Conservation of developmental mechanisms

in evolutionary divergent brain structures

IKUO SUZUKI

DOCTOR OF PHILOSOPHY

Department of Genetics

School of Life Science

The Graduate University for Advanced Studies

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Summary

A remarkable feature of the mammalian evolution is the expansion of the neocortex and emergence of the specific internal cytoarchitecture, the layer structure. All of the mammalian species share the neocortical layer structure, in which a similar type of neurons are arranged in a particular layer parallel to the brain surface. During the neocortical development, the neuronal subtypes in the neocortical layers are produced from neural progenitor cells in a stereotyped temporal sequence from deep to upper layers. This stereotyped sequence of neuronal production is attributed to the temporal restriction of the competence of neural progenitors. The progenitors initially possess the multipotency to generate the neuronal subtypes in all neocortical layers, but gradually loose the potency during the development, and eventually become only able to produce the upper layer neurons.

The dorsal region of the telencephalon called the pallium is the non-mamalian homologue of the neocortex, because the same developmentally important genes are commonly expressed in the mammalian neocortex and the non-mammalian pallium. Although the pallium is completely conserved among the vertebrates, the internal structure is variable. For example, the bird, one of the closest relatives of the mammals, possesses the well-developed pallium packed with distinct subtypes of neurons that are arranged in particular domains. Such observations suggest that the common ancestor of the mammals and the birds had already acquired the pallium, and that the pallial structure has been modified in an animal group-specific manner through alterations in the developmental processes. Therefore, a key event contributing to the evolutionary emergence of

the neocortical layer structure could be found through comparison of the development between the mammalian neocortex and the non-mammalian pallium.

The chick pallium is a good model to approach the problem, because of the closest phylogenetic position to the mammals as well as the convenience of experimental manipulations. For over a century, it has been argued whether the avian pallium has a comparable neuronal repertory to the mammalian neocortex. Therefore, I first checked expression patterns of marker genes for the mammalian neocortical layers in the chick pallium. This analysis revealed that both the deep (layer V) and upper layer (layer II/III) marker genes were expressed in the chick pallium, suggesting that the chick pallium possesses a neuronal repertory similar to the mammalian neocortex. In addition to the molecular expressions, the axon projections were also found to be partially similar between the chick pallial neurons and the corresponding neuronal subtypes of the mammalian neocortex. In spite of the remarkable conservation in the neuronal repertory, spatial distribution patterns of the deep and upper layer neurons were entirely different from the layer arrangement of the mammalian neocortex; in the chick pallium, the deep and upper layer neurons were not arranged in parallel, but distantly located in the medial and lateral side, respectively.

The development of the deep and upper layer neurons in the chick pallium was investigated in detail. First, the birthdate analysis by BrdU pulse-labeling demonstrated that the deep layer neurons were generated earlier than the upper layer neurons in the chick pallium, suggesting that the temporal sequence of the neuron production is evolutionarily conserved between the mammals and the birds. Second, the fate mapping analysis re-

vealed that the deep and upper layer neurons originated from the distinct neural progenitors on the medial and lateral sides in the chick pallium, respectively. This spatially separate production of the neurons is the critical difference from the mammalian neocortical development, in which the deep and upper layer neurons are uniformly produced across the entire neocortex. Probably related to this difference, I found that the late neurogenesis in the chick pallium predominantly occurs on the lateral side. This spatiotemporally biased neuronal production can explain the selective generation of the late-born upper layer neurons only from the lateral side in the chick pallium. Taken together, the distinct neurogenetic properties between the medial and lateral progenitors appeared to be the key to construct the non-layered domain-like cytoarchiteture in the chick pallium.

How then is the medio-lateral difference of neurogenetic properties is instructed in the chick pallium? I cultured neural progenitor cells from the medial and lateral sides of the chick pallium in a clonal density, and surprisingly found that most of the clones derived from a single progenitor cell contained both deep and upper layer neurons, regardless of its origin. This remarkable observation clearly demonstrated that the neural progenitor cells in the medial and lateral sides of the chick pallium intrinsically possess a similar neurogenic competence, and the neurocompetency is extrinsically regulated by the surrounding tissues according to the spatial positions.

On the basis on the results obtained, I propose the following model for the avian pallial development. The avian neural progenitors are intrinsically equivalent to those of mammals and capable of sequentially generating a full repertory of neuronal subtypes. However, the neurogenesis in the avian pallium is extrinsically regulated by two potential

mechanisms. First, the deep layer fate in the early-born neurons on the lateral side is suppressed by environmental factors, and thereby, the lateral neural progenitor cells produce only the upper layer neurons in the later phase of neurogenesis. Second, the medial neural progenitors terminate the neurogenesis precociously before producing the upper layer neurons leading to the preferential generation of deep layer neurons from the medial side.

Lastly, the emergence of the layered neocortex in mammals has been a long-standing mystery in evolutionary biology. The present discovery of the evolutionary conservation in the neural progenitor competence between the mammals and the avian suggests that the common ancestor of the amniotes has already possessed the developmental potential to sequentially produce the multiple neuronal subtypes. During the evolutionary diversification into each lineage of animal groups, alterations in the spatial regulation of the neurogenetic program may have contributed to the emergence of animal group-specific brain structures, such as the layer structure in the mammalian neocortex and the domain structure in the avian pallium.

Abbreviations

VZ   ventricular zone

SVZ   subventricular zone

DL   deep layer

UL   upper layer

MP   medial pallium

DP   dorsal pallium

LP   lateral pallium

VP   ventral pallium

NPC   neural progenitor cell

PH   parahippocampal region

M   mesopallium

H   hyperpallium

N   nidopallium

BrdU   bromodeoxyuridine

EGFP   enhanced green fluorescent protein

pH3   phospho histone H3

Introduction

A remarkable feature of the mammalian evolution is massive enlargement of the neocortex, which accomplishes intellectual properties to humans. The mammals have not only increased the neocortical volume, but also developed a specific internal architecture of the neocortex, the layer structure. In the individual layers, a similar type of neurons are distributed in parallel to the brain surface and serve the stereotypic functions (figure.1) (Paxinos and Franklin 2001; Butler and Hodos 2005). For example, the neurons in the layer IV share the function to receive the input from the thalamus and mainly transmit information to layer II/III neurons. The neurons in the layer II/III connect with cortical neurons in the same and other areas and take part in the higher order information processing, and the cortically processed information is finally output by layer V and VI neurons through their projections to the subcortical targets. This neocortical architecture involving the dense vertical interconnections among the layers constitutes the basis of the functional columnar organization in the mammalian neocortex. The layer structure of the mammalian neocortex is completely conserved among all of the mammals, suggesting that the layer structure has been maintained by selective constraints during the mammalian evolution (Butler and Hodos 2005; Striedter 2005).

The evolutionary origin of the mammalian neocortical layer structure is totally unknown, because the brain structure is not recorded in the fossils. Accordingly, the comparison between the extant mammals and non-mammals is required to approach this problem. The closest relatives of the mammals are the birds and the reptiles; they have diverged from the last common ancestor of the amniotes 310 million years ago (figure.2)

(Kumar and Hedges 1998; Kumazawa and Nishida 1999; Jarvis, Güntürkün et al. 2005). The counterparts of the mammalian neocortex in the birds and reptiles are believed to be the pallium (figure.3). Although this brain field completely lacks the layer structure, it seems functionally equivalent to the mammalian neocortex, commonly processing the same types of information such as visual and somatosensory representations and generation of motor outputs (Karten 1991; Medina and Reiner 2000; Jarvis, Güntürkün et al. 2005; Kaas 2007). Furthermore, resent molecular expression studies have confirmed that the mammalian neocortex and the non-mammalian pallium indeed originate from very similar populations of neural progenitor cells (NPC), which are virtually indistinguishable in the gene expression profiles (Fernandez, Pieau et al. 1998; Puelles, Kuwana et al. 2000; Medina, Brox et al. 2005). These observations suggest that the ancient pallium has been acquired by the last common ancestor of amniotes, and the neocortical layer structure has been emerged at the common ancestor of the mammals sometime before the diversification of mammalian species, because the layer structure is entirely conserved among all of the mammalian species and does not exist in the non-mammalian pallium (figure.2).

During the mammalian neocortical development, the layer structure is constructed in an "inside-out" fashion, in which the deeper neurons are produced earlier than the upper neurons (figure.4) (Rakic 1974; Jackson, Peduzzi et al. 1989). In the beginning of the neocortical development, NPCs actively divide symmetrically and tangentially expand the neocortical area. Then, NPCs begin to divide asymmetrically to produce two different daughter cells, one postmitotic neuron and one progenitor that continues dividing and

producing neurons later on. In this way, a single neocortical NPC undergoes multiple rounds of asymmetric division and generates multiple types of neurons (Noctor, Flint et al. 2001; Götz and Huttner 2005; Kriegstein, Noctor et al. 2006; Shen, Wang et al. 2006). The birth timing is a critical determinant of neuronal subtypes, leading to the stereotyped sequential production of different subtypes. The underlying mechanism seems to involve temporal restriction of the competence of NPCs. More specifically, early progenitors have the potential to produce both deep layer (UL) and upper layer (UL) neurons, whereas, later progenitors loose the potential to produce DL neurons, and only generate UL neurons (figure.4) (McConnell and Kaznowski 1991; Frantz and McConnell 1996; Desai and McConnell 2000).

How has the neocortical layer structure emerged at the stem mammalian ancestor? In principle, morphological evolution results from modifications in the pre-existing developmental programs (Carroll, Grenier et al. 2001; Paxinos and Franklin 2001; Shigetani, Sugahara et al. 2002; Carroll 2008). Therefore, I may find a potential answer in the critical difference of developmental processes between the mammalian layered neocortex and its non-layered homologues in non-mammalian vertebrates. Among many aspects of developmental processes, the neurogenesis deserves a special attention. Because NPCs produce a huge number and variation of neurons, only a slight alternation in the neurogenetic program can lead to a larger impact on the adult brain structure (Chenn and Walsh 2002; Hanashima, Li et al. 2004; Shen, Wang et al. 2006; Sahara and O'Leary 2009).

To explore developmental changes underlying the structural differences between the mammalian neocortex and the non-mammalian pallium, I took comparative approaches. I examined the development of the chick pallium, the homologue of the mammalian neocortex, as a non-mammalian model. Expression patterns of neocortical layer specific marker genes demonstrated that the chick pallium possess a set of neuronal subtypes comparable to those of the mammalian neocortical layers. However, the internal arrangement of the neuronal subtypes within the chick pallium was absolutely different from the neocortical layer arrangement; the individual subtypes were assembled into the domains along the media-lateral axis. In the chick pallium, different neuronal subtypes originated from medio-laterally distinct NPCs, and the spatially biased production of neurons seemed to cause their domain-like arrangement. Surprisingly, in a culture condition, NPCs in the chick pallium had the intrinsic competence to produce a full set of neuronal subtypes equivalent to that of mammalian neocortex, suggesting the extrinsic restriction of the competence of NPCs in the chick pallium. From these results, I hypothesized that the mammalian neocortical layer structure has evolved through alterations in the extrinsic control of the common neurogenetic mechanisms shared by birds and mammals.

Results

Expression of UL and DL markers in the chick pallium

I first asked whether the neuronal subtypes observed in the mammalian neocortex existed in the avian pallium. In the mammalian neocortex, many genes have been found to be expressed in a layer-specific manner (Lein, Hawrylycz et al. 2007; Molyneaux, Arlotta et al. 2007). Among these layer marker genes, I focused on transcriptional regulators, because they can regulate expression of the downstream target genes, and thereby control the neuronal identities. Indeed, some of the layer-specific transcriptional regulators are shown to be critical for determination of neuronal layer fates in mammals; neuronal identities switch to others, even changing their axon projection patterns, when such a regulator is manipulated (Molnár and Cheung 2006; Molyneaux, Arlotta et al. 2007; Leone, Srinivasan et al. 2008). Thus, I chose 19 mammalian layer-specific marker genes that encode transcription regulators, and then, isolated their chicken orthologues to examine their expressions in the chicken pallium (Table.1).

Out of the nine layer II/III (upper layer : UL) marker genes that I examined, six genes, Satb2, FOXP1, Lmo3, Cux2, Brn2 and Mef2c, showed expressions in the lateral region (Figure.5, Figure.6) of the E16 chick pallium. The chick pallium is further subdivided into several regions such as the hyperpallium, mesopallium and parahippocampal region (Jarvis, Güntürkün et al. 2005; Puelles 2007). Strong expressions of the UL marker genes were detected mainly in the mesopallium and the adjacent hyperpallial regions. Shared expressions of all six genes were found in the caudal mesopallium (Figure.5, Figure.6), in which they exhibit the strongest expression in the most ventral edge, presumably meso-

pallial intermediate field (MIF) with a dorsally weakened expression. Apart from the common expression in the caudal mesopallium, three genes, FOXP1, Lmo3 and Mef2c, had further expanded expressions; FOXP1 was expressed in the marginal zone of the hyperpallium (Figure.6 b), and Lmo3 and Mef2c had nidopallial expressions (Figure.6 c, f). At the rostral level, the UL marker genes were also expressed in the lateral mesopallial domain in a more complex nested pattern; Satb2, FOXP1 and Lmo3 showed a similar pattern with the overlapping expression in the central region of the mesopallium and the ventro-lateral part of the hyperpallium (Figure.5 a-c). Brn2 was expressed in a more restricted region of the central mesopallium (Figure.5 e). Cux2 expression weakly delineated ventral margin of the mesopallium (Figure.5 d). Mef2c was found in the middle region of the hyperpallium, presumably the intermediate hyperpallium, in addition to the merginal zone of the mesopallium (Figure.5 f). The ventral extension of the mesopallial Mef2c expression was overlaped with Satb2, FOXP1, and Lmo3 (Figure.5 f).

Only one of the two mammalian layer VI specific marker genes, Ror-beta, was successfully detected in the central region of the mesopallium, where Brn2 was strongly expressed (Figure.7). The caudal expression of this marker in the mesopallium was also similar to that of upper layer markers such as Brn2 and Satb2.

I examined four DL marker genes that are exclusively expressed in the layer V (deep layer : DL) of the mammalian neocortex, and were involved in the specification of neuronal identities. Three DL marker genes, Er81, Fezf2 and CTIP2, were strongly expressed in the medial part of the pallium, the parahippocampal region (PH) in the E16 chick pallium (Figure.8, Figure.9), whereas Otx1 was not detected in this stage. The common me-

dial expression shared by the three DL marker genes continued throughout the entire rostro-caudal axis of the pallium, although they showed slightly different patterns. On the rostral level, all of the three genes were strongly expressed in the dorsal half of the PH, in which the vast majority of neurons appeared to co-express the three genes (Figure.8). Fezf2 and CTIP2 had additional expression in the hyperpallial regions, probably the intermediate hyperpallium, in which the neurons expressing either of the markers were sparsely scattered (Figure.8 b, c). In the caudal level, Er81 and Fezf2 were expressed in a mostly overlapping manner in the PH (Figure.9 a, b). The CTIP2 expression was partially segregated dorsally from that of the other two genes, but still confined to the PH (Figure.9 c).

All of the four layer VI marker genes, Tbr1, Sox5, Tle4 and Foxp2, failed to show expression signals in the E16 chick pallium, although Tbr1 and Sox5 were detected in an earlier developmental stage.

In summary, the expressions of the marker genes for the layer II/III (UL) and layer IV were detected in the lateral part of the DP, the mesopallium and adjacent hyperpallial regions. On the other hand, those for the layer V (DL) of the mammalian neocortex were detected in the medial part of the chick pallium, the PH and the dorso-medial side of the hyperpallium.

Partial conservation of the axon projection patterns between the mammalian neocortex and the chick pallium

I wondered whether the chick pallial neurons expressing the mammalian neocortical layer marker genes had axon projections similar to the corresponding neuronal subtypes in the mammalian neocortex. To visualize the axon projections of the neurons in the chick pallium, a small crystal of lipophilic dye, DiI, was introduced into the E16 chick pallium.

Mammalian UL neurons project intracortically, with some of the axons even reaching the contralateral side through the corpus callosum (Molyneaux, Arlotta et al. 2007; Leone, Srinivasan et al. 2008). When a DiI crystal was introduced into the chick mesopallium where the UL marker genes were expressed, no long axon tracts were visibly labeled, and only short DiI-labeled fibers locally projected within the mesopallium. Because chicken brains lack the corpus callosum, it is not surprising that the chick neurons expressing the UL marker genes do not have the commissural projection homologous to that in mammals (Bock, Cardew et al. 2000; Butler and Hodos 2005; Striedter 2005; Kaas 2007; Puelles 2007). Rather, the pallium-restricted projections might represent the conserved nature of the neurons as a part of the intracortical or intrapallial association systems.

Mammalian DL neurons are heterogenous and project to several distinct targets (Hallman, Schofield et al. 1988; Molnár and Cheung 2006; Molyneaux, Arlotta et al. 2007). Among them, the most characteristic projection is the subcortical pathway descending to the brainstem. When a DiI crystal was introduced into the chick rostral PH where DL

marker genes were expressed, three distinguishable axon bundles were labeled (Figure.10). The most prominent projection was the internal capsule (ic) extending to the thalamus (Figure.10) (Bock, Cardew et al. 2000; Butler and Hodos 2005; Striedter 2005; Kaas 2007; Puelles 2007). Another projection passed through the septum toward the dorsal thalamus and tectum, which is designated as the cortico-septo-mesencephalic (csm) tract (Figure.10 b-d) (Medina and Reiner 2000; Puelles 2007). The third pathway terminated with massive axon branchings in the pallial regions, the mesopallium and nidopallium. The fact that the chick pallial neurons expressing the DL markers had the prominent descending projections to the brainstem supports the functional similarities of these neurons to mammalian DL neurons.

In the mammalian neocortex, afferent projections from the thalamus terminate in the layer IV (Miller 2003). When I labeled chick thalamic axons with DiI or GFP, the axons were found to terminate at several regions in the E16 chick DP, including the mesopallium (Figure.11). However, the terminating regions were distinct from the domain expressing Ror-beta, the neocortical layer IV marker gene. Therefore, the axon tracing experiment suggested that this domain is not a functional counterpart of the layer IV in the mammalian neocortex.

Confinement DL and UL marker-expressing domains in the chick dorsal pallium

The pallial ventricular zone (VZ) containing NPCs is specified into four compartments, the medial pallium (MP), dorsal pallium (DP), lateral pallium (LP) and ventral pallium

(VP). Among them, only the DP produces neocortical neurons in the mammals. The distinct ventricular compartments can be distinguished by the combinatorial expressions of specific marker genes such as Lhx5, Sfrp1, Tlx, Emx1 and PAX6 (Table.2) (Hébert and Fishell 2008). Using these markers, I investigated whether the chick DL and UL neurons originated from the DP progenitors.

The marker genes for the mammalian ventricular compartments were expressed in the VZ of the E7 chick pallium (Table.2). By using those expressions as the landmarks, I defined the four pallial compartments in the chick and checked whether the expression domains of the layer specific marker genes were included in the DP compartmnet (Figure.12). All of the three DL marker genes, Er81, Fezf2 and CTIP2, were expressed in postmitotic neurons that were juxtaposed to the ventricular zone of the DP compartment defined by the marker expressions (Figure.12 a-h). More specifically, the chick DL neurons were located at the most medial region of the DP, which was immediately adjacent to the MP characterized by Lhx5 expression (Figure.12 a). Interestingly, the initial expression of the DL markers at E6 was widespread over the whole DP, but subsequently shrunk to the restricted medial region of the DP by E7 (Figure.13). Because the DP region actually enlarges during the time period by producing an increasing number of neurons, the restriction of the expression domains suggests suppression of the initial gene expression at the later stage.

In the E7 chick pallium, three UL marker genes, Cux2, Satb2 and Mef2c, were co-expressed in the lateral side of the DP (Figure.12 i-k). The ventral edge of the expression domain abutted on the boundary with the LP marked by Sfrp1 expression (Figure.12 b).

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The postmitotic expression of the three UL markers started at a relatively late timing around E7. Later on, the expression domain remained fixed in the same lateral DP but drastically expanded, suggesting massive production of UL neurons in this period (Figure.14). Prior to the postmitotic expression, Cux2 was also expressed in the subventricular zone (SVZ) of the DP, exhibiting a latero-medial gradient at E6-E6.5, (Figure.14). Interestingly, a similar subventricular expression of Cux2 was reported in the mammalian neocortex, in which the gene is expressed by basal progenitors, a unique late population of NPCs that are destined to preferentially produce UL neurons (Nieto, Monuki et al. 2004; Zimmer, Tiveron et al. 2004).

In summary, the DL specific genes are initially expressed widely in the E6 chick DP and gradually confined to the medial region by E7. On the other hand, the expressions of UL specific genes have a relatively late onset and are confined to the lateral side of the DP from the beginning.

Conserved temporal order of neuronal differentiation in the chick pallium and the mammalian neocortex

In the mammalian corticogenesis, DL neurons are born earlier than UL neurons (Figure.4, Figure.17) (Rakic 1974; Jackson, Peduzzi et al. 1989; McConnell and Kaznowski 1991; Frantz and McConnell 1996; Desai and McConnell 2000). To determine the timing of final mitosis of chick DL and UL neurons, I injected a small dose of bromodeoxyuridine (BrdU) into the ventricle of chick brains at various developmental stages

and chased the pulse labeled neurons that had just undergone the final DNA synthesis upon the BrdU application (Nomura, Takahashi et al. 2008). Most of the neurons expressing the DL marker genes were labeled with BrdU when given the BrdU injection at E5 (Figure.15 a-d). When BrdU was injected at E6, only a few of them were BrdU-labeled (Figure.15 e-h). On the other hand, most of the UL neurons were labeled when BrdU was injected at the E7 (Figure.16 c, d), and a smaller proportion was labeled when injection was given at E6 or E8 (Figure.16 a, b, e, f). The results show that the peaks of DL and UL neuron generation are at E5 and E7, respectively, indicating that the temporal order of the differentiation from DL to UL neurons in the mammalian neocortex is conserved in the chick pallium (Figure.17).

Medio-laterally separated origins of DL and UL neurons in the chick pallium

In the mammalian neocortex, a single NPC produces both DL and UL neurons according to the developmental timing, regardless of its location (Figure.4, Figure.20) (Luskin, Pearlman et al. 1988; Price and Thurlow 1988; Austin and Cepko 1990; Shen, Wang et al. 2006). This spatially homogeneous production of a full variety of neurons is the foundation for constructing the uniformly layered mammalian neocortex. In the chicken pallium, on the other hand, DL and UL neurons are distantly located on the medial and lateral sides of the pallium. Therefore, I wondered whether the separation could be attributed to the spatially distinct origins of these two types of neurons and searched for their developmental origins.

I labeled NPCs in a small region of the VZ in the E5 chick pallium by introducing a cDNA expression construct for the enhanced green fluorescent protein (EGFP) through in ovo electroporation (Nomura, Takahashi et al. 2008). When NPCs on the medial side of the pallium were labeled at E5, the resulting EGFP-positive neurons derived from the labeled NPCs were mainly distributed in the medial and dorsal regions of the pallium (Figure.18); many of the EGFP-labeled neurons expressed the DL markers (Figure.18 a-c). A small fraction of EGFP-labeled neurons sparsely spread over the lateral side of the pallium, in which only a few of them expressed the UL markers (Figure.18 d). In contrast, when the NPCs on the lateral side of the pallium were labeled at the same stage, EGFP-positive neurons were exclusively distributed over the lateral side of the pallium, including the mesopallium, where the UL neurons were localized (Figure.19). I also confirmed abundant neurons coexpressing EGFP and the UL markers in the area. The results indicated that chick DL and UL neurons are originated from the distinct populations of NPCs on the medial and lateral sides of the pallium (Figure.20).

Spatiotemporally biased neurogenesis in the chick pallium

The spatiotemporal analyses of DL and UL neuron production in the chick revealed that the DL neurons are earlier generated from the medial NPCs, whereas the UL neurons are later generated from the lateral NPCs. This spatiotemporally biased production of these neurons contrasted with the uniform production in the mammalian cortex and raised an important question of how the spatial and temporal patterns of neuronal production were

coordinated in the chick pallium. Hoping to gain clues, I analyzed the spatiotemporal neurogenetic activities across the chick pallium.

First, mitotic activities were measured in the VZ. In the E5 pallium, phospho-histone H3 (pH3)-positive mitotic cells were fairly evenly distributed along the inner surface of the entire VZ in the pallium (Figure.21 b, e). At this stage, there were substantial mitoses occrring even in the medial quarter with the smallest mitotic activity. At E7, the medio-lateral bias of the mitotic activity was enhanced, suggesting that a much larger number of neurons were produced from the lateral side compared with the medial side. The medial-low lateral-high gradient of NPC mitosis was consistent with the medio-lateral difference in the thickness of the chick pallium (Figure.21 d, f).

Recent studies have identified a unique class of NPC called the basal progenitors in the mammalian neocortex; they reside basally in the SVZ, detached from the apical ventricular surface, and eventually produce two neurons through symmetric cell divisions (Tarabykin, Stoykova et al. 2001; Haubensak, Attardo et al. 2004; Noctor, Martı ́nez-Cerden ̃ o et al. 2004; Zimmer, Tiveron et al. 2004; Wu, Goebbels et al. 2005; Martı ́nez-Cerden ̃ o, Noctor et al. 2006). Because the basal progenitors emerge at later stages of the mammalian corticogenesis and also because some UL markers, such as Cux2, are coincidently expressed in these cells as well, the basal progenitors are supposed to be the major source of UL neurons in the mammalian neocortex (Tarabykin, Stoykova et al. 2001; Zimmer, Tiveron et al. 2004; Wu, Goebbels et al. 2005). The expression of Cux2 in the chick SVZ suggested the existence of basal progenitors in the chick (Figure.14). Furthermore, I found more conclusive evidence that the basal progenitors indeed exist in the

chick DP. In the chick pallium at E7, pH3-positive mitotic cells were observed not only along the apical ventricular surface but also basally displaced from the VZ (Figure.22 a). These basally positioned pH3-positive cells were mostly detected in the lateral and ventral quarters of the pallium. A SVZ marker, TBR2, further confirmed that the chick pallium had a ventrolaterally thicker SVZ (Figure.22 b). When DNA replication was marked by BrdU incorporation for thirty minutes before sacrifice, a significant number of cells were double positive for BrdU and TBR2 in the lateral and ventral quarters of the chick pallium but not in the medial quarter (Figure.22 b'-b''''). Importantly, such Tbr2+/BrdU+ double positive cells were undetectable at an earlier developmental stage, indicating that the basally displaced NPCs detected in the lateral and ventral quarters of the chick pallium indeed correspond to the basal progenitors characterized in the mammalian neocortex.

I next analyzed neuronal production across the chick pallium. BrdU was injected at 24 hours before sacrifice, and the BrdU labeled cells were then assayed for expression of a pan-neuronal marker, β(III)tubulin and an NPC marker, PAX6. In this way, I determined whether the newly produced cells that had completed the DNA synthesis within the previous 24 hours, differentiated into neuron or retained the progenitor characteristics. At E5, in any region of the pallium, most of the newly produced cells maintained the NPC character, indicating that self-renewing symmetric divisions of NPCs were still highly prevalent across the pallium (Figure.23 a, c). Substantial number of neurons were only generated from the lateral and ventral quarter, most of which corresponded to the lateral pallium that eventually produce nidopallial neurons expressing neither DL or UL mark-

ers. In the E7 pallium, we observed many newly differentiated neurons expressing the neuronal marker in the zone loosely surrounding the VZ (Figure.23 b). The cell quantification showed a more prominent neurogenesis occurring in the lateral and ventral quarters, in which neurogenetic cell divisions outnumbered self-renewing NPC divisions (Figure.23 d). Compared with the massive neurogenesis in the ventral and lateral quarters, the proportion of the neurogenetic division was much smaller in the medial quarter.

The medio-laterally biased neurogenesis was also conformed by expression pattern of a neuronal differentiation marker, Ngn2, which is transiently upregulated in newly differentiated neurons immediately after the final mitosis (Miyata, Kawaguchi et al. 2004; Shimojo, Ohtsuka et al. 2008). Compared to the E5, Ngn2 expression exhibited a more significant medial-low lateral-high gradient at E7 (Figure.24). This means that the medio-lateral difference in neuron production activity is enhanced from E5 to E7.

Isolated chick NPCs can produce both DL and UL neurons in vitro

Chick NPCs on the medial and lateral sides of the pallium had different properties in the neurogenetic potential and the neuronal types that they give rise to. This seems the critical difference from the mammalian neocortical NPCs that have nearly identical properties regardless of their spatial location. The spatially different properties of chick NPCs can be explained by two factors; one is the cell intrinsic difference in NPCs themselves, and the other is the extrinsic control over NPCs by surrounding environment. To distinguish the two factors, I cultured chick NPCs in a clonal density. If the medio-lateral dif-

ference is encoded intrinsically in the NPCs, the medial and lateral NPCs should produce different neuronal subtypes according to the spatial origins even in the isolated situation. On the other hand, if environmental factors extrinsically control the NPC activities and create the medio-lateral difference, the medial and lateral NPCs might produce similar neuronal subtypes in the culture condition lacking the surrounding spatial information.

The NPCs isolated from the medial and lateral halves of the E5 chick pallium were cultured for 5 days in vitro at a low cell density (Figure.25 a). Under the culture condition, a single NPC proliferated and constructed an isolated colony. The colonies were then immunostained for β(III)tubulin as a pan-neuronal marker (Figure.25 b, e), FOXP1 as a UL-high and DL-low marker, (Figure.25 b, c) and CTIP2 as a DL-specific marker (Figure.25 b, d). When medial NPCs were clonally cultured, only one third of the colonies contained exclusively DL neurons, whereas two thirds of them contained both UL and DL neurons (Figure.25 f). Even more interestingly, when lateral NPCs were clonally cultured, more than 80% were the mixed colonies containing both DL and UL neurons, although the lateral NPCs never produced neurons expressing a DL marker in vivo (Figure.25 g). This result clearly showed that the extrinsic control is the main factor to create the spatial difference, and that the medial and lateral NPCs have intrinsically similar competence to produce both DL and UL neurons at least in an isolated condition.

Discussion

I found that the avian pallium contains a set of neuron subtypes similar to that of the mammalian neocortex. This evolutionary conservation of neuronal repertory between the mammalian neocortex and the avian pallium is presumably based on the common cellular mechanisms for generating multiple types of neurons from individual NPCs. At the same time, I also found that distributions of the homologous neuronal types were completely different between the birds and mammals. This difference could be attributed to an animal group-specific modification in the spatial regulation of NPC dynamics.

Conservation of intrinsic NPC properties between the mammalian neocortex and the avian pallium

A surprising finding of this study is conservation of NPC competence between the mammalian neocortex and the avian pallium. In clonal culture, chick NPCs isolated from either of the medial or lateral DP generated both DL and UL neurons. This implies that the common ancestor of the mammals and the birds has already acquired the potential to produce multiple types of neocortical neurons, including DL and UL neurons. Indeed, it is reasonable to think that all extant amniotes, the mammals the reptiles and birds, possess a similar set of neocortical neurons that are required for the basic brain function common to these animals. Furthermore, they seem to share the developmental program to sequentially generate the full set of neurons from a single NPC. From an evolutionary viewpoint, such an elegant way of generating neuronal variations from the limited pro-

genitor pool would be beneficial and kept by selective constraints, thereby only allowing slight modifications, such as the spatial regulation for the mechanism. Perhaps, each animal group has evolved the specific brain structure by introducing slight modifications in the conserved pre-existing neurogenetic program, and acquired adaptive benefits.

Extrinsic modifications of the NPC program create the differences between the mammalian neocortex and the chick pallium

What types of modification can create the differences between the mammalian neocortex and the chick pallium? Because chick NPCs had an intrinsic competence to produce multiple neuron subtypes in vitro, similar to the mammalian neocortical NPCs, a potential scenario will be that some extrinsic factors in the chick brain make the differences. The most striking characteristic of the chick pallium is the spatial segregation of DL and UL neurons, which can be attributed to their separated origins, the medial and lateral NPCs, respectively. In addition, the distinct origins had different temporal programs in the neurogenesis. Taken together with these results, I will discuss two scenarios that are required to explain the construction of the chick pallium.

First, in the lateral side of the pallium, DL fates have to be suppressed in early-born neurons. This suppression is only operational in vivo, since the lateral NPCs actually produced DL neurons in clonal culture, suggesting that extrinsic factors in the chick brain are responsible for the suppression. In this regard, one interesting observation was DL markers were initially expressed widely in early stages and subsequently restricted to

the small area on the medial domain of the pallium. This process might represent the actual suppression of DL markers in early-born neurons destined to the lateral side of the pallium. Although the molecular nature of the extrinsic factors is completely unknown so far, I assume the involvement of negative transcription factors in this process, because suppression of DL marker genes is one of the prevailing mechanisms to assign non-DL characters in the mammalian neocortex. For example, expression of DL marker Ctip2 is directly suppressed by UL gene Satb2 (Alcamo, Chirivella et al. 2008; Britanova, de Juan Romero et al. 2008;) and the deepest layer VI gene Sox5 (Alcamo, Chirivella et al. 2008; Britanova, de Juan Romero et al. 2008; Kwan, Lam et al. 2008; Lai, Jabaudon et al. 2008; Leone, Srinivasan et al. 2008). Therefore, a similar negative transcriptional regulation could account for the suppression of DL marker genes in the early-born neurons in the lateral side of the chick pallium.

Second, in the medial side of the chick pallium, the neurogenesis must be inhibited, especially at the late stage, so that UL neurons are not produced after DL neurons. Indeed, the chick NPCs in the medial side hardly proliferated at the later stage and the neurogentic activity was maintained at the extremely low level, resulting in very few of late-born neurons generated from the medial side of the pallium. At the same time, the lateral side of the chick pallium holding basal progenitors produced an expanding number of neurons at the later stage, which would eventually become the vastly outnumbered UL neurons in the lateral domain. This spatially biased neurogenesis is not observed in the mammalian neocortex, in which the NPCs in any regions of the mammalian neocortex produce neurons in all layers (Luskin, Pearlman et al. 1988; Price and Thurlow

1988; Austin and Cepko 1990; Shen, Wang et al. 2006), although the relative thickness of DL and UL are slightly different among the neocortical areas (Paxinos and Franklin 2001; Smart, Dehay et al. 2002). Thus, the early termination of the neurogenesis in the medial side of the chick pallium is a unique characteristic and might require a special mechanism that is missing in mammals.

The low proliferation and neurogenetic activities in the chick medial NPCs may resemble the quiescent state of stem cells in other systems. The cells were not apoptotic and still expressed many molecules, such as PAX6, Hes1, Vimentin, and PCNA, as the adjacent mitotically active NPCs. Of particular interest was that those NPCs expressed a slightly higher level of Hes1, compared to the adjacent NPCs (data not shown), because a recent study suggested that Hes1 is involved in the transition between the quiescent and mitotically active states in some cell lines (Sang, Coller et al. 2008). Although the function of Hes1 in the NPC quiescence is unknown, it is possible that Hes1 is involved in the quiescence of the chicken lateral NPCs.

Functional implications of layer marker expressions in the amniote pallium

In the mammalian neocortex, Satb2 is strongly expressed by callosally projecting neurons in the UL (layer II/III) (Britanova, Akopov et al. 2005; Szemes, Gyorgy et al. 2006). This transcription factor cell-autonomously determines the axon projection pattern of these neurons toward the contralateral cerebral hemisphere through the corpus callosum (Alcamo, Chirivella et al. 2008; Britanova, de Juan Romero et al. 2008). Consequently, in

the Satb2 knockout mice, these axons fail to project through the corpus callosum and ectopically project to the brainstem like the intact DL neurons (Alcamo, Chirivella et al. 2008; Britanova, de Juan Romero et al. 2008). The non-mammalian amniotes, such as the birds, do not have the commissural projections corresponding to the corpus callosum of the mammals (Butler and Hodos 2005; Striedter 2005). Accordingly, the Satb2-expressing neurons in the chick pallium only projected short axons to connect with the nearby neurons within the same hemisphere and lacked long projections to the contralateral side. Therefore, the function of Satb2, to control the callosum projection to the contralateral hemisphere, does not seem to be conserved in the chick pallium, but still could define the intracortical property of the projections shared by chicken and mammals.

The mammalian DL neurons are classified into several subclasses based on the projection targets (Molnár and Cheung 2006). Among the subclasses, Fezf2 and Ctip2 are expressed in the subcortical projecting neurons and commit neurons to project to the brainstem (Arlotta, Molyneaux et al. 2005; Chen, Schaevitz et al. 2005; Molyneaux, Arlotta et al. 2005; Chen, Wang et al. 2008), whereas Lmo4 is expressed in the callosal projection neurons (Arlotta, Molyneaux et al. 2005). Er81, on the other hand, is expressed in all classes of the pyramidal neurons in the DL of the mammalian neocortex (Molnár and Cheung 2006; Yoneshima, Yamasaki et al. 2006). In the chicken DP, all of the three successful DL markers, Fezf2, Ctip2 and Er81 showed expressions in the most medial region of the pallium (Nomura, Takahashi et al. 2008), and Lmo4 is already described for the specific expression in the same region (Abellán and Medina 2009). By labeling with DiI, I

found that this medial domain indeed had the prominent projection to the brainstem, similar to the mammalian subcortical projecting neurons (Figure.10). Therefore, the functional role for the DL markers that commit neurons to project to subcortical targets, may well be conserved among the amniotes. Apart from the common expression domain in the medial side of the pallium, the DL markers exhibited varied expression patterns across the chick pallium. The functional significance of the different patterns remains unclear.

The layer IV of the mammalian neocortex receives the afferent input from the thalamus. In the present study, the only specific marker gene for layer IV, Ror-beta, was detected in the chicken mesopallium. The domain defined by this marker was, however, not layer IV-specific but included in the UL domain. Futhermore, I found that the thalamic afferents do not terminate in the Ror-beta expressing domains but another area in the chick pallium (Figure.11) (Kaas 2007). Thus, the neurons expressing the marker gene do not seem to be the functional homologue of the layer IV neurons in the mammalian neocortex.

Although the expression of mammalian marker genes for layer II-V were detected in the chicken pallium, none of the marker genes for the mammalian deepest layer VI were detected in the chick pallium at the corresponding stage of differentiation. In the mamalian neocortex, Tbr1 is expressed in the layer VI and required for the neurons to project their axons to the thalamus (Bulfone, Smiga et al. 1995; Hevner, Shi et al. 2001). In the chicken, Tbr1 was only transiently expressed in young neurons in the entire pallium. Other layer VI marker genes, Sox5 and Otx1, were also expressed only in immature neu-

rons similarly to Tbr1. Thus, in the chicken pallium, the layer VI fate might be suppressed by unknown mechanisms.

Evolution of pallial structure

What sort of the pallial structure did the last common ancestor of the amniotes have? Because I only compared two animal groups in this study, it is yet impossible to present a conclusive answer to the question. However, a plausible hypothesis is that the last common ancestor of the amniotes had an avian-type brain structure, in which DL and UL neurons were located in the medial and lateral sides, but not in layers parallel to the brain surface. This is because only the mammals are destined to have multiple neuronal layers in the pallium literally (Medina and Reiner 2000). For example, the squamata, a groups of the reptile including lizards and snakes, has a single neuronal layer (Greenberg 1982), while the turtles (Powers and Reiner 1980) and the birds have segregated domains of neurons in the pallium (Bock, Cardew et al. 2000; Medina and Reiner 2000; Butler and Hodos 2005; Striedter 2005; Kaas 2007; Puelles 2007). Therefore, the neocortical layer structure, in which the DL and UL neurons are aligned in parallel to the brain surface, appears to have been innovated in the early mammalian evolution at the Jurassic period. During the same time period, the reptiles and the birds might have created their own specific brain structures by spatiotemporally modifying the NPC dynamics and optimizing distributions and numbers of the DL and UL neurons.

Materials and methods

Animals

Fertilized chicken eggs were purchased from a local farm, Ohata-shaver (Shizuoka, Japan). The day on which eggs were transferred to a 37°C incubator was designated as embryonic day 0 (E0).

cDNA constructs

The chick embryonic brains at E10 and E14 were used for cloning of chicken genes. The whole-brain was dissected from a chicken embryo, and mRNA was purified from it using QuickPrep mRNA purification kit (GE Healthcare). The mRNA was reverse transcribed into cDNA using AMV reverse transcriptase first strand cDNA synthesis kit (LSK1000, TAKARA BIO). The cDNA fragments coding Satb2, Lmo3, Cux2, Brn2, Mef2c, Cux1, Brn1, Lhx2, Ror-beta, Couptf1, Fezf2, Tle4, Foxp2, Lhx5, Tlx, Emx1 and Sfrp1 were amplified by PCR with the primers described in the list below, and subcloned into plasmid vector pTA2 (TOYOBO). The cDNA constructs for Er81, Sox5, Tbr1 Otx1, and Ngn2, were kindly given by collaborators.

List of primers for gene cloning

gene name	forward primer	reverse primer
1. Satb2	ACCAGCACCCACAAGCTATCAACC	ACTCCTCCTCATAGATCACATCCCTCTC
2. Foxp1	AAGATAAAGAGCGCCTGCAA	GGCCTTCATTTTCATCTGGA
3. Lmo3	TCAACTCGGCTCCTCACACG	CCAAAAACCCCAATAGCAAGCAA
4. Cux2	AAGATGAAGCAGTTTCTCTCTGATGAGCAG	GGCTCTTATTTTGACCAGCACTGGG
5. Brn2	CTCTGACGACCTGGAGCAGT	CCACACTCTCACCACCTCCT
6. Mef2c	CGTTGAGAAAGAAAGGACTTAATGG	CCATCAGCCATCTCAACAACATATGGTAC
7. Cux1	CCATCTTCTTCAGAGTATTGGAAAGAGTGG	CCCTGTCCTTCTATAGAGACCAATAAGGA
8. Brn1	CCATATGCTGAGCCATGC	CTGCGAGTAGAGGAGCGACT
9. Lhx2	ATGAGGGCCAGGGATTTGGT	CGGGCACTGACGTCAAGACA
10. Ror-beta	GGTTTACAGCAACAGCATCAGCAAC	GCTTGGAAGTGGTTTTGGTGAGAATGTG
11. Couptf1	GTTTGGCTATTCGGTTCAGC	TTGTGTATCGCATCCTCCAA
12. Er81	(Given by Dr.Nakamura)	
13. Fezf2	CAAGAGCCTGGCCTTCTCCA	TGAGCGTGGAGCTCCTGTTG
14. Otx1	(Given by Dr.Shimamura)	
15. Tbr1	(Given by Dr.Shimamura)	
16. Sox5	(Given by Dr.Wakamatsu)	
17. Tle4	GCCAGATGCAGCCTGTTCCT	TTCTTGTCCCCAGAGCCAGTG
18. Foxp2	CTGTCCAGTCTCTGCCACAA	GCCTGCCTTATGAGAGTTGC
19. Lhx5	CGAGAAATGCTTCTTCAGGG	GAGCCAGGTCCTGAGTTCTG
20. Tlx	CCACCGAATCTGTCTGTGAG	TTTGTACATATCTGAAAGCAGTCTTG
21. Emx1	CCAAGCGCTGCTTCACCATA	TCTCCGCTCGACTGCTTGGT
22. Sfrp1	TGCTCAACAAGAACTGCCAC	AGGTGTTGGGTCTTCACCTG
23. Ngn2	(Given by Dr.Shimamura)	

Histological techniques

Heads of embryos at E5-16 were fixed with 4% paraformaldehyde overnight at 4°C, and soaked in 30% sucrose in PBS (phosphate-buffered saline) overnight at 4°C. After the head sank in the sucrose solution, they were embedded in 66% OCT compound /10% sucrose solution, and frozen in liquid nitrogen. Coronal sections were made using a cryostat (Leica), placed on sterile MAS-coated slide glasses (Matsunami), and dried at room temperature.

Brain sections of three amniotes, chick, mouse and turtle, were first soaked in Hematoxylin solution for 30 seconds, and next treated with Eosin solution for 5 min at room temperature. After washing, the stained sections were mounted with Entellan new (Merck).

in situ hybridization

After complete digestion of the template cDNA with the appropriate restriction enzymes, antisense RNA probes were synthesized with DIG RNA labeling kit (Roche). The transcribed RNA probes were concentrated by ethanol precipitation, followed by purification with Probe Quant G-50 Micro Columns (GE Healthcare).

The sections were soaked in 4% paraformaldehyde in PBS for 20 min, washed with PBS, and treated with ProteinaseK for 10 min. Then, the sections were re-fixed with 4% paraformaldehyde in PBS, and acetylated with 0.25% acetic acid anhydride for 10min. After pre-hybridization with hybridization buffer (50% formamide, 1xDenhardt's (Am-

resco), 0.25 mg/ml RNA (Roche), 1x PE, 100 ug/ml Heparin, 0.1% Tween20, 0.75 M NaCl) for 3 hrs at 60 °C, hybridization was performed with hybridization buffer containing 1 ug/ml DIG-labeled RNA probes at 60 °C overnight. Unhybridized probes were washed out with a series of buffers listed below, and additionally digested with 20 ug/ml RNaseA. After washing, the sections were treated with 5 units/ml anti-DIG antibody conjugated with alkaline phosphatase (Roche) overnight at 4°C. For visualization, the sections were incubated with NBT/BCIP solution (Roche) in NTMT buffer (100 mM Tris9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween20).

List of probes for in situ hybridization

	gene name	restriction enzyme	RNA polymerase
1.	Satb2	BamHI	T7
2.	Foxp1	BamHI	T7
3.	Lmo3	XhoI	T3
4.	Cux2	BamHI	T7
5.	Brn2	XhoI	T3
6.	Mef2c	BamHI	T7
7.	Cux1	XhoI	T3
8.	Brn1	XhoI	T3
9.	Lhx2	BamHI	T7
10.	Ror-beta	XhoI	T3
11.	Couptf1	XhoI	T3
12.	Er81	HindIII	T3
13.	Fezf2	XhoI	T3
14.	Otx1	XhoI	T3
15.	Tbr1	EcoRI	T7
16.	Sox5	XhoI	T7
17.	Tle4	BamHI	T7
18.	Foxp2	XhoI	T3
19.	Lhx5	HindIII	T3
20.	Tlx	XhoI	T3
21.	Emx1	XhoI	T3
22.	Sfrp1	HindIII	T3
23.	Ngn2	HindIII	T3

Immunostaining

The brain sections were washed with TBST (10 mM Tris-HCl, pH 7.4, 130 mM NaCl, 0.1% Tween 20) for 5 min. Then, the sections were incubated with antibodies listed below diluted with 1% skim milk in TBST overnight at 4°C. For visualization, the sections were treated with the secondary antibodies conjugated with fluorescent molecules listed below for 2 hrs at room temperature. After mounting with 90% glycerol in PBS, the stained sections were examined through a fluorescent microscope (Zeiss), and photographed with a digital camera (Olympus).

List of primary antibodies

antibodies	(immunized animal)	concentrations	manufacture	No. (clone name)
1. anti-Pax6 antibody	(rabbit IgG)	1:100	(Given by Dr. Osumi)	
2. anti-MAP2 antibody	(mouse IgG)	1:500	Sigma	M4403 (HM2)
3. anti-CTIP2 antibody	(rat IgG)	1:500	Abcam	ab18465
4. anti-FOXP1 antibody	(rabbit IgG)	1:500	Abcam	ab16645
5. anti-β(III)tubulin antibody	(mouse IgG)	1:500	Covance	MMS-435P (Tuj1)
6. anti-pH3 antibody	(rabbit IgG)	1:1000	Millipore	06-570
7. anti-TBR2 antibody	(rabbit IgG)	1:500	Abcam	ab23345
8. anti-BrdU antibody	(mouse IgG)	1:500	Becton Dickinson	347580 (B44)
9. anti-BrdU antibody	(rat IgG)	1:500	Abcam	ab6326 (BU1/75)

List of secondary antibodies

1. anti-rat IgG-Cy3	(Donkey IgG)	1:500	Jackson	712-165-153
2. anti-mouse IgG -Cy3	(Donkey IgG)	1:500	Jackson	715-165-151
3. anti-rabbit IgG -Cy3	(Donkey IgG)	1:500	Amersham	PA43004
4. anti-rat IgG-Alexa 488	(Goat IgG/IgM)	1:500	Invitrogen	A11006
5. anti-mouse IgG-Alexa 488	(Goat IgG)	1:500	Invitrogen	A11029
6. anti-rabbit IgG-Alexa 488	(Goat IgG)	1:500	Invitrogen	A11034
7. anti-mouse IgG MarinaBlue	(Goat IgG)	1:500	Invitrogen	M10991
8. anti-rabbit IgG MarinaBlue	(Goat IgG)	1:500	Invitrogen	M10992

Axon labeling with DiI (l,l'- dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine)

The E16 chick brain was fixed in 4% paraformaldehyde overnight. A small crystal of DiI (l,l'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine) was introduced into a desired position in the brain with a tungsten needle. The DiI-introduced brain was incubated for 2-3 weeks at 37° C in 4% paraformaldehyde. After the incubation, the brain was sectioned with a vibratome (Leica) in 100-300 um thickness, and the brains sections were mounted on slide glasses. Axon trajectories were examined with a fluorescent microscope (Leica).

BrdU labeling analysis

Three different types of BrdU labeling methods were used in this thesis. First, for birth-dating neurons, a small dose of 40 uM BrdU was injected into the embryonic ventricle at

a particular timing (E5-8) of the chick development, and the injected embryos were incubated until E10. Second, for labeling mitotic (G2/S phase) cells, 40 uM BrdU was injected into the ventricle at 30 min before the sacrifice. Third, for characterization of newly produced cells, 500 uM BrdU was injected into the blood vessel of the extra-embryonic tissue, and the BrdU-injected embryo was incubated for 24 hrs at 37°C. In any of the cases, the BrdU-labeled brains were sectioned and treated with 2 N HCl for 2 hrs at room temperature, neutralized with Tris-buffered saline (pH9.5) for 5min, and immunostained with anti-BrdU antibody.

in ovo electroporation

The expression construct for EGFP, pCAGGS-EGFP, was previously constructed in Hirata lab. The cDNA construct was dissolved in TE at the concentration of 5 ug/ul. The solution was colored with Fast-Green to facilitate injection of the solution in the ventricle. After the injection of a small amount of the DNA solution into the ventricle of E5 chick embryo with a fine glass needle made with a puller (PN-30, Narishige), electric pulses (30 V, three 50-sec pulses with 950 msec intervals) were applied between the tweezer-type electrodes (CUY-650-P0, Nepagene). For the fate mapping analysis, the electroporated embryos were incubated by E10 at 37°C. For the thalamic axon labeling, the electroporated embryos were incubated by E16 at 37°C.

NPC clonal culture

The methods of mouse neural progenitor cell culture (Shen, Wang et al. 2006) were modified. Briefly, the pallium of chicken embryo was isolated and separated into medial and lateral parts. The pallial fragment was enzymatically treated with papain (Sigma) and dissociated into single cells. The cells were washed with DMEM (GIBCO), transferred to a poly-L-lysine-coated chamber slide, and cultured for 5 days in DMEM supplemented with 1 mM L-glutamine, 1 mM sodium pyruvate, 1 mM N-acetyl-cystein (Sigma), 10 ng/ml fibroblast growth factor 2, N2 supplement and B27 supplement (Invitrogen).

Cell counting and statistical analysis

To analyze the distribution of mitotic cells in the pallium of chick embryo, the mitotic cells were labeled with anti-phospho histon H3 (pH3) antibody (Millipore). After taking image data of the slices, the pallium on each image was digitally divided into four sections using Adobe Photoshop software (Adobe Systems, San Jose, CA). The number of pH3 positive cells in each quarter was counted on a computer screen.

For counting the number of basal progenitors, anti-BrdU (BD) and anti-TBR2 (Abcam) antibodies were used to label the mitotically active cells and the SVZ cells, respectively. After obtaining the image data, the pallium was digitally divided into 4 quarters and the number of the TBR2/BrdU double labeled cells were counted in the 100um width on the center of each pallial quarter.

For the characterization of newly produced cells, the BrdU-labeled cells were simultaneously stained for a neural progenitor marker, Pax6 (gift from Dr.Osumi) and a pan-neuronal marker, β(III)tubulin (COVANCE). After obtaining the image data, the pallium was digitally intersected into 4 quarters and the numbers of β(III)tubulin/BrdU and PAX6/BrdU double labeled cell s were counted in the 100um width on the center of each quarter.

In NPC clonal culture, the cultured cells were labeled with three antibodies, anti-β(III)tubulin antibody as a pan-neuronal marker, anti-CTIP2 antibody as a DL specific marker, and anti-FOXP1 antibody that strongly marked UL neurons and weakly DL neurons,. The numbers of DL and UL neurons in a clonal cluster originated from a single NPC were counted, and the ratios of the clones containing both DL and UL neurons, only DL neurons, only UL neurons and neither DL nor UL neurons, were calculated.

All data were represented as mean±s.e.m.

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