Molecular Profiling of the Developing Avian Telencephalon: Regional Timing and Brain Subdivision Continuities

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ABSTRACT

In our companion study (Jarvis et al. [2013] J Comp Neurol. doi: 10.1002/cne.23404) we used quantitative brain molecular profiling to discover that distinct subdivisions in the avian pallium above and below the ventricle and the associated mesopallium lamina have similar molecular profiles, leading to a hypothesis that they may form as continuous subdivisions around the lateral ventricle. To explore this hypothesis, here we profiled the expression of 16 genes at eight developmental stages. The genes included those that define brain subdivisions in the adult and some that are also involved in brain development. We found that phyletic hierarchical cluster and linear regression network analyses of gene expression profiles implicated single and mixed ancestry of these brain regions at early embryonic stages. Most gene expression–defined pallial subdivisions began as one ventral or dorsal domain that later formed specific folds around the lateral ventricle. Subsequently a clear ventricle boundary formed, partitioning them into dorsal and ventral pallial subdivisions surrounding the mesopallium lamina. These subdivisions each included two parts of the mesopallium, the nidopallium and hyperpallium, and the arcopallium and hippocampus, respectively. Each subdivision expression profile had a different temporal order of appearance, similar in timing to the order of analogous cell types of the mammalian cortex. Furthermore, like the mammalian pallium, expression in the ventral pallial subdivisions became distinct during prehatch development, whereas the dorsal portions did so during posthatch development. These findings support the continuum hypothesis of avian brain subdivision development around the ventricle and influence hypotheses on homologies of the avian pallium with other vertebrates. J. Comp. Neurol. 521:3666–3701, 2013.

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INDEXING TERMS: brain evolution; in situ hybridization; zebra finch; embryo; pallium; subpallium; FOXP1; COUP-TF2; ROR-β; LHX9; PPAPPDC1A; anatomical gene expression networks

The telencephalon is regarded as a complex and elaborated structure of the brain. The evolutionary origin and organization of this structure is still unclear. The avian telencephalon organization was recently reclassified into two major regions that contain at least seven major subdivisions: the pallium (containing the hyperpallium, mesopallium, nidopallium, arcopallium, and hippocampus) and the subpallium (containing the striatum and pallidum) (Reiner et al., 2004; Jarvis et al., 2005). The avian supallial subdivisions are considered well conserved with their mammalian counterparts in developmental origin, connectivity, cell types, and cell organization (Medina and Abellan, 2009; Butler et al., 2011). However, the pallial subdivisions are less conserved, and thus our understanding of the organization of the avian pallium and its cellular homologies with mammals is still controversial (Jarvis et al., 2005; Butler et al., 2011; Puelles, 2011; Martinez-Garcia et al., 2012).

There are two main competing hypotheses: 1) the nuclear-to-layered hypothesis posits that the different nuclear subdivisions of the avian pallium contain cells that are homologous to different layers of the...
mammalian cortex (Jarvis et al., 2005; Wang et al., 2010); and 2) the nuclear-to-claustrum/amygdala hypothesis posits that the different nuclear subdivisions of the avian pallium below the lateral ventricle, called the dorsal ventricular ridge (DVR), are homologous to the mammalian amygdala and claustrum (Puelles, 2001; Jarvis et al., 2005; Medina and Abellan, 2009). A less discussed hypothesis is that the avian anterior DVR (minus the arcopallium) has cell types that are homologous as a field between the mammalian cortex and claustrum/amygdala complex (Butler and Molnar, 2002; Butler et al., 2002). All hypotheses treat the DVR below and the dorsal pallial subdivisions above the lateral ventricle as different from each other, and as a result their subdivisions have been given different names in the classical and recently revised terminologies (Reiner et al., 2004; Jarvis et al., 2005). Our companion study (Jarvis et al., 2013) suggests that the dorsal and ventral subdivisions of the avian pallium have gene expression profiles more similar to each other across the ventricle divide than they do on the same side of the ventricle, and thus the DVR should not be thought of as a separate structure from the dorsal pallium (or dorsal cortex). However, this new hypothesis of avian pallium organization is based exclusively on adult features, whereas developmental profiles could help support or refute it.

Here we performed in situ hybridization expression profiling to infer the developmental origin of the adult brain telencephalic subdivisions. We selected genes that were most enriched in different adult pallial subdivisions (COUP-TF2, EMX2, ER81, LH9X, PA6X, PAPDC1A, ROR-β, SEMA6A), subpallial subdivisions (D1B, DLX1, DLX6, LH8X), or a combination of both (FOX2P1, NKX2.1, GRIN2D), and a control gene with enrichment in the brainstem (OTX2). We performed quantitative and qualitative profiling of five embryonic stages, two early posthatch stages, and adulthood of the zebra finch. We used sagittal sections, as it allowed us to assess regional expression continuities that are difficult to discover in coronal sections. Our findings suggest that different adult cell populations may begin in the embryo either below or above the lateral ventricle, and that during development they wrap around the ventricle to expand in the counter territory to form a partial mirror image of gene expression surrounding the lateral ventricle and the adjacent lamina. The results influence hypotheses on vertebrate brain organization between birds and mammals.

**MATERIALS AND METHODS**

**Animals**

Zebra finch eggs were collected within 6 hours after females laid them, and incubated at 37–37.5°C and 45–50% humidity. We then opened eggs at 1–2-day intervals to characterize zebra finch developmental stages that correspond to Hamburger–Hamilton (HH) stages based on embryonic morphology (Fig. 1A; n = 94 embryos total). Embryos used for gene expression analyses were from embryonic day 4 (ED4; n = 6), day 6 (ED6; n = 6), day 8 (ED8; n = 6), day 10 (ED10; n = 6), and day 12 (ED12; n = 6) of incubation. Posthatch day 1 (P1) hatchlings were collected from the incubator and two from the nest that had been raised by their parents (n = 8 total). P6 animals (n = 6) were collected from the nest, and adult male animals (>120 days after hatch; n = 6) were collected from the cages in our aviary. All animal protocols were approved by the Duke University Institutional Animal Care and Use Committee (Protocol A133-11-05).
Radioactive in situ hybridizations

The heads of embryos and hatchlings were dissected and the skin removed; for adults the skull was also removed. All adult brain samples were male. All tissues were immediately frozen in tissue Tek OCT (Sakura, Torrance, Ca) and stored at −80°C. Radioactive in situ hybridization was performed on serial tissue sections using a previously described protocol (Wada et al., 2004; Chen et al., 2012b). Heads of embryos and hatchlings were sagittally sectioned in five alternative series at 10-µm thickness. Adult brains were sagittally sectioned in 15 alternative series at 12-µm thickness. Sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) and hybridized at 65°C with antisense 35S-UTP labeled riboprobes. Riboprobes were synthesized from zebra finch cDNA clones, which were either obtained from our songbird brain transcriptome cDNA collection (SBTD; http://songbirdtranscriptome.net/; Duke University; Wada et al., 2006), purchased from Clemson University genomics institute (CUGI; Replogle et al., 2008), or cloned by using conserved primers against identified

Figure 1. Development of zebra finch embryos. A: Development of zebra finch embryo morphology and the corresponding Hamburger-Hamilton stages. Embryonic day (ED) indicates days of incubation. Each panel includes separated upper and lower limbs on right side, used for staging. B: Correlation between Hamburger-Hamilton stages and zebra finch embryonic days until the hatch day (ED13). Each dot is the value of one embryo (n = 94). A little under half of the dots overlapped due to very similar values at each developmental stage. A reduced version of this plot up to ED4 was reported in Chen et al. (2012a). Scale bar = 2 mm in ED3–P1; 0.5 mm in ED2.
sequences in the zebra finch genome (Warren et al., 2010), respectively in the pFLC1, pBS SK1, or pGEM T-easy vectors. The clone sources, polymerase information, and Genbank accession numbers or gene IDs of the cDNA fragments are listed in Table 1. The hybridized sections were first exposed to x-ray film (Biomax MR; Kodak, Rochester, NY) for 8 hours to 3 days depending on gene expression abundance, then dipped into autoradiographic emulsion (NTB2; Kodak), incubated for 4–16 days at 4°C, processed with Kodak developer (D-19) and fixer, and Nissl-stained with cresyl violet acetate solution (Sigma, St. Louis, MO). Sections were coverslipped and sealed with Permount solution (Fisher Scientific, Fair Lawn, NJ). Darkfield or brightfield pictures were taken with an Olympus macrozoom microscope (MVX10), and images were processed by using Photoshop CS4 (Adobe Systems, San Jose, CA).

### TABLE 1.
List of Genes Used in This Study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Molecular function</th>
<th>GenBank ID</th>
<th>GenBank accession no. or clone ID</th>
<th>Enrichment of brain regions</th>
<th>Polymerase for antisense/sense riboprobes</th>
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| COUPTF2    | Chicken ovalbumin upstream pro-
|            | moter-TF2                       | AB542998.1 | AB542998 (1-45 bp)                | Nidopallium                                | T7/SP6                                   |
| D1B        | Dopamine receptor 1B           | AB372108.1 | AB372108 (1-625 bp)               | Striatium                                   | T7/SP6                                   |
| DLX1       | Distal-less homeobox 1         | CK306520   | CUGI 32K10                        | Subpallium                                  | T3/T7                                    |
| DLX6       | Distal-less homeobox 6         | EH120087   | SBTD 0063P0006H11                 | Subpallium (striatum; pallidum; POA)        | T3/T7                                    |
| EMX2       | Empty spiracles homeobox 2     | XM_002187442 | HM633193 (170 bp) | Pallial ventricular zone                        | SP6/T7                                   |
| ER81       | Ets-related protein 81         | DV582566   | SBTD 0064P0002B03                 | Arcopallium; hippocampus                     | T3/T7                                    |
| FOXP1      | Forkhead box P1                | NM_001076698 | AY549152 (1544–1711 bp)          | Mesopallium; striatum                       | T7/SP6                                   |
| GRIN2D     | NMDA receptor 2D               | AB042759.1 | AB042759 (1–521 bp)              | Subpallium (striatum; pallidum; POA; midbrain; brainstem) | SP6/T7                                   |
| LHX8       | LIM homeobox 8                 | EH122589   | SBTD 0057P0002H01                 | Subpallium (striatum; pallidum; POA)        | T3/T7                                    |
| LHX9       | LIM homeobox 9, zinc finger    | XM_002193946 | AB542999 (1-467 bp)             | Arcopallium; hippocampus                     | SP6/T7                                   |
| NKX2.1     | NK2 homeobox 2.1               | XM_002199787 | DV583144 | Subpallium (pallidum) | T7/SP6 | T3/T7 |
| OTX2       | Orthodenticle homeobox 2       | HM633194 (1-637 bp) | SBTD 0064P0001D02 | Forebrain–midbrain boundary | T7/SP6 | T3/T7 |
| PAX6       | Paired box 6                   | DV580106   | SBTD 0063P0027B10                 | Pallial ventricular zone                     | T3/T7                                    |
| PPAPDC1A   | Phosphatidic acid phosphatase 2 domain containing 1A | CK305549 | CUGI 27G14 | Hyerpallium; nidopallium                                   | T3/T7                                    |
| ROR-β      | RAR-related orphan receptor beta | XM_002190956 | XM_002190956 (702–1102 bp) | Pallial sensory neurons (L2, E, and B) | SP6/T7                                   |
| SEMA6A     | Semaphorin-6A                  | CK308635   | CUGI P45115                      | Hyperpallium; nidopallium; striatum         | T3/T7                                    |

1The zebra finch cDNA fragments are listed with their corresponding region of the gene (base pair) for those which we cloned in this study, GenBank accession number or the clones used, the clone ID, database source, and polymerase for antisense and sense riboprobes.

### Telencephalic phylo-gene expression trees and network analysis
To classify the brain areas by gene expression profiles within and across developmental stages, we quantified the amount of expression in each brain subdivision in images from either x-ray film or emulsion-dipped slides that were digitally converted to grayscale. Regions of interest were outlined with the highlighting tool of Image J 1.43u (NIH), the average pixel density was calculated in at least two adjacent sections, and adjacent background signal from the glass slide was subtracted out. We chose the regions to quantify based on two criteria: 1) topographic match of expression in adult with late embryonic and hatchling brain patterns (11 regions; A, Nc, Na, H, Hp, MD, MV, Pd, Pv, Std, Stv); and 2) Quadrants of regions labeled without bias to the adult patterns as R1–R9 around the lateral ventricle in early embryonic (ED4, ED6, and ED8) brains, as
we were not confident based on the expression patterns alone of the predicted primordial adult regions. R1–R6 designated putative pallial regions and R7–R9 putative supallial regions. We did not include the primary sensory pallial regions (intercalated pallium) in the quantitative analyses, due to the higher uncertainty in locating them with gene expression markers in embryonic brains. We selected 14 genes for quantification: ER81, SEMA6A, GRIN2D, NKX2.1, COUP-TF2, FOXP1, LHX9, DLX6, D1B, ROR-β, LH8X, PPAPDC1A, DLX1, and OTX2. We excluded PAX6 and EMX2 for the quantifications, as they were uninformative for adult brain expression patterns, being expressed almost exclusively in the ventricular zone.

The gene expression values were either linear normalized between 0 and 1 or discretized into three levels (0, 1, or 2; Jarvis et al., 2013). This scaling normalizes differences in expression intensity for different genes due to exposure time and also prevents genes with high abundance (strong signal) in one brain region from dominating the relationships in the quantitative analyses. We applied the values to the R program Pvclust (free software for statistical computing and graphics: http://www.r-project.org/) to determine the relationships of brain regions within each developmental age. We used the bootstrapping hierarchical clustering method on Distance-Correlation values between brain regions, and calculated bootstrapping probability (BP) and the approximately unbiased probability (AU) of the nodes for each branch (Suzuki and Shimodaira, 2006). To generate tree figures of the brain relationships, we used the program FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). The tree infers the phylogenetic relationship of brain regions with different cell types based on gene expression profiles, and thus we call them phyla-gene expression trees.

To determine the relationships of brain regions across development, we constructed a network across ages by using a simple regression model. First, to prevent stronger relationships within each age from biasing and dominating the relationships of a network over those between ages, we merged the linear normalized values of two regions together that had the highest expression vector correlation of all 14 genes within each age at r > 0.7 (Pearson correlation). This threshold value was determined empirically from the adult data of similarities within two locations of the same brain subdivision. We then further normalized the data to eliminate gene expression differences that were enriched overall in one stage over another, by estimating a stage-specific component of gene expression and then used regression to eliminate that source of variation. This procedure made gene expression across stages more comparable, but very similar results were obtained when this step was omitted (data not shown). After normalization, we built a linear model explaining gene expression in each region of the current stage of interest as a linear combination of regions at previous stages. Because of the changing patterns of expression across seven stages and 14 genes, we only considered the four previous stage regions most correlated (Pearson) to the region of interest by using the following equation: R_S ~ X_1R_1S-1 + X_2R_2S-1 + X_3R_3S-1 + X_4R_4S-1, where R is the current region of interest, S is the current stage, S-1 is the previous stage, R1 ... R4 are the regions with the highest correlation (Pearson) to R, and X_1 ... X_4 are the estimated coefficients. The regression ignores the region labels, so that a particular region at one stage will only match that same annotated region at the previous stage if the gene expression vectors are significantly related. An edge was drawn from the brain region of the late stage to the early stage if the two were significantly related (P < 0.05) based on the linear model. The network relationships were visualized with Cytoscape v2.8.2 (Smoot et al., 2011), which we called an anatomical expression network.

**Figure preparation**

Figures for publication were prepared similarly as in Jarvis et al. (2013). The digitized microscope images were adjusted in Adobe Photoshop, the Levels function was used to expand the image information within the 250-pixel range, and then color was adjusted by using the Color Balance function to remove red darkfield reflectance from the Nissl stain overshadowing the white silver grain mRNA signal. The background outside of the tissue was cut out in order to help visualize the brain boundaries. All images of the same experiment were adjusted similarly to avoid creating artificial differences within a serial sectioned set of images of the same gene.

Detailed histological data from this article are available as virtual slides or whole-slide images using Biolumina Cloud image streaming technology from MBF Bioscience. The collection can be accessed at http://Wiley.Biolumina.net/JCN521-16Jarvis_Chen.

**RESULTS**

**Zebra finch embryonic stages**

In order to address our hypothesis on development of avian brain subdivisions (Jarvis et al., 2013) in songbirds, we first needed to determine the zebra finch embryonic stages relative to established chicken stages (Hamburger and Hamilton, 1992). We chose the zebra finch due to it being a popular animal model for vocal
learning with parallels to human speech (Jarvis, 2004; Zeigler and Marler, 2008). Although a classification of some zebra finch embryo stages was previously reported at around the time we began our project (Charvet and Striedter, 2009), that study did not cover the full range of development and differences could occur under different incubation conditions. Thus, we characterized zebra finch embryonic stages under our incubation conditions, using standard limb morphology characteristics, egg teeth, eyelid positions, and feathers (Fig. 1A). We found that the early zebra finch hatching stages corresponded to late chicken embryonic stages (Fig. 1B). Based on the segregation of different brain divisions (e.g., telencephalon, midbrain, and cerebellum) and subdivisions (populations of cells within the divisions), we chose to study comparative gene expression profiles in animals from embryonic days 4, 6, 8, 10, and 12, to P1, corresponding respectively to Hamburger and Hamilton embryonic developmental stages HH24–46. We also performed expression analyses on a subset of genes in animals at P6 for patterns that differed between P1 and adults, and included expression analyses of all genes in adults. Although most past studies used frontal sections to analyze forebrain development, we used sagittal sections, which we found gave a novel perspective on the anterior-to-posterior forebrain developmental organization around the ventricles.

The primordial songbird pallium and subpallium

In order to track adult brain anatomical expression profiles back to embryonic development, we next needed to identify the primordial pallium and subpallium at the earliest developmental stage studied, ED4. We found that similar to early chicken HH26 and mouse ED12 embryos (Garcia-Lopez et al., 2008), zebra finch ED4 embryos contained a dorsal ventricular zone region in the telencephalic neural tube (upside-down U-shaped label in sagittal sections) that expressed high levels of early pallial-specific markers, the transcription factors EMX2 (Fig. 2A) and PAX6 (Fig. 2B). There were some differences between genes. EMX2 had a gradient of lower expression into the anterior primordial subpallium, whereas PAX6 had sharp boundaries with the subpallium. We followed the developmental expression profiles into adulthood and found that enriched expression levels of both genes remained mostly restricted to the ventricular zone of the telencephalon (Fig. 3). However, for EMX2, from ED6 to P1, there was a posterior region of labeled cells that emanated from the caudal lateral ventricle near the pallium–subpallium boundary (Fig. 3A2–A6; yellow arrows), which has been proposed to be migrating cells moving into the anterior pallium (Cobos et al., 2001; Medina and Abellan, 2009). In adults, EMX2 expression was mainly enriched in the dorsal part of the pallial ventricular zone (Fig. 3A7), indicating that expression was shut down in the ventral pallial ventricular zone at an earlier age. For PAX6, an isolated region of the caudal striatum had high expression in adults that could be traced back to ED10 (Fig. 3B4–B7; yellow arrowheads). In adults, both genes had lower expression throughout the ventricular zone relative to embryonic animals, suggesting a reduced, but still present role of these genes.

We found, also similar to chicken and mouse (Bardet et al., 2010), a ventral region of the zebra finch telencephalic zone at ED4 that expressed the subpallial marker transcription factors NKX2.1, LHX8, DLX1, and DLX6 (Fig. 2C–F). NKX2.1 was expressed throughout the subpallium, whereas LHX8, DLX1, and DLX6 were restricted to a thin layer in the superficial ventral part of the subpallium. The developmental expression pattern of these genes into adulthood was not restricted to the ventricles, but was mainly in the subpallium (discussed in subpallium section below).

Together, these findings suggest a well-conserved pattern of expression from early embryonic stages to adulthood, and identify primordial pallial and subpallial regions during all embryonic stages from which we can anchor specific subdivision markers to test our hypothesis. In the remainder of this study, we first present our quantitative analyses within and across each developmental stage, then a detailed qualitative analysis of the developing pallium and subpallium subdivision profiles, and end with possible discovery of an embryonic appearance of the motor song production nucleus of the arcopallium.

Quantitative brain phylo-gene expression trees analyses within ages reveal shared relationships of regions above and below the ventricle during development

To conduct a quantitative assessment of the brain expression profiles within each age, we applied our brain phylo-gene expression approach on 14 genes, with defined adult brain subdivision patterns, in 9 (ED4–ED8) to 11 (ED10–P1) embryonic brain regions (tree results in Figs. 4 and 5; gene expression profiles in Figs. 7–15, 18). Each brain region measured at each age was represented by a vector of expression levels for the 14 genes. The difference in the number of brain regions measured for different developmental ages was because we could readily locate brain subdivisions that resembled the adult forms back to ED10, and thus labeled those regions with the presumed adult names as a hypothesis, but we could not readily locate such regions from ED4 to ED8 as the brain topography was more different than in adults, and thus for these earlier
ages we measured gene expression in nine consecutive regions surrounding the ventricle, as a discovery approach (see Materials and Methods; Fig. 4H–J). This means that the numbered regions have the possibility of not corresponding to each other for each age from ED4 to ED8. In selecting the regions, we noted that nearly all pallial markers had at least two expression domains below and above the lateral ventricle, with lags in expression time for some of them (shown for all 14 genes in Figs. 7–9, 18). Furthermore, these dorsal and ventral pallial regions had points of anatomical continuities with each other seen in medial-to-lateral sagittal sections (shown for canonical subdivision–specific genes in Figs. 10–15).

By using the above approach, first we confirmed that phylo-gene expression tree analyses of the 14 selected genes on the more reduced set of 11 brain regions in the adult resulted in trees that were consistent with the analyses of 50 genes and 23 brain regions in our companion study (Jarvis et al., 2013). Like that study, we found that with these 14 genes, in the adult pallium, the dorsal and ventral mesopallium (MD and MV), the nidopallium and hyperpallium (N and H), the arcopallium and hippocampus (A and Hp), each above and
below the ventricle and adjacent lamina mesopallium intermediate (LMI), were more similar to each other than they were to any other brain subdivision (Fig. 4A). In the subpallium, the dorsal and ventral striatum (Std and Stv) and the dorsal and ventral pallidum (Pd and Pv) were each more similar to each other than they were to any other brain subdivision (Fig. 4A; according to our revised nomenclature). The pallial regions also separated from the subpallial regions. These relationships held regardless of whether non-normalized, normalized, or discretized values were used (Fig. 5A–C). The normalized values resulted in higher bootstrap (BP) and approximately unbiased (AU) probabilities; but even for BP below 70 and AU below 95 the tree structure was similar. Removal test of one or more genes showed that the tree structure was relatively stable until we went below 10 or less genes (Fig. 5G–J). We believe that the overall stability of the tree structure with fewer genes than in our adult study (14 vs. 50) occurred because we included fewer brain regions (11 vs. 23). Thus, this set of 14 genes was sufficient to reliably quantify stable relationships of the quantified brain regions.

With the adult tree structure confirmed, we went backward from P1 to ED10, and found similar strongly supported relationships with the regions we hypothesized to be the adult precursors (Fig. 4B–D). A difference was that the region we proposed to be hyperpallium and nidopallium in the embryos for these ages, based on adult topography and gene expression, split up, where our proposed embryonic hyperpallium grouped closer with the two mesopallium regions (MV and MD) at all three ages, and our proposed nidopallium grouped closer with the arcopallium and hippocampus (Fig. 4B–D). We believe this is due to mixed cellular ancestry, as highlighted in our network analyses below. The trees from ED4 to ED8 indicated shared relationships as in the older embryos and adults, but the topology of the relationships with our numbering across ages was not consistent (Fig. 4E–G) as expected. Importantly, though, we noted that unlike ED10–P1, where regions above and below the ventricle had shared relationships as in adults, from ED4 to ED8,

Figure 3. Time series expression of pallial ventricular zone genes. The panels from right to left across each page are sagittal sections from ED4 to adult. Right is oriented rostrally. A,B: *EMX2* (A) and *PAX6* (B) are expressed strongly in the dorsal ventricular zone (white arrowheads) through all development stages. Yellow arrow points to junction between the posterior ventricle location and brain where *EMX2* cells are thought to migrate out of; yellow arrowhead points to dissociated cells for *PAX6*. C: Brightfield Nissl-stained sections of in situ hybridizations with *EMX2* showing the Nissl-defined regions examined in this study. The magnifications of C1 and C2 are different, in order to highlight the regions of interest. For abbreviations, see list. Scale bar = 500 μm; 2 mm in adult panels.
the shared relationships were mostly local (closer R numbers to each other).

Quantitative temporal networks analyses across ages suggest origins of adult brain subdivisions

To determine the relationships across ages, we performed a linear network analysis using the same expression values applied to the tree approach (see Materials and Methods). We first merged up to two regions at regression $r > 0.7$ ($P < 0.05$) within each age to reduce the possibility that strong attraction of regions within one brain subdivision would overshadow relationships across time. This merging resulted in identifying and confirming supported similarities of gene expression profiles within each age group, including the relationships of our hypothesized adult and embryonic dorsal and ventral mesopallium (MD and MV), the nidopallium and hyperpallium, the arcopallium and hippocampus, the two different striatal regions (Std and Stv), and the two pallidal regions (Pd and Pv), from ED10 onward (Fig. 6). Before ED10, regions merged as well, which was expected considering that we separated them into segments that had shared expression profiles. However, the merged regions did not always have the same-labeled numbers, consistent with the numbering not necessarily being the same region across ages (Fig. 6).

Next we used the merged values to generate networks with the linear method blind to brain region identity. We found that the hypothesized subpallial regions all linked the strongest together in time and not to pallial regions, where they began as one merged region we labeled R5 and R7 at ED4, followed by R7 and R8 from ED6 to ED8, that then split into striatal and pallidal subdivisions by ED10 and stayed that way into adulthood (Fig. 6). The more dorsal R7 region (at ED6–ED8) predominantly gave rise to the striatum, and the more ventral R8 region gave rise to the pallidum. Within the pallium, R2 and R3 in the embryonic caudal pallium had the strongest links with the adult hippocampus and arcopallium from ED4 onward, even though R6 was in the topographic position of the arcopallium containing high levels of COUP-TF2 expression (Figs. 4H–J, 6). The adult nidopallium did not show links to regions before ED10, but it did approach statistical significance ($P < 0.07$) for R4 and R5 in the pallium below the ventricle at ED8 (data not shown). R1 in the anterior dorsal...
Figure 4. Phylo-gene expression tree analyses of telencephalic organization at different development stages. A: Phylo-gene expression tree and heat map of adult brain regions (y-axis) for 14 genes (x-axis). In the tree, similar colors indicate shared expression profiles between brain areas, such as yellow for the hippocampus and arcopallium, with the topography indicated in the diagram in the upper left of an adult avian sagittal brain section. In the heat map, the color of the boxes indicates relative expression levels (red, higher than the average for that region relative to other regions; blue, lower than the average). The bootstrap probability (BP; green) values and the approximately unbiased probability (AU; red) values are listed in each branch, as well as distances at each node. A small correlation distance between areas indicates a high similarity in brain gene expression patterns (≈ 1-correlation values). The scale bar below the phylo-gene expression tree shows the branch distance. B–G: Phylo-gene expression trees for the same genes at (B) P1, (C) ED12, (D) ED10, (E) ED8, (F) ED6, and (G) ED4. H–J: Sagittal sections using the COUP-TF2 pattern to show the numbered regions (R1–R9) we measured telencephalic gene expression at (H) ED8, (I) ED6, and (J) ED4. For abbreviations, see list. Scale bar = 500 µm in H–J.
Figure 5. Telencephalic phylo-gene expression trees from different methods and numbers of genes. A–F: Comparison of trees generated with the continuous (A,D), normalized (B,E) or discretized (C,F) gene expression data at two example ages, adult and ED10. G–J: Adult trees with fewer genes than 14, minus (G) OTX2, (H) OTX2, DLX1, and SEMA6A, (I) OTX2, DLX1, SEMA6A, and LHX8, or (J) OTX2, DLX1, SEMA6A, LHX8 and ER81. For abbreviations, see list.
pallium above the ventricle starting at ED8 was most strongly associated with the dorsal and ventral mesopallium combined (MD and MV) into adulthood, but in a transient relationship with the hyperpallium at P1. On either side of P1, the hypothesized hyperpallium was partly linked in the network with the anterior nidopallium. There was not a link of the pallidal domains from P1 to adulthood, which we think was weakened by the migrating patterns of subpallial \( \gamma \)-aminobutyric acid (GABA)ergic cells into the striatum and pallium (described in supallial section below).

Overall, both the phylo-gene expression tree and the network analyses indicate that adult brain subdivisions can have either local or mixed embryonic ancestry, with dual relationships to regions above and below the lateral ventricle. We next asked how such mixed and dual relationships could arise. To answer this question, we analyzed the temporal-anatomical patterns of the canonical markers that defined the adult subdivisions in the chronological order that their expression profiles appeared in the embryo (Table 2), starting with pallial regions and designating the R1–R9 regions by their adult inferred regions according to the network analyses.

Qualitative temporal–anatomical analyses of pallial development reveal continuities around ventricle folds

**Development of arcopallium and hippocampus**

In our companion study (Jarvis et al., 2013), we found that core portions of the arcopallium had the most similarities with the hippocampus. Consistent with this finding, our quantitative analyses in this study linked the arcopallium and hippocampus mainly to R3 around a ventricle fold of the caudal pallium (Figs. 4,6). The
expression markers for these regions, the LHX9 and ER81 transcription factors, had already appeared at ED4. For LHX9, there was high expression throughout the pallial ventricular zone that overlapped with the EMX2 and Pax6 expression region (compare Fig. 7A1,B1 with Fig. 3A1,B1). However, from ED6 onward, a region of high continuous LHX9 expression was restricted around the caudal–lateral ventricle fold, with the dorsal part (R3) later becoming the hippocampus (Fig. 7A2–A7) and the ventral part (R6) later becoming the arcopallium (Fig. 7B2–B7). This continuity between hippocampus and arcopallium LHX9 expression could be easily seen in medial-to-lateral sections from ED6 to P1 (Figs. 11A–15A; brightfield section example in Fig. 16A). At some time between P1 to adulthood, LHX9 expression was shut down in the anterior arcopallium nucleus (Fig. 7B6–B7). LHX9 expression in other brain areas, such as the dorsal thalamus and optic tectum, was consistent throughout embryonic development to adulthood (Fig. 7A,B).

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1Black text, stable enriched gene expression profile found into adulthood. Red text, transient enriched gene expression profiles that do not last into adulthood. N/P, not present.
ER81 expression during embryonic development was not as topologically consistent (Fig. 7C,D). Instead, at ED6, high levels of ER81 expression appeared first in a layer of cells within the dorsal pallium and in cluster of cells within the primordial basal ganglia (Fig. 7C2,D2). By ED10, expression was still high in the primordial striatum and pallidum, but there were scattered labeled cells in the dorsal pallium and in the primordial nidopallium (Fig. 7D3). ED10 was the first age at which we could see signs of higher levels of ER81 in the hippocampus, and ED12 was the first to display signs in the arcopallium, both contained within the LHX9-labeled region (Fig. 7B4–B5,D4–D5). However, there were still scattered labeled cells in the basal ganglia (Fig. 7D5). This pattern was still present in P1 animals (Fig. 7C6–D6). By P6, there was a greater enrichment of expression in the arcopallium and hippocampus with decreasing levels in the basal ganglia (Fig. 17B). By adulthood, ER81 label was almost entirely enriched in the arcopallium (except the anterior arcopallium from P1 onward), hippocampus, and a part of the medial nidopallium (Fig. 7D7). These findings suggest that ER81 does not label a specific embryonic brain subdivision developmentally, but labels cells with either transient expression or that migrate. The latter hypothesis is consistent with previous developmental studies suggestion of ER81 in the mammalian cortex and its eventual enriched expression in layer V cells (Yoneshima et al., 2006; Teissier et al., 2010). Together the above findings suggest that the arcopallium and hippocampus develop early around the caudal pole of the lateral ventricle, potentially with ER81 cells migrating in from other pallial regions, or turning on later during development.

Development of nidopallium and hyperpallium

In our companion study (Jarvis et al., 2013), we found that the adult hyperpallium (formally named hyperpallium apicale; Reiner et al., 2004; Jarvis et al., 2005) had very similar molecular and Nissl profiles as the adult nidopallium (minus the sensory input fields of L2, E, B, and I). These two regions showed continuity of expression at the anterior pole of the lateral ventricle, but only when the ventricle was in the posterior part of the cerebrum. An exception was the COUP-TF2 nuclear receptor transcription factor, which was enriched in both the nidopallium and arcopallium more so than in the hyperpallium. Based on these and other findings, we had hypothesized that the hyperpallium and nidopallium might derive from the same area of the embryo, forming as a continuous domain that wraps around the lateral ventricle, with later differentiation of COUP-TF2, or that the hyperpallium and nidopallium develop separately, but with similar molecular profiles (Jarvis et al., 2013). Our quantitative analysis of the embryonic expression profiles in this study was consistent with either hypothesis, in that they linked the adult hyperpallium and nidopallium to regions above and below the ventricle close to significance from ED8 onward, but there was also a suggestion of mixed ancestry with the mesopallium (Fig. 4). To test these hypotheses, we analyzed the temporal–anatomical profile of their strongest markers: the COUP-TF2 transcription factor, the PPAPDC1A phosphatase, and the SEMA6A axon guidance receptor.

For COUP-TF2, an adult-like nidopallium-shaped region that overlapped with a PPAPDC1A region and not with LHX9 in the arcopallium was seen earliest in the ventral pallium at ED6 (Figs. 8A2–A7,B2–B7, 11A–C). This nidopallium-like COUP-TF2 and PPAPDC1A labeled region appeared to expand into a bulge in the lateral ventricle as part of the DVR from ED6 to ED8 and then remained that way into adulthood (Fig. 8A2–B3, white arrows). From ED10 onward, COUP-TF2 was also highly expressed in the anterior part of the third ventricle that later become the boundary between the optic tectum and thalamus (Fig. 8A4–A7, yellow arrows). We did not find a COUP-TF2 region at any age in the dorsal pallium above the lateral ventricle where the hyperpallium was expected to form (Figs. 8A, 10B–15B, 17A).

In contrast, the PPAPDC1A region that overlapped with COUP-TF2 expression at ED6 in the ventral pallium had cells that extended from it dorsally at the anterior pole of the ventricle by ED8 at the same location where COUP-TF2 expression stopped (Fig. 8A2–A3,B2–B3). By ED10, this continuous region of PPAPDC1A expression extended and wrapped around the ventricle to reach the dorsal pallium (Fig. 8B4). This continuity between the primordial nidopallium and hyperpallium could be readily seen in medial-to-lateral sections at all ages from ED8 to P1, but with an intermediate bridge region of slightly lower PPAPDC1A expression (Figs. 12C–15C, white arrows).

Although SEMA6A labeled the adult nidopallium and hyperpallium (as well as the anterior arcopallium and lateral striatum [Fig. 8C7; Jarvis et al, 2013]), like ER81, its expression topologically changed locations and was thus more difficult to interpret (Fig. 8C). Nevertheless, wherever there was enriched PPAPDC1A expression in the embryonic pallial regions, the same region had high SEMA6A expression (Fig. 8B,C). These findings suggest that the reason that the nidopallium and hyperpallium are similar to each other is that they are continuous with each other around the ventricle, but with still some molecular differences that allow them to be distinguished during development.
Development of mesopallium

In our companion (Jarvis et al., 2013) and other studies (Haesler et al., 2004; Mouritsen et al., 2005; Feenders et al., 2008; Horita et al., 2010), the two regions whose names were revised to hyperpallium dorsale and mesopallium in the 2004–2005 nomenclature (Reiner et al., 2004; Jarvis et al., 2005) were proposed instead to be two subdivisions of the mesopallium on either side of the lateral ventricle. Evidence that they might be continuous was that the two domains had a thin region of continuous gene expression in the anterior pole of the LMI lamina that was once the lateral ventricle space during development. Our quantitative analyses in this study linked the two adult regions (MD and MV) to one region above the embryonic ventricle that we labeled R1 at ED8 (Fig. 6). This link was best revealed by the marker that we used to identify the mesopallium, the FOXP1 transcription factor.

From ED4 to ED6, there was no detectable differential high expression of FOXP1 in the telencephalon (Figs. 8D1–2, 10D, 11D). By ED8, high differential FOXP1 expression occurred in the pallium in a strip of cells in R1 above the medial half of the ventricle (Figs. 8D3, 12D1–D3; located according to EMX2 in Fig. 3A3). High expression also appeared in the embryonic dorsal thalamus and striatum as in adults. The dorsal pallial
strip extended into the anterior pole of the telencephalon above a proliferative zone where one would expect to find adult hyperpallium dorsale in the 2004–2005 nomenclature (Reiner et al., 2004) or dorsal mesopallium of our revised nomenclature (Jarvis et al., 2013). There was no other pallial brain region at ED8 with high FOXP1 expression, including the eventual location of the ventral mesopallium below the ventricle (Fig. 12D1–D4). By ED10, the anterior part of this dorsal pallial region had proliferated (enlarged) and begun to extend into the DVR in front of the ventricle (Figs. 8D4, 13D). By ED12, the anterior part of the FOXP1 region had wrapped around the ventricle and turned into a caudal portion of the DVR (Figs. 8D5, 14D, 16B) in a location where one would find adult mesopallium of the 2004–2005 nomenclature (Reiner et al., 2004) or ventral mesopallium of our revised nomenclature (Jarvis et al., 2013). After hatching, from P1 to P6 the two FOXP1-expressing areas were still in the dorsal and ventral pallium above and below the ventricle, and began to take on the adult shapes (Figs. 8D6, 15D; P6 in Fig. 17C). Simultaneously, from ED12 to P6, as the ventral FOXP1 expression area moved caudally into the DVR, the anterior two-thirds of the lateral ventricle sealed up on itself, with the two FOXP1 areas sealing with it to form the LMI lamina of low FOXP1 expression that separates them (Fig. 17C) as seen in adults (Fig. 8D7); there was still clear continuity of expression that wrapped around the anterior pole of LMI at P6 (Fig. 17C). By adulthood, the seal was complete and extended so far anterior near the venicula blood vessel at the brain’s surface that we could barely detect the FOXP1 continuity around LMI (Fig. 8D7, white arrow and Jarvis et al., 2013), making it appear as if these two regions were...
entirely separate unless examined at higher magnification (Fig. 16E,F). When doing so, we noted that even though the MOAB gene is expressed in the ventricular zone cells and the LMI lamina (Jarvis et al., 2013), PAX6 expression and the darker Nissl-stained ependymal epithelial cells remained only in the ventricular zone (Figs. 3B4–B7, 16E,F). The FOXP1 developmental pattern was not seen with non-mesopallium stably expressed genes, including those highly enriched in the striatum (D1B and DLX6; see subpallium section) like FOXP1 and in other pallial divisions (COUP-TF2, PPAPDC1A, and LHX9) not like FOXP1 (Fig. 8). This pattern was seen in all animals at each developmental time point after ED8.

Interestingly, from ED10 to P1, contained within a subportion of the FOXP1 dorsal expression region was the primordial hyperpallium marker PPAPDC1A (Fig. 8B,D), indicating a potential source of the mix ancestry result seen in our quantitative analyses (Fig. 4B–D). Supporting this possibility are four additional qualitative results: 1) PPAPDC1A appeared at ED6 in the ventral pallium before FOXP1 did at ED8, where they did not overlap; 2) the subsequent migration of the PPAPDC1A expression into the dorsal pallium at ED8 did not overlap with the FOXP1 region at that time (Fig. 12C,D); 3) in the most lateral sections at ED10, the dorsal PPAPDC1A expression was found without FOXP1 expression (Fig. 13C,D) and in the most medial sections
the reverse pattern was found (Fig. 13C1,D1); and 4) the overlap between PPAPDC1A expression and FOXP1 expression in midsagittal sections was only seen from ED12 to P1, and it was more patchy for PPAPDC1A and more uniform for FOXP1 (Figs. 14C1–C3,D1–D3, 15C1–C3,D1–D3). After P6, the dorsal PPAPDC1A and FOXP1 labels started to further segregate into their adult-like forms in the hyperpallium and dorsal mesopallium, respectively (Fig. 8A7,B7, 17C,D). These findings suggest that nidopallium–hyperpallium markers develop first more laterally in the pallium below the ventricle, the mesopallium marker develops first more medially in the pallium above the ventricle, and the two cross each other’s domains more centrally as they enter the respective opposite territories above and below the ventricle.

Development of primary sensory telencephalic fields

In our companion study (Jarvis et al., 2013), we found that the sensory input fields share similar molecular identities. These fields were: 1) IH (visual and somatosensory parts) sandwiched between the hyperpallium and dorsal mesopallium; and 2) field L2 (auditory), E (visual), and B (somatosensory) sandwiched between the nidopallium and striatum (except L2 within the nidopallium). L2, E, and B formed a continuous strip of differentially expressed cells that we proposed to call the intercalated nidopallium (IN) as a complement to the IH. These findings suggested that all of these regions could have the same embryonic origin. Although we could not reliably quantify thinly labeled IN and IH regions in the small embryonic brains for the phylo-
gene expression tree and network analyses, we were still able to partially test this hypothesis by examining the temporal–anatomical profiles of two genes, GRIN2D and ROR-β that were expressed at high levels in parts of adult IH and IN relative to the rest of the pallium. ROR-β, however, is expressed at intermediate levels in the adult mesopallium (Fig. 9A7; Jarvis et al., 2013).

We found that during embryonic development, there were regions with enriched ROR-β expression that were similarly located and shaped as adult IH and IN relative to the rest of the pallium. ROR-β, however, is expressed at intermediate levels in the adult mesopallium (Fig. 9A7; Jarvis et al., 2013).

For abbreviations, see list. Scale bar = 500 μm; 2 mm in adult panels.
that were later more segregated during posthatch development. We next examined the subpallial regions to determine whether they had similar properties.

**Qualitative temporal–anatomical analyses of subpallial development reveal mixed ancestries**

**Development of striatum**

In adults, *DLX6* and *D1B* had the most enriched expression in the striatum (Fig. 18A7,B7) and they overlapped with *FOXP1* striatal expression (Fig. 8D7; Jarvis et al., 2013). In embryos, from ED4 onward, we found a clear pattern of enriched *DLX6* expression in the subpallium ventral to the *PAX6* pallial region (Fig. 18A), defined as R7 and R8 in our network analyses (Fig. 6). By ED8, both *D1B* (Fig. 18B3) and *FOXP1* (Fig. 8D3) expression appeared in the dorsal part of this region (R7) with stable pallial–subpallial expression boundaries into adulthood, and thus it appears to be the primordial striatum. The ventral part of the *DLX6*-enriched region without *FOXP1* and *D1B* expression (R8) could be the primordial pallidum (see next section). *DLX6* also had a stable enriched expression pattern in the thalamus from ED6 into adulthood (Fig. 18A2–A7).

**Development of pallidum**

In adults, three genes we examined, *LHX8*, *GRIN2D*, and *NKX2.1*, had enriched expression in the pallidum (Figs. 9C, 18C–E). Several of these genes, such as *DLX1*, were also previously found in the avian embryonic subpallium (Abellan and Medina, 2009; Fragkouli et al., 2009). Here, we found that the patterns of some of these genes varied from embryonic to adult stages. From ED4 to P1, *LHX8* was highly and equally expressed in the primordial striatum (*FOXP1*- and *D1B*-defined) and pallidum (*DLX6*-defined; Fig. 18C1–C6), and was then turned down in the striatum by adulthood (Fig. 18C7). *GRIN2D* was turned on in the pallidum by ED6 (Fig. 9C2) and increased in this same region from P1 onward to become more enriched in the pallidum from P1 to adulthood (Fig. 9C6–C7). The *DLX1* pattern, in contrast, migrated from the highest levels in the subpallium at ED4 to the highest levels in the caudal nidopallium from ED12 to P1, to low expression throughout the telencephalon except in the hippocampus in adults (Fig. 18D). *NKX2.1* started with high expression in the entire extent of the primordial subpallial ventricle from ED4 to ED6, was localized in the primordial pallidum at ED8, spread to the primordial striatum by ED10, and...
finally spread with relatively uniform labeling throughout the subpallium and pallium, except for the two mesopallium domains (MV and MD), in adulthood (Fig. 18E). Similar to some of the pallial expression patterns around folds in the ventricle, the striatum and medial septum region also showed continuous high expression of \textit{NKX2.1} around a posterior ventral fold in the ventricle (Fig. 16C). Although \textit{OTX2} showed some enriched expression in the subpallium at ED4 (Fig. 18F1), it did not last and was mainly restricted to the brainstem from ED6 onward (Fig. 18F2–F7).

The above findings suggest that striatal subpallial markers may be more relatively stable throughout development, whereas pallidal subpallial markers might have a higher propensity to migrate. The latter finding is consistent with known migration of \textit{NKX2.1} GABAergic-positive interneurons from the pallidum to the rest of the telencephalon in birds, reptiles, and mammals (Puelles et al., 2000; Bardet et al., 2006; Metin et al., 2007; Garcia-Lopez et al., 2008; van den Akker et al., 2008; Medina and Abellan, 2009; Bardet et al., 2010). Our findings further indicate that the mesopallium might not receive a high concentration of \textit{NKX2.1} GABAergic neurons from the pallidum.

**Possible origin of the RA song nucleus**

Gene expression specializations in song nuclei have been found to begin occurring mainly after P10 (Konishi and Akutagawa, 1990; Nixdorf-Bergweiler, 1996; Gahr and Metzdorf, 1999). These findings have led to the belief that the specialized song nuclei of vocal learning birds appear days after posthatch development. To explore possible development of song system nuclei earlier than P10, we examined the developmental profiles of the subset of genes we used with specialized expression in adult song nuclei. \textit{DLX6} was highly expressed in the adult area X, but we did not find this feature from ED4 to P6 (Fig. 19A). \textit{EMX2} was expressed at a slightly higher level and \textit{LHX9} was downregulated in adult RA relative to the surrounding arcopallium, and here we noted comparable differential expression of these two genes in a topologically similar position from ED12 to P1 (Fig. 19B,C). While our paper was in press, a recent discovery was made of calbindin specialization
in the area X song nucleus of the striatum at ED13 (García-Calero and Scharff, 2013), which is the same as our P1 animals under our incubation conditions. These findings suggest that development of song nuclei specializations could start earlier than P10, toward the end of embryonic development.

**DISCUSSION**

Here we performed quantitative and qualitative analyses of expression profiles of genes enriched in each subdivision of the avian telencephalon throughout embryonic development to adulthood. Our findings lead us to make the following conclusions: 1) the partial mirror image of subdivision expression profiles found in adults can be best explained as continuous zones that develop around different sectors of the ventricle (Fig. 20); 2) these developmental expression profiles cannot be mapped onto the commonly Cartesian-defined coordinate system, two perpendicular axes in the coronal plane to define the telencephalic subdivisions of ventral, dorsal, medial, and lateral pallium relative to the ventricle (Fig. 21); and 3) the pallial subdivisions form sequentially, which may occur in a comparable order to mammalian cortical layers. Below we discuss the implications of these findings for understanding overall avian telencephalic development and impact on hypotheses of homologies with the mammalian brain.

![Figure 11. Medial–lateral anatomical profiles of adult pallial enriched genes at ED6. Sagittal brain sections of zebra finch embryos from medial (top) to lateral (bottom) shows development of (A) LHX9, (B) COUP-TF2, (C) PPAPDC1A, (D) FOXP1, and (E) ROR-β gene expression. Note that PPAPDC1A begins to show high expression in the ventral–lateral pallium, where the caudal nidopallium (Nc) appears to begin. For abbreviations, see list. Scale bar = 1 mm at lower left (applies to all).](image-url)
A temporal developmental model of avian pallial organization

In our companion study (Jarvis et al., 2013), based on shared gene expression and known connectivity evidence, we proposed that the avian telencephalon consists of four major pallial populations: the IH and IN regions as 1st-pallium, the hyperpallium and nidopallium as 2nd-pallium, the dorsal and ventral mesopallium as 3rd-pallium, and the arcopallium as a 4th-pallium. We hypothesized that this brain organization might form by continuous domains that wrap around the telencephalic ventricle during embryonic development. Our developmental findings in this study are consistent with this hypothesis.

To explain these findings, we propose the model shown in Figure 20, which considers the position of the labeled cell population around the ventricle in all planes, development to adulthood, function, and connectivity. In this model, the 4th-pallium (arcopallium) and hippocampal markers appear first between ED4 and ED6 located in the posterior ventral–dorsal pallium before the DVR bulge into the ventricle, FOXP1 begins to show high expression in the dorsal pallium where the primordial dorsal mesopallium appears to begin (yellow arrows), and ROR-β starts to label the primary sensory intercalated pallium zones in the most anterior part of the telencephalon (white arrowhead). For abbreviations, see list. Scale bar = 1 mm at lower left (applies to all).

Figure 12. Medial–lateral anatomical profiles of adult pallial enriched genes at ED8. Sagittal brain sections of zebra finch embryos from medial (top) to lateral (bottom) show development of (A) LHX9, (B) COUP-TF2, (C) PPAPDC1A, (D) FOXP1, and (E) ROR-β gene expression. Note that PPAPDC1A expression begins to migrate into the dorsal pallium above the ventricle, FOXP1 begins to show high expression in the dorsal pallium where the primordial dorsal mesopallium appears to begin (yellow arrows), and ROR-β starts to label the primary sensory intercalated pallium zones in the most anterior part of the telencephalon (white arrowhead). For abbreviations, see list. Scale bar = 1 mm at lower left (applies to all).
then wraps around the developing LMI lamina as the ventricle folds to become the ventral mesopallium from ED12 onward (Fig. 20E,F, red). Finally, the 1\textsuperscript{st}-pallium (IH/IN) markers appear with weak expression first by ED8 as a curved strip in the anterior ventral–dorsal pole of the telencephalon (Fig. 20C, orange), followed by caudal extensions both ventrally and dorsally from ED12 onward to become respectively IN inserted between the nidopallium and striatum and IH inserted between the dorsal mesopallium and hyperpallium (Fig. 20D–F, orange). Because the 1\textsuperscript{st}-pallium pattern shows up weakly at first, we believe additional gene expression markers will be necessary to verify its timing. It may also be that it is simply a diffuse set of cells that overlap in timing with the appearance of other subdivision populations. Some of the timing we found confirms a recent study showing that ER81-labeled lower pallial cells appear before FOXP1-labeled upper pallial cells in chicken embryos (Suzuki et al., 2012). It is possible and probable that some of the timing details will change when more genes are analyzed, in that some brain subdivisions may appear earlier than the expression of two or more of its markers, as in the case of FOXP1 in the striatum. At least the existing data give us a temporal model to start working with, and one that is consistent with the adult organization.

This model could explain the partial mirror image organization of brain subdivisions. However, the model...
is not a simple wrapping of continuous regions around the ventricle folds for all regions. The invasion of the primordial hyperpallium-like expression of PPAPDC1A from the nidopallium would mean that it mixes with the developing mesopallium cells or is coexpressed with the mesopallium marker (FOXP1) in late embryonic stages (Fig. 20D,E, green dots). It is only after hatching that the well-defined boundaries and enlargement of hyperpallium occur. The migration of the mesopallium expression pattern below the anterior ventricle fold in medial-to-central pallial sectors would require that it displace the migration of the nidopallium-hyperpallium regions that would wrap around the same ventricle fold in central pallial sections.

Such complex moving patterns are consistent with the known more massive anterior-posterior tangential migration of cells in the developing avian telencephalon relative to other migration patterns (Striedter and Keefer, 2000; Dugas-Ford et al., 2012). The moving patterns are also consistent with radial migration of cells away from the ventricle to the surface of the avian telencephalon (Nomura et al., 2009; Tanaka et al., 2011; Suzuki et al., 2012). However, in our model this radial migration would be an expansion of a brain subdivision, not a mechanism to move cells between different dorsal and ventral pallial subdivisions. An alternative explanation is that the pallial populations above and below the lateral ventricle do not

Figure 14. Medial-lateral anatomical profiles of adult pallial enriched genes at ED12. Sagittal brain sections of zebra finch embryos from medial (top) to lateral (bottom) show development of (A) LHX9, (B) COUP-TF2, (C) PPAPDC1A, (D) FOXP1, and (E) ROR-β gene expression. Note that PPAPDC1A dorsal hyperpallium labeled region is still mixed in with the FOXP1 dorsal mesopallium region (yellow arrows), and the anterior-ventral portion of the FOXP1 region has expanded to where the future ventral mesopallium is located, taking on the MV shape as seen in adults. Also seen is a further expansion of the ROR-β region into the primary sensory intercalated pallium zones dorsally (white arrowhead), but still with some regions contained within the FOXP1 mesopallium-like region. For abbreviations, see list. Scale bar = 1 mm at lower left (applies to all).
have a shared direct developmental origin through migration, but have a parallel independently evolved developmental program. A third alternative is transdifferentiation, whereby nonmigrating cells influence adjacent proliferating cells to convert to a similar cell type. Of these three possibilities, we believe that the continuum hypothesis due to migration is the most parsimonious, as independent evolution would require independent mutations for large transcriptomes of genes in different subdivisions to change in the same way and transdifferentiation would not require migration, but it is already known that there is a significant amount of cell migration in the developing avian telencephalon.

These hypotheses can be validated or falsified by testing more gene expression markers and performing developmental fate mapping of labeled cells in vivo, with existing tools (Striedter and Keefer, 2000; Suzuki et al., 2012). One such experiment would be to locally transfect embryonic cells in a ventral or dorsal region relative to the embryonic ventricle with a viral genomic integration vector containing a promoter for the gene marker of that region upstream of a fluorescent protein (e.g., green fluorescent protein), and then perform time-lapse microscopy recordings of the developing embryos, which is possible with birds before hatching (Kulesa et al., 2009). If fluorescently labeled cells are seen migrating and expanding from the opposite end of the

Figure 15. Medial–lateral anatomical profiles of adult pallial enriched genes at P1. Sagittal brain sections of zebra finch embryos from medial (top) to lateral (bottom) show development of (A) LHX9, (B) COUP-TF2, (C) PPAPDC1A, (D) FOXP1, and (E) ROR-β gene expression. Note that the PPAPDC1A dorsal primordial hyperpallium region starts to show decreased FOXP1 expression, but the majority is still mixed in with the FOXP1 dorsal mesopallium-like region (yellow arrows); the FOXP1 ventral mesopallium region is more similar to its adult form and does not overlap with PPAPDC1A. Also seen is a weak expansion of the ROR-β primary sensory intercalated pallium zones (white arrowhead). For abbreviations, see list. Scale bar = 1 mm at lower left (applies to all).
ventricle, around the ventricle folds, forming the same pattern as the endogenous gene expression patterns, then this would support the continuum hypothesis of development of the same brain subdivisions around the ventricle folds. However, if fluorescent labeled cells are seen to only develop locally and not migrate into the region with shared gene expression markers above or below the ventricle, then this would support the independent origins or transdifferentiation hypothesis.

Contrast to the common Cartesian-defined model of developmental brain organization

Our model differs from some past developmental models in important ways. Vertebrate telencephalic gene expression profiles and cellular fate mapping has most often been examined in the coronal view, where the pallium has been categorized into four perpendicular Cartesian sectors: dorsal, ventral, lateral, and medial pallium with the center of the coordinates in...
the middle of the ventricle (Puelles, 2001, 2011). These four Cartesian sectors have been equated with the adult brain subdivisions, usually with the center of the coordinates at the level of the LMI lamina (Fig. 21A,C), but with differences among studies. One of the latest views is that the embryonic Cartesian-defined avian dorsal pallium gives rise to the hyperpallium (hyperpallium and dorsal mesopallium of this study), the ventral pallium gives rise to the nidopallium and arcopallium, the lateral pallium to the mesopallium (ventral mesopallium of this study), and the medial pallium to the hippocampus (Medina and Abel­lan, 2009). However, this model is not consistent with the topographic gene expression relationships we discovered in the developing and adult pallium. For example, the mesopallium marker \textit{FOXP1} forms at the dorsalmost edge of the medial half of the lateral pallium first before it expands further laterally above the ventricle and ventrally below it. The hyperpallium–nidopallium marker \textit{PPAPDC1A} first forms in the ventral pallium above the striatum but in a lateral location, mixes with the invading mesopallium region around an anterior fold in the ventricle, and then seg­regates dorsally above the ventricle. By adulthood, the hyperpallium, nidopallium, dorsal mesopallium, and ventral mesopallium subdivisions all extend from the lateral surface of the ventricle to the lateral edge of the telencephalon, with no one subdivision more medial or lateral to each other in the Cartesian coordinate system depending on anterior–posterior location (Fig. 21B,D). That is, the mesopallium topographically cannot be called lateral pallium relative to other pallial subdivisions because it is not more lateral to them. Rather, it so far appears that the nidopal­lium+hyperpallium develops more laterally to the mes­opallium (Fig. 21B). Finally, neither the “dorsal, ventral, lateral, and medial pallium” model nor its predecessor, the “dorsal pallium and DVR” model, can explain our observation of dorsal–ventral continuities around the ventricle in the developing avian telencephalon. Instead, our findings suggest that avian telencephalic organization is more contorted and continuous (Fig. 21C,D). We believe that the more complex, but visible organization may have been easily missed in past studies for several reasons: viewing brain sections mainly in the coronal plane where some

\textbf{Figure 17.} Pallial subdivision defining genes at P6. \textbf{A:} \textit{ER81} at P6 is expressed in the hippocampus and arcopallium, but scattered labeled cells are found in the pallidum, striatum, and non-mesopallium parts of the pallium, approaching the pattern found in adulthood. \textbf{B:} \textit{COUP-TF2} expression in the nidopallium, arcopallium, and hippocampus. \textbf{C:} \textit{FOXP1} expression with now clearly defined dorsal and ventral mesopallium label around the LMI lamina and ventricle, separate from the hyperpallium. White arrow, continuity between dorsal and ventral mesopallium. \textbf{D:} \textit{PPAPDC1A} starts to show more enriched expression in anterior parts of the hyperpallium and nidopallium. For abbrevia­tions, see list. Scale bar = 1 mm in A–D.
Figure 18. Time series expression of subpallial enriched genes. A: DLX6 expression showing pallidal (white arrowhead) and striatal (white arrow) locations from ED4 to adulthood, with reduced expression in the adult pallidum. DLX6 also has some expression in caudal nidopallium (yellow arrow) and the thalamus from ED6 to P1, but is reduced in these regions in adults. B: D1B expression at high levels mainly in the striatum from ED8 onward. C: LHX8 expression at high levels in the pallidum (white arrowhead) and striatum from ED4 to P1 (C1–7), and then downregulated in the striatum of adults. D: DLX1 shows a migrating pattern of expression from the primordial pallidum at ED4 (D1), to the striatum, then nidopallium and arcopallium, and then finally enrichment in the hippocampus in adulthood (D7). E: NKX2.1 shows a migrating, spreading pattern from the primordial pallidum at ED4 (E1, white arrowhead), to the striatum (white arrow), to ventral pallial regions, and finally to dorsal pallial regions in adults, except for the mesopallium. F: OTX2 shows high levels in the brainstem from ED4 (F1) to adulthood (F7), demarcating the boundary between the telencephalon and thalamus (white arrows). For abbreviations, see list. Scale bar = 500 μm; 2 mm in adult panels.
of the anterior-to-posterior continuities cannot be found in single sections; not having a sufficient number of gene expression markers for each brain subdivision; and/or viewing the position of the ventricle as an absolute border where cells on one side were not considered directly related to cells on the other side.
Interpretations relative to homologies with mammals

Our findings impact the two major competing hypotheses of avian and mammalian pallium homologies (Medina and Reiner, 2000; Jarvis et al., 2005; Medina and Abellan, 2009; Wang et al., 2010; Butler et al., 2011; Puelles, 2011): 1) the nuclear-to-layered hypothesis, which claims that the avian pallial regions ventral to the LMI lamina (old LFS) are homologous to different mammalian cortical layers (Fig. 22A vs. 22B; red, green,
yellow, orange); and 2) the claustrum–amygdala hypothesis, which instead claims that avian pallial regions ventral to the LMI lamina (old LFS) are homologous to the mammalian claustrum and amygdala (Fig. 22A vs. 22C; red, green, yellow; Jarvis et al., 2013). Both hypotheses claim that the subdivisions above the LMI lamina are homologous to the mammalian cortex. However, in order for either of these hypotheses to be true in the context of our findings, all of the avian 1\textsuperscript{o}–4\textsuperscript{o} pallial regions above and below the LMI will have to be homologous to either the six-layered cortex or the claustrum/amygdala. The nuclear-to-layered hypothesis does satisfy this requirement, but only partly, as the homologies with specific cortical layers in the dorsal pallium above the LMI need to be further specified with those below the LMI. To specify one possibility, our designated 1\textsuperscript{o}-pallium would be homologous to mammalian cortex layer IV neurons, the 2\textsuperscript{o}-pallium to layer III, the 3\textsuperscript{o}-pallium to higher order layer II and III neurons, and 4\textsuperscript{o}-pallium to layer V and VI neurons (Fig. 22B; Jarvis et al., 2013). Consistent with this modified nuclear-to-layered hypothesis is that the avian pallial sequential development we found thus far overlaps with the order of the mammalian cortical development from deeper to upper layers, in the order of layers VI, V, IV, III, and II (Molyneaux et al., 2007; Boyle et al., 2011). Our timing findings are also consistent with overall pallial development in mammals, which occurs sequentially from ventral to dorsal maturation of regions (Bayer and Altman, 1991). The major difference is that in mammals layer IV neurons form before layers III and II, and the markers of avian 1\textsuperscript{o}-pallium that we studied appear either simultaneously or soon after the 3\textsuperscript{o}-pallium markers. However, more markers and finer time course analyses are necessary to validate this difference. An issue with this model is that there is nothing yet that distinguishes relationships with the claustrum and amygdala separate from the cortex homologies.

In order for the claustrum–amygdala hypothesis to hold in the context of our findings, the mammalian claustrum and amygdala would have to be considered extensions of specific layers of the cortex. Here, the
avian pallial regions dorsal to the LMI would be homologous to different layers of the cortex as described above, but the regions ventral to the LMI would be continuous extensions homologous to the claustrum and amygdala. Consistent with this idea, the pallial amygdala has been proposed to be an extension of cortex layers V and VI (Swanson, 2000). The developing claustrum and mammalian upper cortical layer neurons both express FOXP1 and EMX1 (Smith-Fernandez et al., 1998; Puelles et al., 2000; Aboitiz, 2011; Boyle et al., 2011) as does the developing avian mesopallium (3°-pallium). The developing mammalian amygdala and different layers of the cortex express LMO4, LHX9, and COUP-TF2 (Allen Brain Institute Mouse Atlas; http://mouse.brain-map.org), genes originally used to claim that the amygdala is homologous to the avian arcopallium (Yamamoto et al., 2005; Molnar and Cheung, 2006; Watakabe et al., 2007; Garcia-Lopez et al., 2008; Butler et al., 2011; Puelles, 2011; Dugas-Ford et al., 2012; Tang et al., 2012). Difficulties with this idea are that the amygdala has also been found to develop both from cells that migrate from the six-layered cortex and from nonlayered cortical regions (Deussing and Wurst, 2007; Remedios et al., 2007; Soma et al., 2009), and the avian mesopallium appears to begin in the dorsal pallium, whereas the nidopallium–hyperpallium cells appear to begin in the ventral pallium.

We also found that the adult layer V marker, ER81, was not anatomically consistent through all developmental stages in birds, but spreads from two separate zones, the subpallium to the pallium and the dorsal pallium to other parts of the pallium. This finding indicates that the ER81-expressing cells possibly migrate tangentially, as has been suggested previously for mammals (Teissier et al., 2010). It is not clear whether layer V ER81 cells migrate into their cortical regions by a mechanism separate from radial migration, an idea that would contradict the dominant inside-out pattern of excitatory neuron development away from the ventricle. However, we note that ER81 is also expressed at comparably high levels in the basolateral amygdala (Allen Brain Institute Mouse Atlas), and amygdala development does include tangential migration of cells (Deussing

Figure 22. Avian cerebral organization and its mammalian homologies according to different hypotheses. A: Color-coded scheme of the avian brain in our organization model. B: Color-coded scheme of the rodent brain according to the nuclear-to-layered hypothesis of homology with the avian brain. C: Color-coded scheme of the rodent brain according to the nuclear-to-claustrum/amygda hypothesis of homology with the avian brain. D: Color-coded scheme of the rodent brain according to a field hypothesis of homology with the avian brain proposed in this study. For abbreviations, see list.
and Wurst, 2007; Remedios et al., 2007; Soma et al., 2009). We were also surprised to find that the gene expression profiles of the arcopallium were most closely related to the avian hippocampus, also around a fold in the ventricle. Like the mammalian hippocampus, the avian hippocampus contains multiple, segregated cell types, but more nuclear-like in organization than the layered formation in mammals (Atoji and Wild, 2006). Finally, we believe that a sufficient set of gene expression markers has not yet been identified that distinguish the claustrum and amygdala from the six-layered cortex, and that have been analyzed also in the avian brain.

At present, until further evidence can support either hypothesis or the modifications mentioned above, we believe that our adult (Jarvis et al., 2013) and developmental (this study) data on the avian brain compared with expression profiles in the mammalian brain are best explained in the context of a field homology hypothesis (Fig. 22A vs. 22D; Butler and Molnar, 2002; Butler et al., 2002; Jarvis et al., 2013). In this hypothesis, the 1<sup>st</sup>-4<sup>th</sup> cells in the avian pallium would be homologous as a field to cells in the mammalian cortex, claustrum, and amygdala. The avian arcopallium (4<sup>th</sup>-pal- lium) would be homologous to both the mammalian cortex layer V and pallial amygdala; the avian mesopallium (3<sup>rd</sup>-pallium) would be homologous to both the mammalian cortex layers I–II and the claustrum. Testing this hypothesis would require further resolution of cell-type relationships first within mammalian and avian brains, and then between mammalian and avian brains.

In summary, our findings suggest that a new model is necessary for explaining avian brain organization, evolution, and homologies with other vertebrates. We have attempted to propose such a model that is consistent with our current findings and the previously published data. We caution that the details of this model will probably require revision and fine tuning as more expression data are gathered and other types of analyses and more functional experiments are performed. However, we believe that the fundamental principles are unlikely to change. We hope that this study can serve to germinate novel ideas and approaches to further decipher the general principles of our understanding of comparative vertebrate brain organization.

ACKNOWLEDGMENTS

We thank Tony Reiner, Harvey Karten, Ann Butler, Loreta Medina, Luis Puelles, Martin Wild, Toru Shimizu, and Claudio Mello for their comments and critical discussions.

CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest in this work.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. CCC and EDJ performed analyses and quantifications and wrote the paper. CCC and CMW performed experiments and quantified images; ARP and CCC performed the computational analyses.

LITERATURE CITED


Suzuki IK, Kawasaki T, Gogobori T, Hirata T. 2012. The temporal sequence of the mammalian neocortical neurogenic


