukuda[1], Yuki Hattori[1], Fujio Murakami[1], Daisuke Nakajima[2], Manabu Nakayama[2] and Nobuhiko Yamamoto[1]

[1]Neuroscience Laboratories, Graduate School of Frontier Biosciences, Osaka University, Toyonaka, Osaka 560-8531, Japan and [2]Department of Human Gene Research, Kazusa DNA Research Institute, 2-6-7 Kazusa-kamatari, Chiba 292-0818 Japan

[3]Present address: The Rockefeller University, Box 57, 1230 York Avenue, New York, NY 10021, USA

Laminar specificity is one of the most striking features of neocortical circuitry. To explore the molecular basis of this specificity, particularly in relation to thalamocortical connectivity, we searched for the genes expressed in the upper cortical layers by constructing a subtraction cDNA library that was enriched for genes expressed in layer 4 of perinatal rat somatosensory cortex. Differential screening, sequence analysis and *in situ* hybridization demonstrated that a new unc5 family member (*unc5h4*), *deltex-like* gene, stem cell factor (*SCF*) and myocyte-specific enhancer factor-2C (*MEF-2C*) were specifically expressed in layer 4 or layers 2/3–4 at postnatal day 7, by when laminar organization and fundamental cortical circuitries have been established. In terms of regional specificity, *unc5h4* and *SCF* signals were stronger in sensory cortices, whereas *MEF-2C* and *deltex-like* gene were expressed rather uniformly in all neocortical regions. Analysis during development demonstrated that expression of these genes was pronounced between late embryonic and early postnatal developmental stages, except for *MEF-2C* expression, which continued in later stages. These results demonstrate that certain types of molecules including transcription factors, receptor and ligand molecules, are expressed specifically in the upper layers of the developing neocortex, suggesting a role in laminar specification of cortical cells and circuitry.

Keywords: cerebral cortex, gene expression, layer specificity, thalamocortical projection, unc5h4

Introduction

The neocortex is fundamentally composed of six cell layers, which are distinguishable by cellular morphology and the extrinsic and intrinsic connections they make (McConnell, 1989). An intriguing question is how cortical neurons differentiate into a particular laminar type, and are connected with a specific population of subcortical and cortical neurons. A plausible mechanism is that a set of transcriptional factors expressed in a given layer regulates laminar fate, and expression of downstream molecules including ligand and receptor molecules, is required for cell type specification and axonal guidance of cortical afferents and efferents. Therefore, a key approach is to identify the molecules that are expressed with laminar specificity.

Several molecules with lamina-specific expression patterns in the cortex have been identified. For instance, transcription factors of *Otx1* and *Id2* are primarily distributed in layer 5, and *Tbr1*, a T box gene, in layer 6 (Bulfone *et al.*, 1995; Frantz *et al.*, 1994; Hevner *et al.*, 2001). Moreover, retinoid Z receptor (*RZR*-β) and chick ovalbumin upstream transcription factor 1 (*COUP-TF1*) are expressed rather specifically in layer 4 (Becker-Andre *et al.*, 1994; Park *et al.*, 1997; Liu *et al.*, 2000). It has also been demonstrated that *cadherin-6* and *rCNL3* – homologous to G-protein-coupled receptors – are expressed in the upper layers (Suzuki *et al.*, 1997; Chenn *et al.*, 2001). Although molecular identification has progressed, a further understanding of gene expression in the developing cortex is necessary to pursue the mechanisms of laminar specification.

In the present study, we attempted to identify lamina-specific genes, focusing on thalamocortical connectivity. The thalamocortical projection exhibits typical laminar specificity (Jones, 1981; Gilbert, 1983) and has also been well characterized during development (Lund and Mustari, 1977; Ghosh and Shatz, 1992; Agmon *et al.*, 1993; Kageyama and Robertson, 1993; Catalano *et al.*, 1996; Molnár *et al.*, 1998). To date, *in vitro* studies using organotypic co-cultures of the cortex with the thalamus have demonstrated that there is a target recognition mechanism by which thalamocortical axons recognize layer 4, their target (Yamamoto *et al.*, 1989, 1992; Molnár and Blakemore, 1991, 1999; Bolz *et al.*, 1992). Our previous investigations have further suggested that thalamocortical axon branching is induced by membrane-associated molecules in layer 4, whereas termination of axonal growth is regulated by growth-inhibitory molecules in layers 2/3 and 4 (Yamamoto *et al.*, 1997, 2000a, 2000b). These findings suggest that the factors regulating thalamocortical axon targeting and differentiation of target layer cells are expressed specifically in the upper layers. Based on this hypothesis, we searched for the molecules expressed specifically in these layers, in particular, layer 4, by constructing a subtraction cDNA library. Four genes, including a new unc5 family member, were obtained. Their expression patterns were further investigated to gain an insight into their possible roles in thalamocortical connectivity and/or cortical cell identity.

Materials and Methods

Animals

Sprague–Dawley rats were used for all experiments (Nihon Animals, Osaka, Japan). The day of vaginal plug detection was designated as E0, and the day of birth as postnatal day 0 (P0).

Construction of a Subtraction cDNA Library

The whole brain was removed from P7 rats. Coronal slices (250 μm thickness) were cut with a microtome in ice-cold Hanks' solution. Layer 4 strips, ~500 μm in length, were dissected from the somatosensory cortex with a small scissors (Fig. 1). The cortical barrel structures, which were visible under a trans-illuminating microscope, were used as a landmark for layer 4 (Fig. 1). Virtually the same size of layer 5 strip was dissected beneath layer 4 (Fig. 1). Three or four pieces for each layer were collected, from which total RNAs were extracted (RNeasy Mini Kit, Qiagen, Tokyo, Japan). Approximately 10–20 ng/μl of total RNA (30 μl) was obtained. Layer 4 and layer 5 cDNAs were

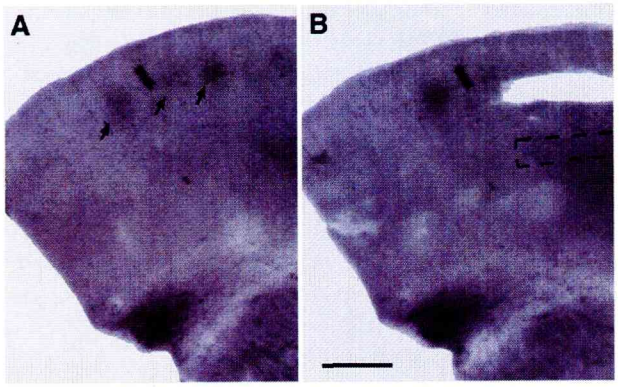

Figure 1. Dissection of layer 4 and 5 strips from P7 rat somatosensory cortex. (*A*) Somatosensory cortical slice under transilluminating light before dissection. Arrows indicate the barrels. (*B*) The same cortical slice after dissection of layer 4 strip. The area surrounding the dashed lines represents dissected layer 5 strip. Bar indicates 0.5 mm.

synthesized from ~50 ng of these RNAs with reverse transcriptase (SMART PCR cDNA Synthesis Kit, Clontech, Tokyo, Japan). These cDNAs were amplified with a primer (AAGCAGTGGTAACAACGCAGAGT), and subjected to *Rsa*I digestion for the following hybridization. The layer 4 DNA fragments were hybridized with an excess amount of the layer 5 DNA fragments (PCR Select cDNA Subtraction Kit, Clontech, Tokyo, Japan). Thereafter, unhybridized DNA fragments were further amplified, cloned into pGEM-T vectors (Promega, Tokyo, Japan), and stored as a subtraction cDNA library (layer 4 minus layer 5). Likewise, a reverse subtraction library (layer 5 minus layer 4) was produced.

Differential Screening and Sequence Analysis

To eliminate false-positive clones a differential screening was performed prior to *in situ* hybridization. After transformation of the subtraction cDNA library, colonies were picked randomly and grown in a 96-well plate. Each insert was amplified by polymerase chain reaction (PCR) from the bacterial culture solution. Amplified DNAs were denatured with 0.6 M NaOH and duplicated onto two nylon membranes. Two kinds of DNA probes (layer 4 and layer 5 probes) were produced with Digoxigenin (DIG)-labeled nucleotides (Roche, Tokyo, Japan) by amplifying inserts from the two subtraction cDNA libraries (layer 4 minus layer 5 and layer 5 minus layer 4). The duplicate membranes were subjected to hybridization with each probe and detected with chemilluminescence reaction. The clones showing >2-fold intensity with layer 4 probes were selected and subjected to *in situ* hybridization.

The selected clones were applied to sequence analysis with the plasmid-specific sequences. In some cases, extended DNA fragments were obtained by 5′ race method (Smart race kit, Clontech, Tokyo, Japan). In a few cases, a human brain cDNA library was used to obtain full-length cDNAs (Nagase *et al.*, 1997).

In Situ Hybridization

DIG-labeled RNA probes were used for hybridization. To produce linearized templates for the synthesis of riboprobes, inserts in pGEM-T vectors were amplified by PCR using oligonucleotides that contain T7 and SP6 promoter sequences. The PCR products were purified (PCR purification kit, Qiagen, Tokyo), and *in vitro* transcription was carried out (DIG-RNA Synthesis Kit, Roche, Tokyo). Finally, these probes were purified with gel filtration columns (Quick Spin Columns, Roche, Tokyo) and kept at -80°C.

Rats were decapitated after anesthesia to obtain whole brains from postnatal animals (P0, P3, P7 and P14). Embryonic brains were taken from fetuses under deep anesthesia. The brains were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h at room temperature and then 2 h at 4°C. After overnight incubation in PBS containing 20% sucrose, the brains were frozen and then cut into 10 μm sections (frontal or sagittal sections) with a cryostat.

Sections were refixed in 4% paraformaldehyde in 0.1 M phosphate buffer, washed with distilled water and 0.1 M triethanolamine, then acetylated in 0.25% acetic acid in 0.1 M TAE, followed by a final wash in PBS. Prehybridization was carried out for 1 h in hybridization buffer (50% formamide, 5% SDS, 5× SSPE, 1 mg/ml tRNA), followed by hybridization overnight at 60°C in hybridization buffer containing 1 μg/ml DIG-labeled RNA probe. After three washes in 50% formamide and 2 × SSC at 60°C, these sections were subjected to blocking (blocking regent, Roche, Tokyo, Japan) for 1–4 h at room temperature, and then incubated overnight at 4°C with alkaline phosphatase-conjugated anti-DIG antibody (1:2000, Roche, Tokyo, Japan). After washing five times at room temperature, the color reaction was carried out at room temperature or 4°C in BM Purple (Roche, Tokyo, Japan). The reaction was terminated by immersing the sections in 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min. Then sections were treated in 70%, 80%, 90% and 100% ethanol, and xylene, and then embedded.

For Nissl staining, adjacent sections were used. These sections were immersed in 0.1% methylene blue for 15–60 s and then subjected to the ethanol series and embedding.

Results

Expression of Upper Layer-specific Genes in the Neocortex

To identify the genes that are expressed in layer 4 or layer 2/3–4, we constructed a subtraction cDNA library in which cDNAs derived from layer 4 strips of P7 rat somatosensory cortex was enriched by subtracting cDNAs from layer 5 strips (see Materials and Methods). Approximately 1000 clones from the subtraction library were subjected to the differential screening in the first-round screening. As a result, we obtained 130 positive clones, which showed stronger signals to the layer 4 probe than the layer 5 probe. *In situ* hybridization was then performed with each of the 130 clones, in order to examine laminar specificity on P7 rat brain, in which neocortical laminar configuration is established. Although most of the clones tested showed virtually no or very faint signals, four clones (571, 585, 746 and 846) exhibited specific expression in layer 4 or layer 2/3–4.

Figure 2 shows the laminar expression pattern of these genes in sensory cortices. The expression of 571 was highly restricted to layer 4 of the visual and somatosensory cortices, with almost no expression in any other layers (Fig. 2*A*,*E*). The expression of 585 was also strong in layer 4, although it expanded slightly to the adjacent layers (Fig. 2*B*,*F*). On the other hand, the expression of 746 was distributed in layers 2/3–4, with no detectable expression in the deep layers (Fig. 2*C*,*G*). The expression of 846 was also localized in the upper layers, but faint signals were also detected in the deep layers (Fig. 2*D*,*H*).

Identification of the Lamina-specific Genes

Sequence analysis was performed for the four DNA fragments. The sequences of 746 and 846 have high homology to mouse stem cell factor (85 %), and mouse myocyte-specific enhancer factor (95 %), respectively. Taking into consideration the difference between species, 746 and 846 were thought to be rat homologs of these two genes.

On the other hand, DNA fragment 571 showed no homology to any known gene, though there were expression sequence tags (ESTs) that exactly matched these clones. However, an extended product of 571 from the 5′ race method was homolo-

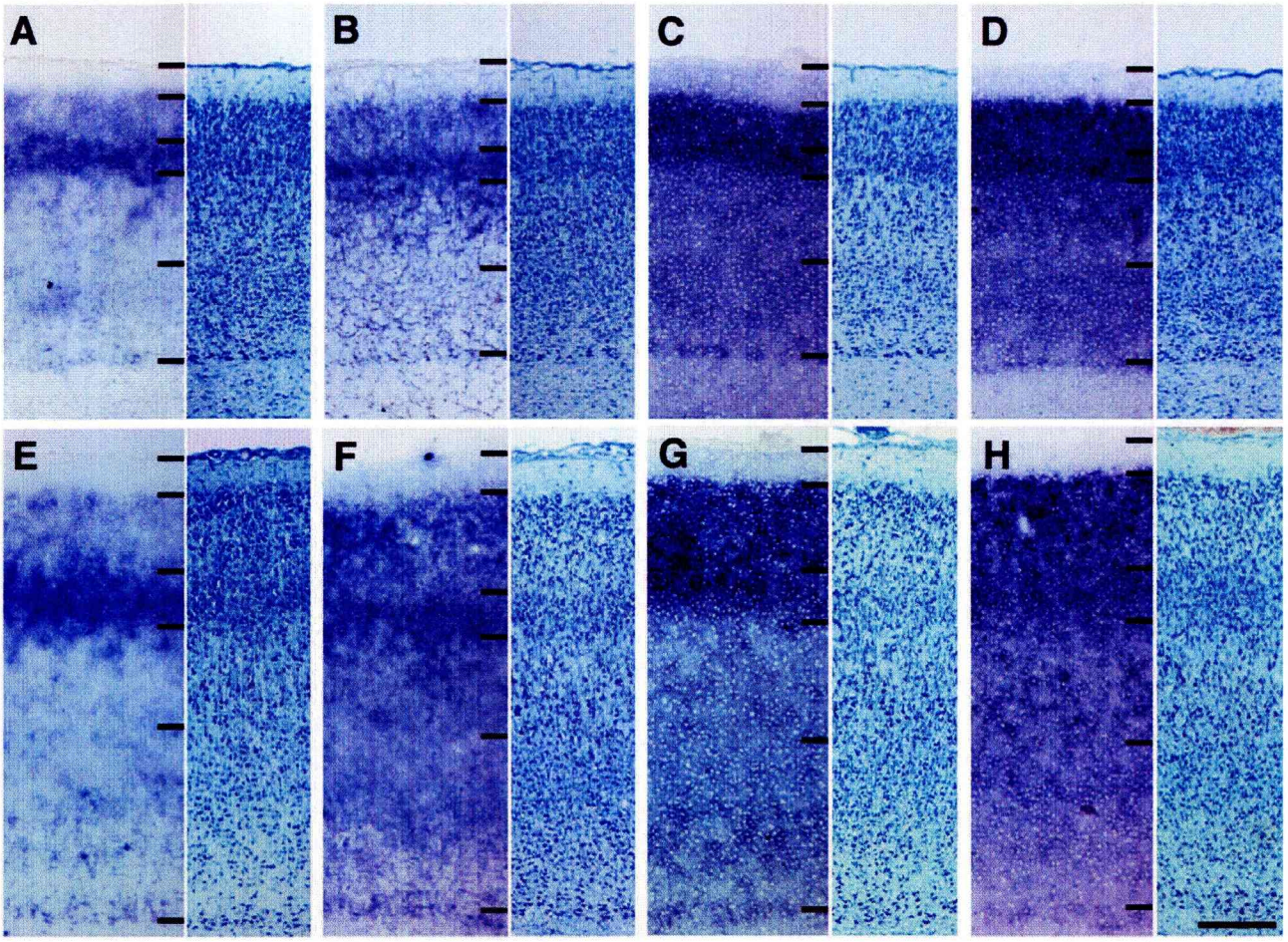

Figure 2. Four clones which are expressed in layer 4 or in layer 2/3–4. *In situ* hybridization shows laminar expression patterns in the visual (*A–D*) and somatosensory (*E–H*) cortices of P7 rat. DIG-labeled probes were used. In each panel, *in situ* hybridization is shown to the left, and Nissl staining in adjacent sections is to the right. 571 (*A, D*) and 585 (*B, E*) genes were more specifically expressed in layer 4, while 746 (*C, G*) and 846 (*D, H*) genes were expressed in layer 2/3–4. Bar represents 0.2 mm.

gous to an unreported human cDNA, which was obtained from a human brain cDNA library (Nagase *et al.*, 1997, 1999). The transcript contained an open reading frame (2847 bp) and 3′ untranslated region (UTR) of >4000 bp. The deduced amino acid sequence (948 aa) revealed its features to be a transmembrane protein, including a signal peptide sequence, two immunoglobulin and thrombospondin domains (Fig. 3). Its cytoplasmic region consists of ZU5 and death domains, which are common to unc5-like netrin receptors (Ackerman *et al.*, 1997). We hereafter designate this novel member of the unc5 family as unc5h4. The nucleotide sequence of human *unc5h4/KIAA1777* was deposited into DDBJ/GenBank/EMBL DNA databases (accession no. AB055056). *KIAA1777* is an alias for this new gene in the human brain cDNA database.

DNA fragment 585 was also extended by the 5′ race method and found to highly match a coding region of *KIAA0937*, which was obtained from the sequence analysis of human cDNAs (Nagase *et al.*, 1999). The deduced amino acid sequence (653 aa) contained two WWE domains, which are predicted to mediate specific protein–protein interactions (Aravind, 2001), and had high homology (58 %) to human deltex. Therefore, *KIAA0937* is referred to as *deltex-like* gene.

Regional Specificity and Expression in Other Brain Regions

Expression patterns of the four obtained genes were further studied in P7 rat brain. In particular, areal specificity was examined in order to understand whether these genes are involved in more generalized features of cortical laminar organization.

Unc5h4/KIAA1777 (571) exhibited a highly restricted expression in layer 4 across all neocortical regions. However, its expression was particularly strong in sensory cortices including somatosensory and visual areas (Fig. 4*A,B*). Another characteristic of this gene was its prominent expression in the amygdala and hippocampus, especially in CA3 and dentate gyrus. Moderate expression was also found in the hypothalamus. In the thalamus, weak expression was observed in the ventral part of the lateral geniculate nucleus. In other brain regions, it was expressed in mitral cell layers of the olfactory bulb and Purkinje cells of the cerebellum.

The *deltex-like gene* (585) was also expressed in layer 4 of all neocortical areas. However, in the somatosensory area, the signal was not only distributed in layer 4 but also in the upper part of layer 2/3 (Fig. 4*C,D*). No apparent signal was found in areas other than the neocortex, except for weak expression in the granule cell layer of the cerebellum.

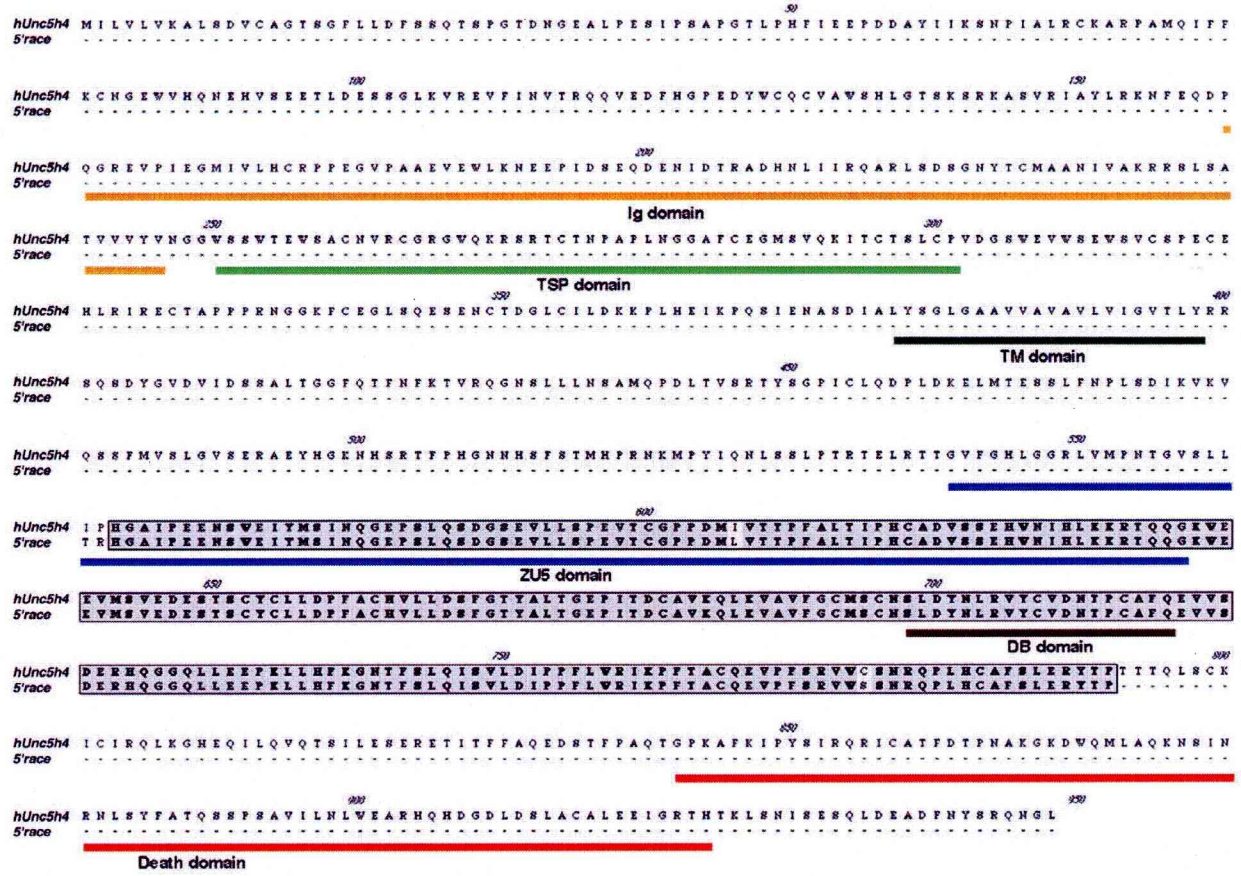

Figure 3. Amino acid sequence of human unc5h4/KIAA1777 and extended product of 571 by 5′ race method. TSP, thrombospondin; TM, transmembrane.

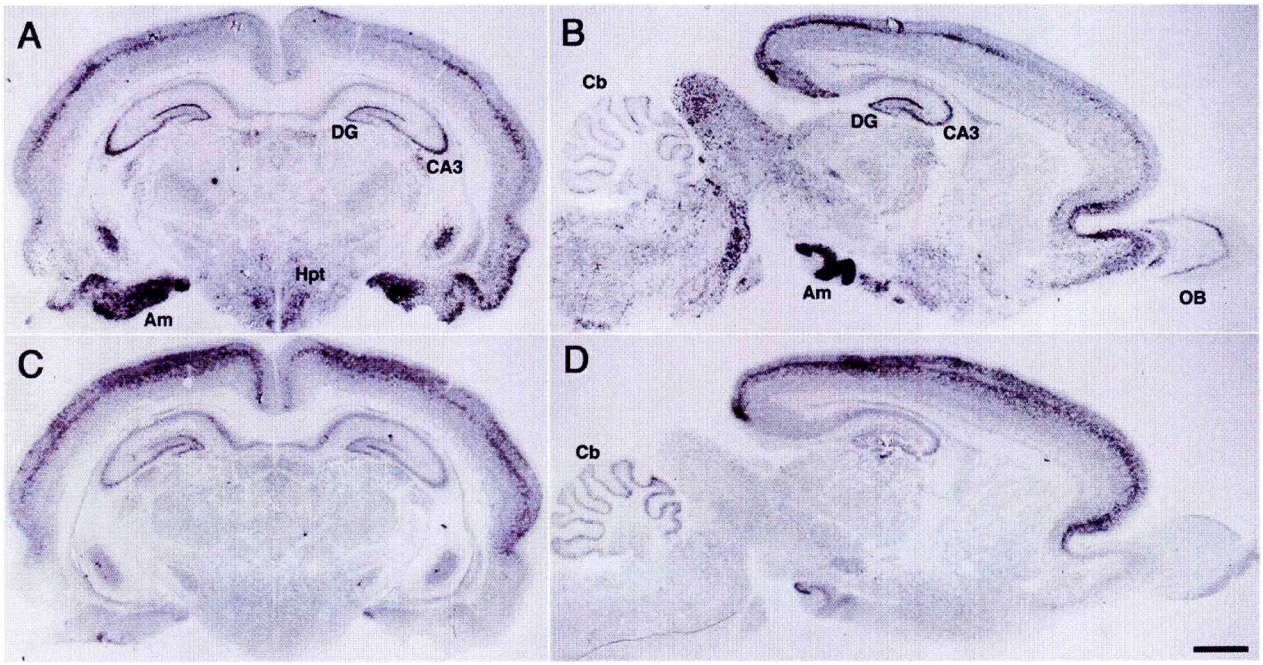

Figure 4. Layer 4-specific genes. Upper (*A*, *B*) and lower (*C*, *D*) panels show expression patterns of *unc5h4* and *deltex-like* gene, respectively. Am, amygdala; Cb, cerebellum; DG, dentate gyrus; Hpt, hypothalamus; OB, olfactory bulb. Bar indicates 1 mm.

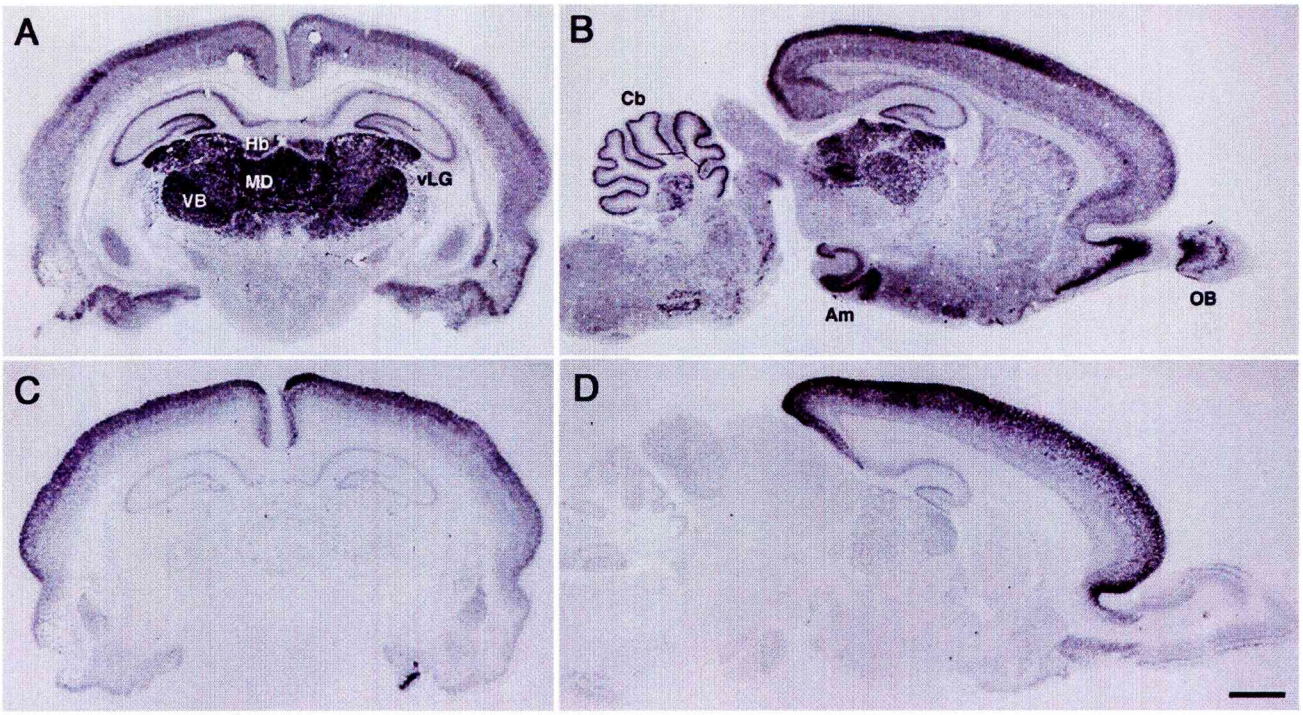

Figure 5. Layer 2/3–4-specific genes. Upper (*A*, *B*) and lower (*C*, *D*) panels show *SCF* and *MEF-2C* expressions, respectively. Am, amygdala; Cb, cerebellum; Hb, habenular nucleus; MD, mediodorsal thalamus; OB, olfactory bulb; VB, ventrobasal complex of thalamus; vLG, ventral part of the lateral geniculate nucleus. Bar indicates 1 mm.

SCF (746) showed strong expression between layers 2/3 and 4 across all neocortical regions (Fig. 5*A*,*B*). As observed for *unc5h4/KIAA1777*, *SCF* expression was strong in the somatosensory and visual areas. Its strong expression in the limbic region was another characteristic it shared with *unc5h4/KIAA1777*. The most striking aspect was the highly specific and strong expression in the thalamus. The expression was located in the dorsal lateral geniculate nucleus and the lateral posterior nucleus, which project to visual areas, and in the ventral basal thalamic nucleus, which projects to somatosensory areas. Strong signals were also found in the habenula, central lateral nucleus, central medial nucleus, parafascicular nucleus and intermediodorsal nucleus. In addition, this gene was expressed in the granule cells of the olfactory bulb and Purkinje cells of the cerebellum.

The expression of *MEF-2C* (846) was highly restricted to the neocortex (Fig. 5*C*,*D*). Its expression in layers 2/3–4 was uniform across the whole neocortex, but no expression was observed in other brain regions.

Developmental Changes of Laminar Expression Patterns

To gain an insight into how each gene is associated with laminar property, cellular differentiation and afferent invasion, their expression patterns were studied in the developing somatosensory cortex.

The expression of *unc5h4/KIAA1777* (571) was observed in the subventricular and intermediate zones at E18 (Fig. 6*A*). At P0, the expression appeared just beneath the marginal zone, and then in slightly lower layers at P3. At P7, the message was restricted to layer 4 (Fig. 6*A*). Thus, the expression pattern of this gene during development was closely related to the laminar locations of the cells destined to layer 4, but the signal was rather weakened after P14.

The message of *deltex-like* gene (585) was expressed in both the ventricular zone and the cortical plate (CP) at E18, but was distributed in the upper part of the CP from P0 to P3 (Fig. 6*B*), which is similar to *unc5h4* expression pattern. At P7, the expression was observed in layer 4 although it was slightly diffuse. Moreover, the message was also observed in the upper part of layer 2/3 in the somatosensory cortex. At P14, almost no expression was retained.

The message of *SCF* (746) was localized in the deepest part of the CP at E18 (Fig. 7*A*). At P0, the expression in layer 6 was weaker, and even more so at P3, although the signal began to appear in the upper part of the CP. At P7, the expression was strong in layers 2/3 and 4, whereas it had virtually disappeared in layer 6. At P14, almost no expression was observed.

MEF-2C (846) was strongly expressed in the upper part of the CP from E18 to P3 (Fig. 7*B*). At P7, the expression was primarily observed in layers 2/3 and 4, and slightly in the upper part of layer 5. Unlike the former three clones, the message was retained at P14 to a great extent.

Discussion

We obtained four genes that were expressed in the upper layers in P7 rat cortex when laminar configuration is established. *Unc5h4/KIAA1777* and *deltex-like* gene/*KIAA0937* were expressed specifically in layer 4, whereas *SCF* and *MEF-2C* were expressed in layers 2/3–4. As for area specificity, *unc5h4* and *SCF* signals were stronger in sensory cortices, but the messages of *MEF-2C* and *deltex-like* gene were distributed rather broadly in the neocortex. All of the genes were strongly

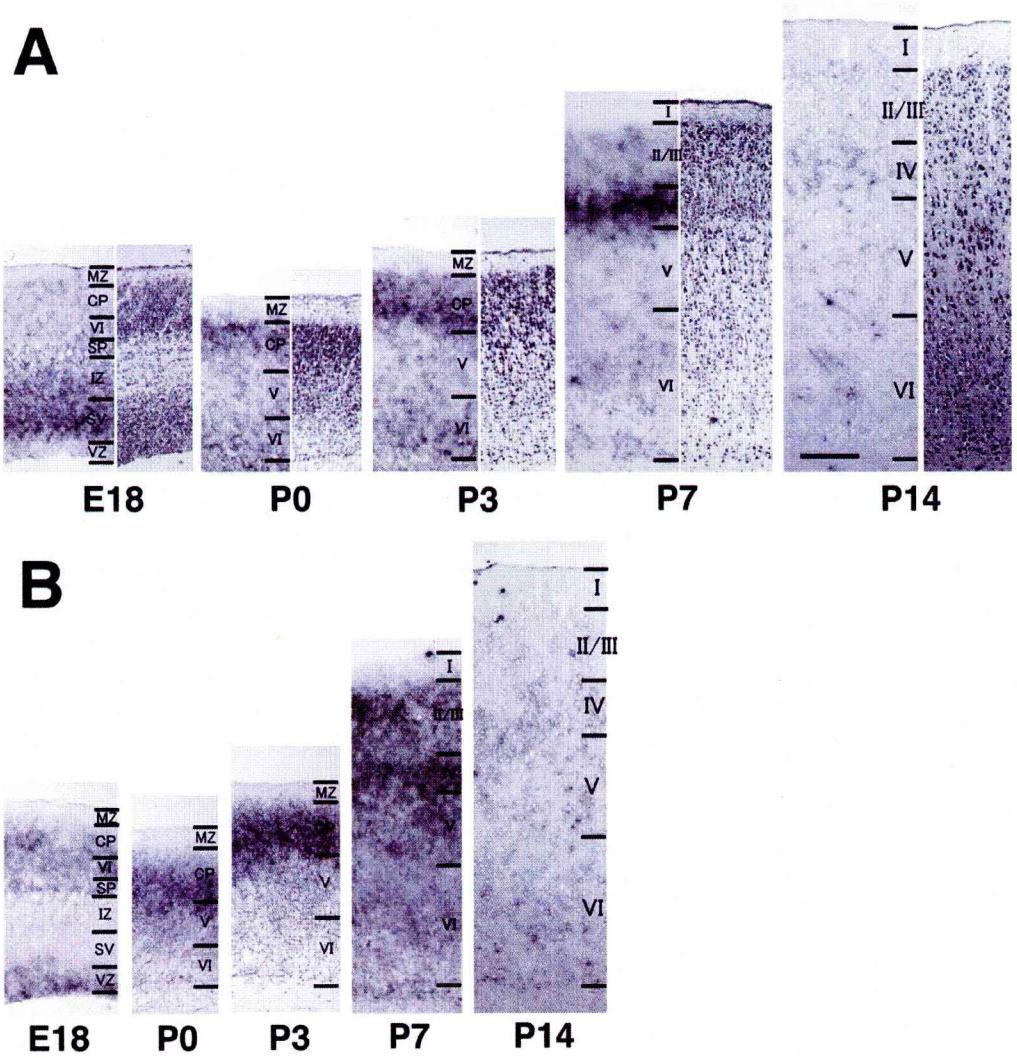

Figure 6. Developmental changes of laminar expression of layer 4-specific genes. *Unc5h4* (A) and *deltex like* gene (B) expressions in the parietal region (above the hippocampus) are shown. In (A), *in situ* signals are shown to the left of each stage, and Nissl staining is shown to the right. MZ, marginal zone; CP, cell-dense cortical plate; SP, subplate; SV, subventricular zone; VZ, ventricular zone. Bar represents 0.2 mm.

expressed in embryonic and early postnatal stages, except that *MEF-2C* messages were still present in later stages. Thus, the present study using systematic screening of the subtraction cDNA library clearly demonstrates that certain types of molecules including extracellular proteins and transcription factors, which might be involved in cellular differentiation and neural circuit formation, are expressed in the upper layers of the developing cortex.

Expression and Molecular Properties of Upper Layer-specific Genes

Unc5h4, a new member of unc5 family, was expressed most specifically in layer 4 of P7 rat cortex. After the sequence of human *unc5h4/KIAA1777* had been registered in GenBank/DDBJ database, its mouse homolog was reported (Engelkamp, 2002). However, the present study is the first to demonstrate its molecular characteristics and expression pattern in the brain. The expression profile during development further showed a migrating behavior of *unc5h4* expression. Layer 4 cells are born at E16–17 in the ventricular zone and migrate to the subventricular and intermediate zones at E18. At P0, they move to the most superficial part of the CP, and gradually settle in layer 4 by P6 (Berry and Rogers, 1965; Lund and Mustari, 1977). *Unc5h4* expression closely resembles this migration pattern, suggesting that *unc5h4* is expressed during development by the cells destined to form layer 4 of the cortex. Weak expression in the frontal lobe is consistent with this view, as granular cells, the major population in layer 4, are scarce in the motor cortex.

It has been shown that unc5 family members are involved in axonal elongation and cell migration as netrin-1 receptors (Leonardo *et al.*, 1997). In addition, these members have been shown to suppress neuronal apoptosis, upon receiving a ligand signal (Hofmann and Tschopp, 1995; Llambi *et al.*, 2001). Although the molecules that interact with *unc5h4* have not yet been identified, it is possible that the ligand may influence

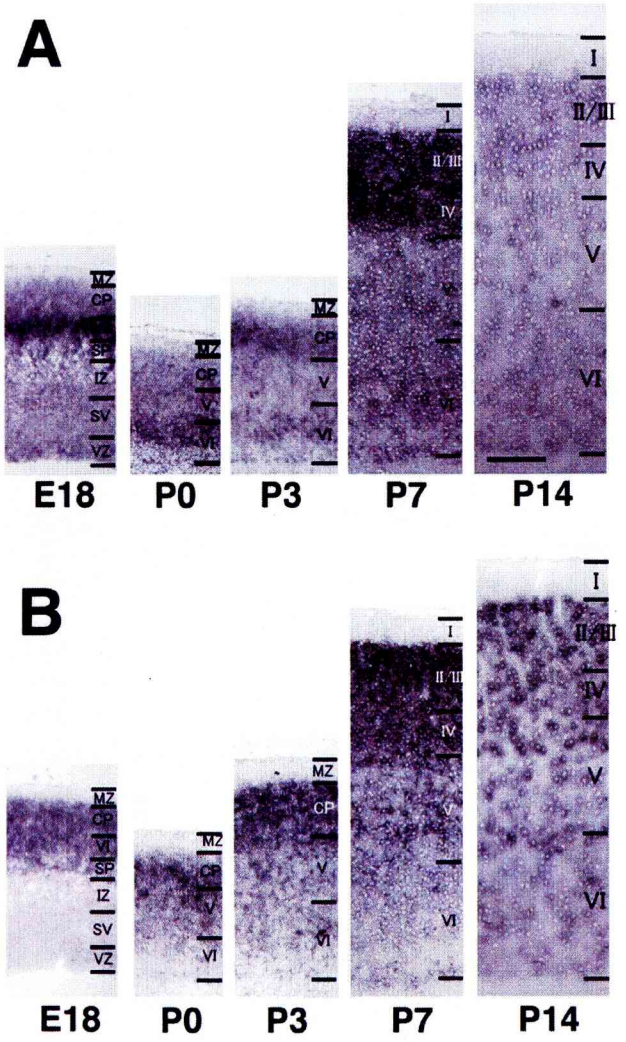

Figure 7. Developmental changes of laminar expression of upper layer-specific genes. *SCF* (A) and *MEF-2C* (B) expressions in parietal cortex are shown. Abbreviations are the same as those in Figure 6. Bar represents 0.2 mm.

neuronal survival of layer 4 cells expressing *unc5h4*. The survival effect might further influence neuronal connectivity. Another possibility is that *unc5h4* acts as a ligand molecule. The extracellular domains containing immunoglobulin and thrombospondin domains might directly influence ingrowing thalamic axons. Identification of the molecules that interact with *unc5h4* would be necessary to elucidate its role to a great extent.

We cloned and characterized another layer-4-specific molecule, *deltex-like* gene/*KIAA0937*, although its expression was not as localized as the *unc5h4* message. Deltex has been shown to mediate the Notch signaling pathway in the nucleus as well as in the cytoplasmic region (Matsuno *et al.*, 1995; Kishi *et al.*, 2001; Yamamoto *et al.*, 2001). Therefore, deltex-like protein may be involved in the differentiation of layer 4 cells by regulating Notch activity transcriptionally and/or through direct binding.

As for *MEF-2C*, Leifer *et al.* (1993) have shown that *MEF-2C* mRNA is expressed in the upper layers of the developing cortex. Our results not only confirm their findings but also demonstrate that this lamina-specific expression is present throughout the neocortex, suggesting that *MEF-2C* may be involved in the upper cortical neuron identity. Although it has been demonstrated that *MEF-2C* supports cell survival of postmitotic neurons (Mao *et al.*, 1999), the prolonged and rather uniform expression in the neocortex indicates the possibility that *MEF-2C* may contribute to the maintenance and differentiation of upper cortical cells rather than simply to the differentiation of postmitotic neurons.

On the other hand, *SCF* expression during cortical development was more complicated, but seems to be related to thalamic axon invasion. Since the tips of thalamic axons start to invade the CP at E18, extend into layer 5 at P0, and reach immature layer 4 at P3 (Lund and Mustari, 1977; Kageyama and Robertson, 1993; Catalano *et al.*, 1996; Molnár *et al.*, 1998), a correlation might exist between the expression of *SCF* and ingrowth of thalamic axons in the neocortex. In accordance with thalamic axon extension, *SCF* expression gradually became weaker in the deep layers and increased in the upper layers. Indeed, immunohistochemistry with an antibody against c-kit, the receptor of *SCF*, showed that c-kit was expressed in thalamic nuclei and axons (not shown), indicating the possibility that thalamic axons respond to SCF distributed in the developing cortex. The weaker expression of *SCF* in the frontal cortex might also be related to the lack of sensory thalamic axons in this area.

Implications of the Presence of Upper Layer-specific Genes

To date, it has been shown that putative regulatory genes, *RZR-β* (Becker-Andre *et al.*, 1994; Park *et al.*, 1997) and *COUP-TF1* (Liu *et al.*, 2000) are expressed in layer 4 of the neocortex, although the functions of these genes are not clear. Thalamocortical projections are disrupted in *COUP-TF1* mutants, but it is likely that the phenotype is attributable to the lack of subplate neurons rather than its direct influences on thalamocortical axon targeting in layer 4 (Zhou *et al.*, 1999). The present results further demonstrate the presence of another putative regulatory gene, *deltex-like* gene, which is expressed primarily in layer 4 and might act as a regulator of cellular differentiation (see above).

It is also important to reveal extracellular molecules such as cell surface or extracellular matrix molecules, in order to understand the molecular basis of cellular interactions and circuit formation (Yamamoto, 2002). In this sense, *unc5h4* is the gene that encodes a cell surface protein and is expressed rather specifically in cortical cells destined for layer 4 during development. Although how *unc5h4* contributes to cellular differentiation and connectivity in the cortex is unknown, its molecular characteristics raise the possibility that it is involved in the interactions between layer 4 cells, or between layer 4 cells and thalamocortical fibers. Recent studies have shown that *ephrin-A5*, an Eph ligand, affects thalamocortical axon behavior by its expression in layer 4 (Mann *et al.*, 2002), but this is unlikely to be the case with the entire sensory thalamocortical projections (Donoghue and Rakic, 1999; Mackarehtschian *et al.*, 1999; Vanderhaeghen *et al.*, 2000; Yabuta *et al.*, 2000). In contrast, localization of *unc5h4/KIAA1777* in layer 4 is found throughout the sensory cortices.

Cadherin-6 and *rCNL3* are known cell surface molecules that are expressed in the upper layers of the developing cortex

(Suzuki *et al.*, 1997; Chenn *et al.*, 2001). Phosphacan, a proteoglycan (Maeda and Noda, 1996), and SemaK1, a GPI-anchored semaphorin (Xu *et al.*, 1998), are also expressed in the upper layers of early postnatal stages, though their expression patterns during development are not well characterized. It would be worthwhile to examine whether these molecules affect thalamic axonal growth, as previous investigations have indicated that thalamocortical axon termination is governed by molecules distributed in layers 2/3–4. (Yamamoto *et al.*, 2000a,b; Noctor *et al.*, 2001; Yamamoto, 2002). In addition, the present findings of *SCF* expression pattern raises the possibility that a developmental change of laminar expression may influence afferent fiber ingrowth (see above). A similar laminar transition has been reported in the expression of *Sema3A*, a potential molecule, which might be responsible for the formation of thalamocortical projections (Skaliora *et al.*, 1998).

Several transcriptional factors have been shown to be expressed in a lamina-specific fashion: *Otx1* (Frantz *et al.*, 1994) and *Id2* (Bulfone *et al.*, 1995) in layer 5 and *Tbr-1* (Bulfone *et al.*, 1995; Hevner *et al.*, 2001) in layer 6. As for layer 4, *COUP-TF1* and *RZR*-β are expressed in layer 4, as described above. The present results show that *deltex-like* gene and *MEF-2C* are expressed in layer 4 and layer 2/3–4 as regulatory factors, respectively. Determining the relation between these regulatory genes and their downstream molecules would also be useful in elucidating the molecular basis of cortical cell differentiation and neural circuit formation.

Notes

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Y. Zhong and M. Takemoto contributed equally to this study.

Address correspondence to Nobuhiko Yamamoto, Neuroscience Laboratories, Graduate School of Frontier Biosciences, Osaka University, Toyonaka, Osaka 560-8531, Japan. Email: nobuhiko@fbs.osaka-u.ac.jp.

References

Ackerman SL, Kozak LP, Przyborski SA, Rund LA, Boyer BB, Knowles BB (1997) The mouse rostral cerebellar malformation gene encodes an UNC-5-like protein. Nature 386:838–842.

Agmon A, Yang LT, O'Dowd DK, Jones EG (1993) Organized growth of thalamocortical axons from the deep tier of terminations into layer IV of developing mouse barrel cortex. J Neurosci 13: 5365–5382

Aravind L (2001) The WWE domain: a common interaction module in protein ubiquitination and ADP ribosylation. Trends Biochem Sci 26:273–275.

Becker-Andre M, Wiesenberg I, Schaeren-Wiemers N, Andre E, Missbach M, Saurat JH, Carlberg C (1994) Pineal gland hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. J Biol Chem 269:28531–28534.

Berry M, Rogers AW (1965) The migration of neuroblasts in the developing cerebral cortex. J Anat 99:691–709.

Bolz J, Novak N, Staiger V (1992) Formation of specific afferent connections in organotypic slice cultures from rat visual cortex with lateral geniculate nucleus. J Neurosci 12:3054–3070.

Bulfone A, Smiga SM, Shimamura K, Peterson A, Puelles L, Rubenstein JL (1995) T-brain-1: a homolog of Brachyury whose expression defines molecularly distinct domains within the cerebral cortex. Neuron 15:63–78

Catalano S, Robertson RT, Killackey H (1996) Individual axon morphology and thalamocortical topography in developing rat somatosensory cortex. J Comp Neurol 366:36–53.

Chenn A, Levin ME, McConnell SK (2001) Temporally and spatially regulated expression of a candidate G-protein-coupled receptor during cerebral cortical development. J Neurobiol 46:167–177. Erratum J Neurobiol 47:244.

Donoghue MJ, Rakic P (1999) Molecular evidence for the early specification of presumptive functional domains in the embryonic primate cerebral cortex. J Neurosci 19:5967–79

Engelkamp D (2002) Cloning of three mouse Unc5 genes and their expression patterns at mid-gestation. Mech Dev 118:191–197.

Frantz GD, Weimann JM, Levin ME, McConnell SK (1994) Otx1 and Otx2 define layers and regions in developing cerebral cortex and cerebellum. J Neurosci 14:5725–5740.

Ghosh A, Shatz, CJ (1992) Pathfinding and target selection by developing geniculocortical axons. J Neurosci 12:39–55.

Gilbert CD (1983) Microcircuitry of the visual cortex. Annu Rev Neurosci 6:217–247.

Hevner RF, Shi L, Justice N, Hsueh Y, Sheng M, Smiga S, Bulfone A, Goffinet AM, Campagnoni AT, Rubenstein JL (2001) Tbr1 regulates differentiation of the preplate and layer 6. Neuron 29:353–366.

Hofmann K, Tschopp J (1995) The death domain motif found in Fas (Apo-1) and TNF receptor is present in proteins involved in apoptosis and axonal guidance. FEBS Lett 371:321–323.

Jones EG (1981) Anatomy of cerebral cortex: Columnar input–output organization. In: The organization of the cerebral cortex (Schmitt FO, Worden FG, Adelman G, Dennis SG, eds), pp. 199–235. Cambridge, MA: The MIT press.

Kageyama, GH and Robertson, RT (1993) Development of geniculocortical projections to visual cortex in rat. J Comp Neurol 335:123–148.

Kishi N, Tang Z, Maeda Y, Hirai A, Mo R, Ito M, Suzuki S, Nakao K, Kinoshita T, Kadesch T, Hui C, Artavanis-Tsakonas S, Okano H, Matsuno K (2001) Murine homologs of deltex define a novel gene family involved in vertebrate Notch signaling and neurogenesis. Int J Dev Neurosci 19:21–35.

Leifer D, Krainc D, Yu YT, McDermott J, Breitbart RE, Heng J, Neve RL, Kosofsky B, Nadal-Ginard B, Lipton SA (1993) MEF2C, a MADS/MEF2-family transcription factor expressed in a laminar distribution in cerebral cortex. Proc Natl Acad Sci U S A 90:1546–1550.

Leonardo ED, Hinck L, Masu M, Keino-Masu K, Ackerman SL, Tessier-Lavigne M (1997) Vertebrate homologues of C. elegans UNC-5 are candidate netrin receptors. Nature 386:833–838.

Liu Q, Dwyer ND, O'Leary DD (2000) Differential expression of COUP-TFI, CHL1, and two novel genes in developing neocortex identified by differential display PCR. J Neurosci 20:7682–7690.

Llambi F, Causeret F, Bloch-Gallego E, Mehlen P (2001) Netrin-1 acts as a survival factor via its receptors UNC5H and DCC. EMBO J 20:2715–2722.

Lund RD, Mustari MJ (1977) Development of geniculocortical pathway in rats. J Comp Neurol 173:289–306.

Mackarehtschian K, Lau CK, Caras I, McConnell SK (1999) Regional differences in the developing cerebral cortex revealed by ephrin-A5 expression. Cereb Cortex 9:601–610.

Maeda N, Noda M (1996) 6B4 proteoglycan/phosphacan is a repulsive substratum but promotes morphological differentiation of cortical neurons. Development 122:647–658.

Mann F, Peuckert C, Dehner F, Zhou R, Bolz J (2002) Ephrins regulate the formation of terminal axonal arbors during the development of thalamocortical projections. Development 129:3945–3955.

Mao Z, Bonni A, Xia F, Nadal-Vicens M, Greenberg ME (1999) Neuronal activity-dependent cell survival mediated by transcription factor MEF2. Science 286:785–790.

Matsuno K, Diederich RJ, Go MJ, Blaumueller CM, Artavanis-Tsakonas S (1995) Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. Development 121:2633–2644.

McConnell SK (1989) The determination of neuronal fate in the cerebral cortex. Trends Neurosci 12:342–349.

Molnár Z, Blakemore C (1991) Lack of regional specificity for connections formed between thalamus and cortex in coculture. Nature 351:475–477.

Molnár Z, Blakemore C (1999) Development of signals influencing the growth and termination of thalamocortical axons in organotypic culture. Exp Neurol 156:363–393.

Molnár Z, Adams R, Blakemore C (1998) Mechanisms underlying the early establishment of thalamocortical connections in the rat. J Neurosci 18:5723–5745.

Nagase T, Ishikawa K, Nakajima D, Ohira M, Seki N, Miyajima N, Tanaka A, Kotani H, Nomura N, Ohara O (1997) Prediction of the coding sequences of unidentified human genes. VII. The complete sequences of 100 new cDNA clones from brain which can code for large proteins in vitro. DNA Res 4:141–150.

Nagase T, Ishikawa K, Suyama M, Kikuno R, Hirosawa M, Miyajima N, Tanaka A, Kotani H, Nomura N, Ohara O (1999) Prediction of the coding sequences of unidentified human genes. XIII. The complete sequence of 100 new cDNA clones from brain which code for large proteins in vitro. DNA Res 6:63–70.

Noctor SC, Palmer SL, McLaughlin DF, Juliano SL (2001) Disruption of layers 3 and 4 during development results in altered thalamocortical projections in ferret somatosensory cortex. J Neurosci 21:3184–3195.

Park HT, Kim YJ, Yoon S, Kim JB, Kim JJ (1997) Distributional characteristics of the mRNA for retinoid Z receptor beta (RZR beta), a putative nuclear melatonin receptor, in the rat brain and spinal cord. Brain Res 747:332–337.

Skaliora I, Singer W, Betz H, Püschel AW (1998) Differential patterns of semaphorin expression in the developing rat brain. Eur J Neurosci 10:1215–1229.

Suzuki SC, Inoue T, Kimura Y, Tanaka T, Takeichi M (1997) Neuronal circuits are subdivided by differential expression of type-II classic cadherins in postnatal mouse brains. Mol Cell Neurosci 9:433–447.

Vanderhaeghen P, Lu Q, Prakash N, Frisén J, Walsh CA, Frostig RD, Flanagan JG (2000) A mapping label required for normal scale of body representation in the cortex. Nat Neurosci 3:358–365.

Xu X, Ng S, Wu ZL, Nguyen D, Homburger S, Seidel-Dugan C, Ebens A, Luo Y (1998) Human semaphorin K1 is glycosylphosphatidylinositol-linked and defines a new subfamily of viral-related semaphorins. J Biol Chem 273:22428–22434.

Yabuta NH, Butler AK, Callaway EM (2000) Laminar specificity of local circuits in barrel cortex of ephrin-a5 knockout mice. J Neurosci 20(RC88):1–4.

Yamamoto N (2002) Cellular and molecular basis for the formation of lamina-specific thalamocortical projections. Neurosci Res 42:167–173.

Yamamoto N, Kurotani T, Toyama K (1989) Neural connections between the lateral geniculate nucleus and visual cortex in vitro. Science 245:192–194.

Yamamoto N, Yamada K, Kurotani T, Toyama K (1992) Laminar specificity of extrinsic cortical connections studied in coculture preparations. Neuron 9:217–228.

Yamamoto N, Higashi S, Toyama K (1997) Stop and branch behaviors of geniculocortical axons: A time-lapse study in organotypic cocultures. J Neurosci 17:3653–3663.

Yamamoto N, Inui K, Matsuyama Y, Harada A, Hanamura K, Murakami F, Ruthazer ES, Rutishauser U and Seki T (2000a) Inhibitory mechanism by polysialic acid for lamina-specific branch formation of thalamocortical axons. J Neurosci 20:9145–9151.

Yamamoto N, Matsuyama Y, Harada A, Inui K, Murakami F, Hanamura K (2000b) Characterization of factors regulating lamina-specific growth of thalamocortical axon. J Neurobiol 42:56–68.

Yamamoto N, Yamamoto S, Inagaki F, Kawaichi M, Fukamizu A, Kishi N, Matsuno K, Nakamura K, Weinmaster G, Okano H, Nakafuku M (2001) Role of Deltex-1 as a transcriptional regulator downstream of the Notch receptor. J Biol Chem. 276:45031–45040.

Zhou C, Qiu Y, Pereira FA, Crair MC, Tsai SY, Tsai MJ (1999) The nuclear orphan receptor COUP-TFI is required for differentiation of subplate neurons and guidance of thalamocortical axons. Neuron 24:847–859.