doi:10.1111/j.1558-5646.2010.01126.x

# SPECIES DIFFERENCES IN EARLY PATTERNING OF THE AVIAN BRAIN

**Luke McGowan,[1,2] Eric Kuo,[3] Arnaud Martin,[4] Edwin S. Monuki,[5] and Georg Striedter[1,4]**

[1]*Department of Neurobiology and Behavior, University of California, Irvine (UCI), California 92697*

[2]*E-mail: lukemcgowan1@yahoo.com*

[3]*Biomedical Engineering, UCI, California 92697*

[4]*Department of Ecology and Evolutionary Biology, UCI, California 92697*

[5]*Pathology and Laboratory Medicine, Developmental and Cell Biology, UCI, California 92697*

Received May 26, 2010
Accepted August 24, 2010

**The telencephalon is proportionately larger in parrots than in galliformes (chicken-like birds), whereas the midbrain tectum is proportionately smaller. We here test the hypothesis that the adult species difference in midbrain proportion is due to an evolutionary change in early brain patterning. In particular, we compare the size of the early embryonic midbrain between parakeets (*Melopsittacus undulatus*) and bobwhite quail (*Colinus virgianus*) by examining the expression domains of transcription factors Pax6 and Gbx2, which are expressed in the forebrain and hindbrain, respectively. Because these expression domains form rostral and caudal borders with the presumptive midbrain when this region is specified (Hamburger-Hamilton stages 9–11), they allow us to measure and compare the sizes of a molecularly defined presumptive midbrain in the two species. Based on published data from older embryos, we predicted that the molecularly defined midbrain territory is significantly larger in quail than parakeets. Indeed, our data show that normalized midbrain length is 33% greater in quail and that the midbrain to forebrain ratio is 28% greater. This is strong evidence of a significant species difference in early brain patterning.**

**KEY WORDS:** **Aves, brain evolution, brain patterning, evo–devo, midbrain.**

With the rise of evo–devo biology, it has become widely accepted that species differences in adult phenotypes are due to evolutionary changes in development (Carroll 2005). However, the evo–devo approach is just beginning to be applied to species differences in brains. Therefore, it is still largely unclear what kinds of changes in development account for evolutionary changes in brain structure and function. Previous work in our laboratory and a handful of others has shown that many species differences in adult brain region proportions are due to differences in cell cycle rates and/or the timing of neurogenesis (Finlay et al. 2001; Charvet and Striedter 2008). However, alterations of early brain patterning as a mechanism of brain evolution have scarcely been explored. The only documented example of a change in brain morphology resulting from a change in early patterning focused on forebrain evolution in cichlid fish (Sylvester et al. 2010).

We here provide evidence for another instance of an evolutionary change in early brain patterning, this time involving the midbrain of birds. Specifically, we compare the amount of early embryonic brain tissue that is allocated to the developing midbrain in parakeets and bobwhite quail, two species that have similar overall adult brain volumes but vary in the size of their optic tectum, which is by far the largest midbrain component in birds (Striedter and Charvet 2008). Published morphometric data from our laboratory show that growth curves for the optic tectum in parakeets and bobwhite quail run roughly parallel all the way back to the time when tectal morphology first becomes apparent (Striedter and Charvet 2008). Backward extrapolation from these data suggested that the parakeet's tectum is smaller than the quail's from the outset—that is, from the time of initial tectal territory allocation or regionalization.

To test this hypothesis, we examine the expression domains of *Pax6* and *Gbx2*, which form sharp rostral and caudal borders with the presumptive midbrain at Hamburger Hamilton stages 9–11, when the tectum is specified (Nakamura 2001a,b). Using this molecular definition, we find that the early presumptive midbrain is significantly larger in bobwhite quail than parakeets.

## Materials and Methods

Fertile eggs of Northern bobwhite quail (*Colinus virginianus*) were obtained from a commercial supplier. Parakeet eggs (*Melopsittacus undulatus*) were obtained from breeding pairs maintained at the University of California, Irvine. All experimental procedures were approved by the local Institutional Animal Care and Use Committee.

### CHOICE OF GENES AND ANATOMICAL RATIONALE

The molecular mechanisms of midbrain specification have been studied in considerable detail (Watanabe et al. 2000; Nakamura 2001a,b). For our purposes, the most useful genes are those expressed at the time of midbrain specification and with sharp boundaries. These criteria are met by *Pax6*, which is expressed quite homogeneously in the forebrain down to the forebrain-midbrain boundary (FMB) at the stages we are examining (Walther and Gruss 1991; Kawakami et al. 1997), and by *Gbx2*, which is expressed in the hindbrain up to the midbrain-hindbrain boundary (MHB) (Katahira et al. 2000; Hidalgo-Sanchez et al. 2005). By labeling embryos with separate probes against both genes, we can identify the midbrain by exclusion (Fig. 1A). Of course, the optic tectum is just part of the midbrain, which also contains the tectal gray, the torus semicircularis, a preisthmic domain, and the basal (ventral) midbrain (Diaz et al. 2000; Hidalgo-Sanchez et al. 2005). However, the optic tectum is by far the largest component of the midbrain and our previous observations have not shown the torus to be reduced in species with a large midbrain. Therefore, if species differences in tectum size are due to a change in embryonic patterning, then this is likely to be associated with a species difference in the size of the entire presumptive midbrain.

### PROBE CONSTRUCTION AND HYBRIDIZATION

*Colinus virginianus* (*Cv*) and *M. undulatus* (*Mu*) first-strand cDNA pools were synthesized from Hamburger-Hamilton (HH) stage 12 embryonic total RNA using M-MLV reverse transcriptase (Promega®). Partial *Cv_Pax6*, *Mu_Pax6, Cv_Gbx2 and Mu_Gbx2* cDNAs were amplified using a Touchdown PCR protocol and primers designed in conserved regions (*Aves_Pax6_F* 5′-GAAGCAAGGATACAGGTGTGG-3′; *Aves_Pax6_R* 5′-AAATGAGACCTGTGGAAGTGGT-3′; *Aves_Gbx2_F* 5′-TTCACCAGCGAGCAGCTGCTG-3′; *Aves_Gbx2_R* 5′- TGCTCCAGCTGCTGGTGCTG-3′). cDNA fragments were cloned in the pCRII vector (InVitrogen®) and sequenced on both strands. Genbank accession numbers are *Cv_Pax6* (HM014447), *Mu_Pax6* (HM014448), *Cv_Gbx2* (HM014449), and *Mu_Gbx2* (HM014450). Chicken *Pax6* clone was a kind gift from J. Rubenstein (Puelles et al. 2000). These cDNAs were then linearized and used for DIG-labeled riboprobe synthesis (Roche AS®).

Parakeet and quail embryos were collected between HH stages 9 and 11 and fixed by immersion in 4% paraformaldehyde in PBS at 4°C overnight. After fixation, they were dehydrated and stored at −20°C in 100% methanol until use. For whole mount

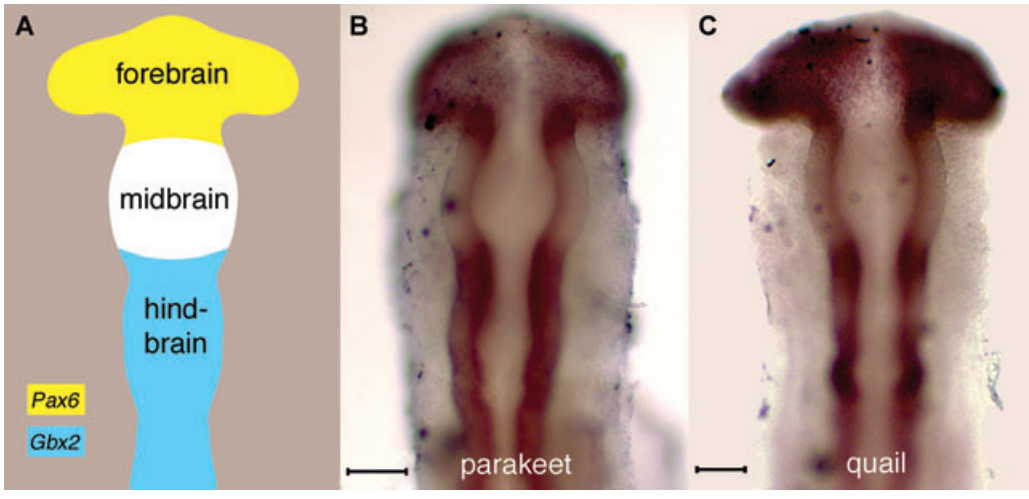


**Figure 1.** Expression patterns of *Pax6* and *Gbx2*. (A) In chickens at HH stages 9–11, around the time of brain regionalization, the expression territories of *Pax6* and *Gbx2* are known to form sharp boundaries with the midbrain (Watanabe et al. 2000; Nakamura 2001a,b). (B and C) *Pax6* and *Gbx2 d*ouble whole-mount in situ hybridization for (A) a parakeet at HH 10 and (B) a bobwhite at HH 10.5. Both specimens reveal the midbrain territory as an unstained region. Scale bars = 100 μm.

in situ hybridization, we followed a slightly modified version of Wilkinson (1993). Parakeet embryos were hybridized with riboprobes made from our parakeet *Pax6* and *Gbx2* clones. Quail embryos were hybridized with riboprobe made from our quail *Gbx2* and with the probe against chicken *Pax6*.

### MEASUREMENTS AND ANALYSIS

The whole mount double in situ embryos were photographed from a dorsal perspective using a digital color camera (Spot Insight; Diagnostics Instruments, Sterling Heights, MI) attached to an Olympus BH-2 microscope (Olympus, Center Valley, PA). Although our double in situ hybridizations resulted in clearly visible *Pax6* and *Gbx2* expression territory borders (Fig. 1), it is difficult to delineate the midbrain boundaries objectively. To overcome this problem, we converted our images to grayscale and used Metamorph software (Molecular Devices, Sunnyvale, CA) to obtain pixel intensity profiles along line scans spanning the putative border location in the inner and outer regions of the neural tube (Fig. 2A). We then drew definitive borders by connecting the points along those transects where pixel intensity dropped off most steeply (Figs. 2B, C). The person drawing the borders was blind with respect to the species being examined. With the border determinations in hand, we measured the length of the midbrain from the MHB along the rostral-caudal axis up to the FMB (Fig. 2D, length $m$), the diameter of the midbrain at the point of its greatest extent (Fig. 2D, length $d$), the length of the forebrain from FMB to the forebrain's rostral edge (Fig. 2D, length $f$), and the thickness of the neural tube wall (Fig. 2C, length $w$). Lateral views were obtained for some embryos to verify that dorsoventral curvature of the midbrain at the studied stages is minimal (Fig. 2E).

The obtained measurements were compared across species using Student's $t$-tests. Analysis of variance (ANOVA) tests were used to explore whether any of the measurements varied significantly with embryonic stage. All statistical analyses were performed in JMP 8 (SAS, Cary, NC).

## Results

Because our embryos covered a small range of stages (HH 9–11; in accord with Hamburger and Hamilton 1951), we first explored whether any of our measurements were significantly correlated with stage. Because no significant trends were found, all embryos from a given species (10 parakeets and 10 quail) were pooled in our comparisons.

A second concern was that quail embryos are generally larger than parakeet embryos at the same stage of development. Therefore, a species difference in absolute midbrain size need not represent a species difference in proportional midbrain size, which

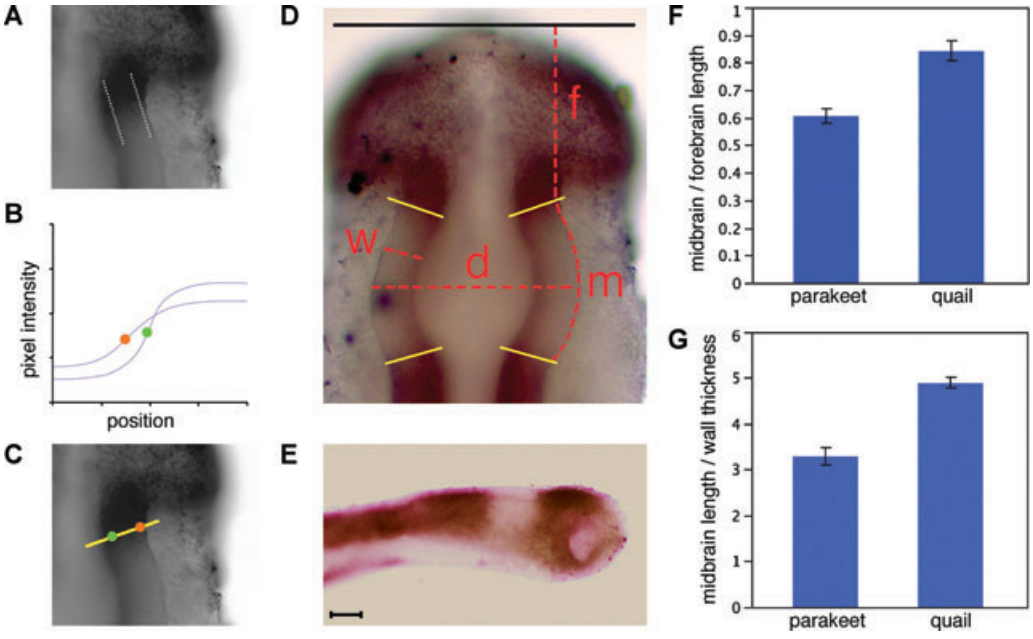


**Figure 2.** Measurements and species differences. (A) Line scans across the midbrain borders were used to analyze changes in pixel intensity. (B) The locations of the steepest changes in pixel intensity are indicated with orange and green points. (C) The orange and green points were connected to delineate the midbrain borders (yellow lines). (D) The following length measurements were made: $f$ = length of forebrain; $m$ = length of midbrain; $w$ = thickness of neural tube wall; $d$ = midbrain diameter. (E) A lateral view of an HH 10 parakeet shows the lack of midbrain curvature. (F) The midbrain–forebrain ratio is 28% greater in quail than parakeet (±SEM included; $t(18) = 5.12$; $P < 0.0001$; $n = 20$). (G) The ratio of midbrain length to wall thickness is 33% greater in quail than parakeets (±SEM included; $t(18) = 7.00$; $P < 0.0001$; $n = 20$). Scale bar = 100 μm.

is what we are interested in. Unfortunately, we could not assess proportional midbrain size directly because our preparations did not permit the drawing of reliable boundaries between brain and spinal cord. Nor did they allow for volume estimates.

Therefore, we developed two alternate approaches to estimating proportional midbrain size. In one approach, we examined the ratio of midbrain length to forebrain length. This ratio was 28% greater in quail than parakeet ($t(18) = 5.12$; $P < 0.0001$; $n = 20$) (Fig. 2F). In the other approach, we divided absolute midbrain length and diameter, as well as forebrain length, by the thickness of the neural tube wall (Fig. 2D, line w), which is slightly larger in quail than in the parakeets. The resultant ratio of midbrain length to wall thickness was 33% greater in quail than parakeets ($t(18) = 7.00$; $P < 0.0001$; $n = 20$) (Fig. 2G). As expected, no significant species differences were found in either normalized midbrain diameter ($t(18) = 0.22$, $P < 0.83$; $n = 20$) or normalized forebrain length ($t(18) = 1.00$; $P < 0.30$; $n = 20$). Because we found no significant species differences in forebrain length when it is normalized to wall thickness, we can conclude that the observed difference in midbrain–forebrain ratio is due to a species difference in embryonic midbrain length, rather than forebrain length.

## Discussion

Evolution has produced an enormous diversity of adult brain region sizes and proportions (Striedter 2005). Our results suggest that one way to achieve these species differences is through alterations of early brain patterning, when gene expression patterns first specify the regional fates of precursor cell populations. In particular, we observed species differences in the size of the molecularly defined presumptive midbrain territory of parakeets and quail. From the time of brain regionalization around stage HH10, the normalized length of the midbrain is already 33% greater in quail than parakeets, which is consistent with our prediction (Striedter and Charvet 2008). A parsimony analysis of presumptive tectum size in various birds, reptiles, and a monotreme has shown that the large presumptive midbrain of chicken-like birds is probably a derived condition within birds (Charvet et al. 2010). Thus, quail and their relatives probably enlarged their presumptive tectum 77–94 million years ago, when their lineage first split from other birds (Kan et al. 2010). Unfortunately, the functional correlates of this phylogenetic change remain unclear.

Because we observed no species differences in normalized midbrain diameter, the difference in midbrain territory size must be due to shifts in the position of the MHB, the FMB, or both. Our observation of a significant species difference in normalized midbrain, but not forebrain, length suggests a shift in the MHB. If the FMB had shifted, then we should have observed a species difference in normalized forebrain length. The alternative hypothesis that the midbrain is simply growing at different rates in the two species is contradicted by the absence of significant trends in length measurements across the stages we examined. Similarly, the lack of a species difference in normalized midbrain diameter suggests that the midbrain is not bulging differentially in the two species, as it probably would if the midbrain's specific growth rate varied between the species. Overall, these data are strong evidence that the observed differences in midbrain territory size are due to a difference in brain patterning and not to differences in cell cycle rates.

We do not yet know which genes are involved in generating the observed difference in midbrain territory size. However, misexpression studies in chicken and mice have shown that *Gbx2* and *Otx2* (which is expressed in both forebrain and midbrain) corepress each other, and that this repressive interaction probably positions the MHB (Millett et al. 1999). Furthermore, overexpression of *Otx2* in chickens results in a caudal shift of the MHB, whereas overexpression of *Gbx2* results in a rostral shift of the MHB (Katahira et al. 2000). Therefore, species differences in the relative expression of these genes could explain the postulated MHB shift.

Changes in patterning are just one way to alter brain development in the service of changing adult brain region proportions—changes in cell cycle rates and neurogenesis timing are two additional mechanisms (Charvet and Striedter 2008). Why would evolution alter one developmental process rather than another in any given case? Chance and phylogenetic inertia aside, the downstream correlates of the various changes are likely part of the answer. For example, changing neurogenesis timing alters the time course of neuronal maturation, including axon outgrowth and synapse formation. Such differences in timing (heterochrony) might well bias competitive interactions between developing axons and, consequently, alter circuit formation. In contrast, evolutionary changes in brain patterning (heterotopy) would not alter the timing of neuronal maturation. However, changes in brain patterning always make one region larger at the expense of another. Therefore, such changes are likely to be relatively small. For larger changes in adult brain proportions, changes in cell cycle rate and neurogenesis timing would be more effective. In sum, nature has at its disposal a multitude of developmental mechanism with which to alter brain region proportions—which mechanisms are ultimately tweaked depends on the larger developmental and functional context of the organism.

### ACKNOWLEDGMENTS

This work was supported by NSF grant IOS-0744332. We would like to thank C. Charvet and members of the UC Irvine Developmental Neurobiology Consortium for useful comments. We would like to thank J. McGowan for help with figure design.

## LITERATURE CITED


Carroll, N. 2005. Endless forms most beautiful: the new science of evo devo and the making of the animal kingdom. W. W. Norton & Company, New York, NY.

Charvet, C. J., A. L. Sandoval, and G. F. Striedter. 2010. Early alterations in brain region proportions. Brain Behav. Evol. 75:104–110.

Charvet, C. J., and G. F. Striedter. 2008. Developmental species differences in brain cell cycle rates between northern bobwhite quail and parakeets. Brain Behav. Evol. 72:295–306.

Diaz, C., C. Yanes, C. Trujillo, and L. Puelles. 2000. Cytoarchitectonic subdivision in the subtectal midbrain of the lizard Gallotia galloti. J. Neurocytol. 29:569–593.

Finlay, B. L., R. B. Darlington, and N. Nicastro. 2001. Developmental structure in brain evolution. Behav. Brain Sci. 24:263–308.

Hamburger, V., and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88:231–272.

Hidalgo-Sanchez, M., S. Millet, E. Bloch-Gallego, and R. M. Alvarado-Mallart. 2005. Specification of the meso-isthmo-cerebellar region: the *Otx2/Gbx2* boundary. Brain Res. Rev. 49:134–149.

Kan, X., Xi-Feng Li, Zu-Pei Lei, L. Chen, H. Gao, Z. Yang, J. Yang, L. Yu, L. Zhang, and C. Qian. 2010. Estimation of divergence times for major lineages of galliform birds: evidence from complete mitochondrial genome sequences. Afr. J. Biotechnol. 9:3073–3078.

Katahira, T., T. Sato, S. Sugiyama, T. Okafuji, I. Araki, J. Funahashi, and H. Nakamura. 2000. Interaction between Otx2 and Gbx2 defines the organizing center for the optic tectum. Mech. Dev. 91:43–52.

Kawakami, A., M. Kimura-Kawakami, T. Nomura, and H. Fujisawa. 1997. Distributions of PAX6 and PAX7 proteins suggest their involvement in both early and late phases of chick brain development. Mech. Dev. 66:119–130.

Nakamura, H. 2001a. Regionalization of the optic tectum: combinations of gene expression that define the tectum. Trends Neurosci. 24:32–39.

———. 2001b. Regionalization and acquisition of polarity in the optic tectum. Prog. Neurobiol. 65:473–488.

Millett, S., K. Campbell, D. Epstein, K. Losos, E. Harris, and A. Joyner. 1999. A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer. Nature 401:161–164.

Puelles, L., E. Kuwana, E. Puelles, A. Bulfone, K. Shimamura, J. Keleher, S. Smiga, and J. L. Rubenstein. 2000. Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes Dlx-2, Emx-1, Nkx-2.1, Pax-6, and Tbr-1. J. Comp. Neurol. 424:409–438.

Striedter, G. F. 2005. Principles of brain evolution. Sinauer Assoc, Sunderland, MA.

Striedter, G. F., and C. J. Charvet. 2008. Developmental origins of species differences in telencephalon and tectum size: morphometric comparisons between a parakeet and a quail. J. Comp. Neurol. 507:1663–1675.

Sylvester, J. B., C. A. Rich, H. Y. Loh, M. J. van Staaden, G. J. Fraser, and J. T. Streelman. 2010. Brain diversity evolves via differences in patterning. Proc. Natl. Acad. Sci. USA 107:9718–9723.

Walther, C., and V. Gruss. 1991. Pax-6, a murine pair-box gene, is expressed in the developing CNS. Development 113:1435–1449.

Watanabe, Y., T. Katahira, T. Sato, and H. Nakamura. 2000. Molecular mechanisms for defining tectum territory in chick embryogenesis. Acta Histochem. Cytoc. 33:1–4.

Wilkinson, D. G. 1993. In situ hybridization. Pp. 256–274 *in* C. D. Stern and P. W. H. Holland, eds. Essential developmental biology: a practical approach. IRL Press at Oxford Univ. Press, Oxford.


Associate Editor: A. Monteiro