

# Brain diversity evolves via differences in patterning

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Differences in brain region size among species are thought to arise late in development via adaptive control over neurogenesis, as cells of previously patterned compartments proliferate, die, and/or differentiate into neurons. Here we investigate comparative brain development in ecologically distinct cichlid fishes from Lake Malawi and demonstrate that brains vary among recently evolved lineages because of early patterning. Divergence among rock-dwellers and sand-dwellers in the relative size of the telencephalon versus the thalamus is correlated with gene expression variation in a regulatory circuit (composed of *six3*, *fezf2*, *shh*, *irx1b*, and *wnt1*) known from model organisms to specify anterior-posterior (AP) brain polarity and position the *shh*-positive signaling boundary *zona limitans intrathalamica* (ZLI) in the forebrain. To confirm that changes in this co-expression network are sufficient to produce the differences we observe, we manipulated WNT signaling *in vivo* by treating rock-dwelling cichlid embryos with temporally precise doses of LiCl. Chemically treated rock-dwellers develop gene expression patterns, ZLIs, and forebrains distinct from controls and untreated conspecifics, but strongly resembling those of sand-dwellers. Notably, endemic Malawi rock- and sand-dwelling lineages are alternately fixed for an SNP in *irx1b*, a mediator of WNT signaling required for proper thalamus and ZLI. Together, these natural experiments in neuroanatomy, development, and genomics suggest that evolutionary changes in AP patterning establish ecologically relevant differences in the elaboration of cichlid forebrain compartments. In general, variation in developmental patterning might lay the foundations on which neurogenesis erects diverse brain architectures.

cichlid | development | evolution | forebrain | WNT signaling

Arguably the most-studied vertebrate organ, the brain has played an important role in the evolution of our own species. Modifications of brain structure are responsible for novel behaviors that galvanized evolutionary radiation of the major vertebrate groups (1). Following decades of research in model organisms, we now know a great deal about how the process of development makes a brain (2). We know much less about evolutionary mechanisms of brain diversification.

The brain develops under the iterative influence of antagonistic anterior and posterior signaling molecules, inductive and repressive transcription factors that receive those signals, and lineage restriction boundaries that define compartments (2, 3). Just after gastrulation, the initial anterior-posterior (AP) polarity of the brain is established by a tug-of-war between posteriorizing signals (e.g., *wnt1*) secreted from the midbrain–hindbrain boundary (MHB) and WNT antagonists (e.g., *six3*, *tlc*) expressed from the anterior neural ridge (ANR). The MHB develops to demarcate the hindbrain from the fore- plus midbrain (Fig. S1). With the subsequent formation of the diencephalon–midbrain boundary and the *zona limitans intrathalamica* (ZLI), the forebrain and midbrain begin to follow separate paths of development.

These initial boundaries and signaling centers (i.e., ANR, MHB, ZLI) continue to direct additional patterning and morphogenesis within the three major brain regions. For example, the forebrain differentiates into rostral (e.g., telencephalon, hypothalamus) and caudal (e.g., thalamus) domains, mediated in part by the ZLI (4). As brain compartments are patterned, proliferating cells

within each compartment undergo neurogenesis, maturing and differentiating into functional neurons. Because brain patterning demarcates one region from another, specific compartments may initiate, prolong, and/or terminate neurogenesis independently (1, 5, 6). Given the continuum of patterning and neurogenesis in brain development, vertebrate lineages might evolve brain diversity by (i) varying the strength or timing of signals from the ANR and/or MHB; (ii) shifting the position of early patterning boundaries; (iii) altering the timing, rate, or extent of neurogenesis; or (iv) some combination thereof. Expectations from the field of evolutionary developmental biology suggest a focus on early patterning events, because such differences prefigure the diversity of animal body plans (7), jaws (8, 9), and dentitions (10). In contrast, our understanding of the brain departs from this notion, as an extensive literature highlights the role of neurogenesis in brain diversification. Most prominently, the “late equals large” model explains how the neocortex (i.e., telencephalon) has evolved to dominate the mammalian brain, and how individual lineages (e.g., primates) have further elaborated this region by tipping the balance among neural cell proliferation, differentiation, and apoptosis (5, 6, 11, 12). Addressing the genetic and developmental mechanisms of brain diversification in nature has been difficult, however, because few systems offer the necessary combination of a wide range of brain phenotypes and tractable experimentation with embryos, against a background of genomic and developmental similarity.

## Results and Discussion

We used cichlid fishes from Lake Malawi to ask when and how brains develop diversity in recently evolved lineages. Cichlid adult brain variation is appreciable (Fig. 1A) and is correlated with ecology and behavior (13–15). For example, algal scrapers exhibit small optic lobes and large telencephala (and olfactory bulbs; Fig. 1B), whereas planktivores have enlarged optic lobes (Fig. 1D). “Sonar” hunters—species that feed by sensing vibrations—have large telencephala and cerebella. This diversity, similar to that observed across seven orders of mammals, including primates, insectivores, marsupials, cetaceans, and bats (16), has evolved rapidly. Hundreds of Lake Malawi cichlid species have diverged from a common ancestor in the last 500,000 years; their genomes are highly similar and retain ancestral polymorphism (17). Malawi cichlid brains are thus as different as those of long-diverged mammals [~150 million years (18)], but their genomes are comparable to those of any two humans.

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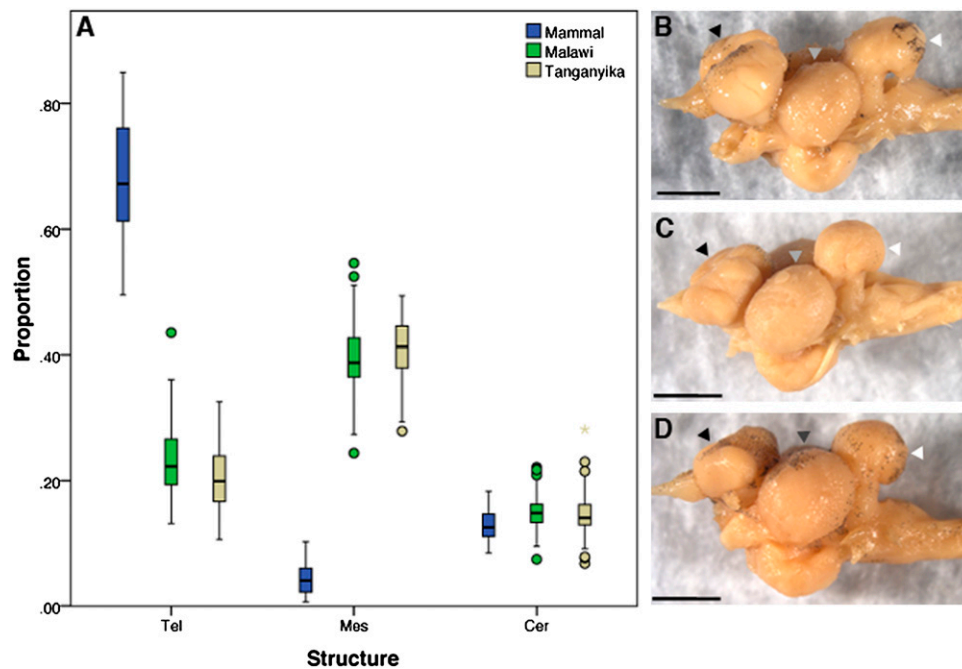
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**Fig. 1.** Cichlid brains are diverse. (A) Cerebrotype box plots of mammals (blue), Lake Malawi cichlids (green), and Lake Tanganyika cichlids (tan), grouped by brain proportions of the telencephalon (forebrain), mesencephalon (midbrain), and cerebellum (hindbrain). The heavy line in the middle of the box is the median value, the box itself is the 25–75% interval around the median, the bars are the 10–90% interval, and dots represent data points outside the 95% interval. Mammals have invested heavily in the neocortex (tel); cichlid brains are more proportional in their regional allocations. Despite an order of magnitude difference in divergence time, the range of variation in brain proportions is comparable from mammals to cichlids. Brains of rock-dwelling (mbuna) cichlids. (B) *L. fuelleborni* (LF, algivore). (C) *M. zebra* (MZ, generalist). (D) *C. afra* (CA, planktivore) in lateral view, anterior is to the left. Black arrowheads, telencephala; gray arrowheads, optic tecta; white arrowheads, cerebella. (Scale bar: 2 mm.) Note that the major midbrain structure of fishes (optic tectum) is of uncertain homology to mesencephalic derivatives in mammals.

We focused on species representing the range of ecotypes in Lake Malawi. We studied brain development of three rock-dwelling (mbuna) cichlids—*Labeotropheus fuelleborni* (LF; obligate algal scraper), *Maylandia zebra* (MZ; generalist), and *Cynotilapia afra* (CA; planktivore)—as well as three sand-dwelling nonmbuna—*Copadichromis borleyi* (CB; planktivore), *Mchenga conophorus* (MC, generalist), and *Aulonocara jacobfreibergeri* (AJ; sonar hunter). Mbuna and nonmbuna represent distinct evolutionary groups, each containing hundreds of species, with generally contrasting lifestyles, body forms, visual systems, pigment patterns, and trophic adaptations (19–21).

**Cichlid Forebrains Differ Early in Development.** By stage 16 (Fig. S1D), Lake Malawi cichlid forebrains have been partitioned into several compartments, visualized in the parasagittal section with the greatest dorsoventral extent (designated “parasagittal section” hereinafter). This is the first stage at which these regions can be reliably measured (Methods and Fig. S2). We quantified the area of forebrain compartments in replicate embryos of the mbuna LF, MZ, and CA and of the nonmbuna CB, MC, and AJ. Embryonic brains show clear divergence between rock-dwelling and sand-dwelling groups (Table 1 and Fig. S3). Rock-dwellers exhibit forebrains with relatively larger telencephala and smaller thalami, and sand-dwellers display the converse pattern. Adult rock-dwelling cichlids have larger telencephala than other habitat specialists on average, perhaps because they spend their lives navigating complex 3D habitats and/or engaging in complex social interactions (13–15). Thalami have been less well studied in fishes, but the vertebrate thalamus is a well-known “relay station” integrating sensory (particularly visual) stimuli (22). Our data demonstrate that differences in cichlid forebrains are apparent early in development, and that these differences might represent a trade-off between rostral and caudal compartments

corresponding to adult structures evolved for contrasting ecological demands.

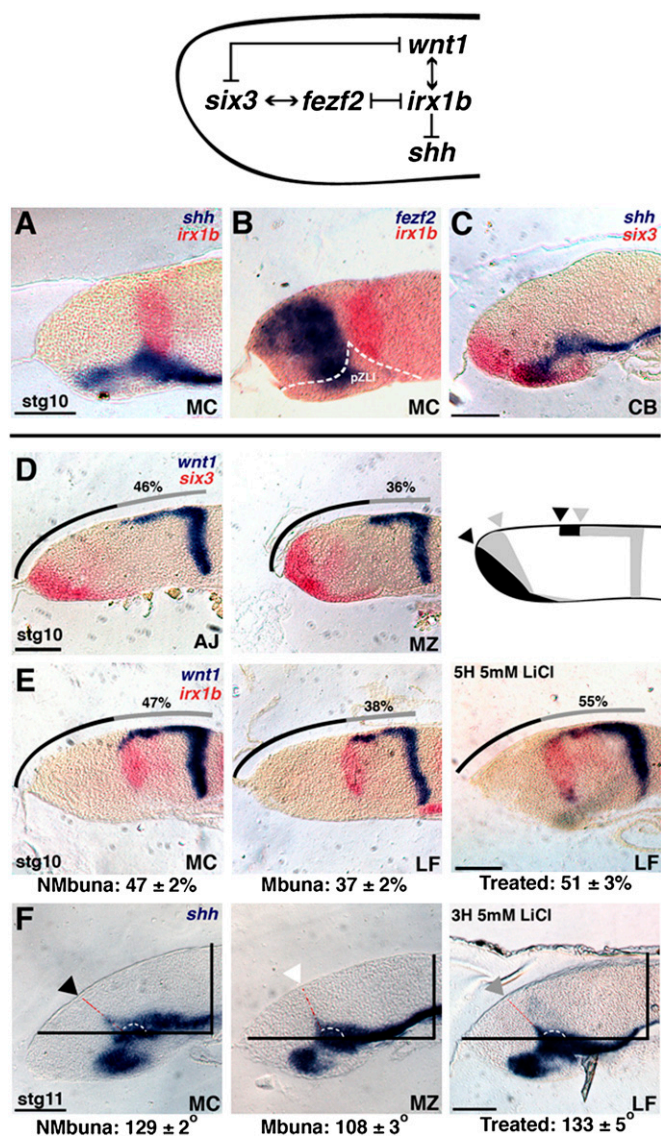
#### Variation in Forebrain Patterning Prefigures Morphological Differences.

We sought to identify the developmental signals that initiate differences in Malawi cichlid forebrains. Studies in developmental models—zebrafish, frog, and mouse—set the context for our experiments (Fig. 2; network). We focused on a gene circuit known to (i) establish anterior (e.g., telencephalon) versus posterior (e.g., thalamus) fate and (ii) position the signaling boundary ZLI within the forebrain. The transcription factor *six3* is a WNT antagonist expressed from the ANR, required for the formation of the telencephalon and ZLI (23). *wnt1* is a posteriorizing signal expressed from the MHB; knockout of *Wnt1* in mouse results in

**Table 1.** Composition of the cichlid embryonic forebrain at stage 16

	% Tel	% Thal	% Prethal	% Hypothal
MZ ( <i>n</i> = 5)	35.5 ± 0.8	18.1 ± 0.7	15.3 ± 1.4	31.1 ± 1.5
CA ( <i>n</i> = 4)	33.9 ± 2.2	19.4 ± 0.4	16.2 ± 0.5	30.5 ± 2.5
LF ( <i>n</i> = 5)	32.8 ± 0.5	20.9 ± 0.5	15.3 ± 2.1	30.9 ± 2
LF <sub>DMSO</sub> ( <i>n</i> = 3)	31.7 ± 0.6	20.6 ± 0.6	16.1 ± 1.2	31.6 ± 0.6
LF <sub>LiCl</sub> ( <i>n</i> = 5)	27.7 ± 1.3	24.7 ± 0.9	15.7 ± 0.3	31.9 ± 0.8
MC ( <i>n</i> = 5)	28.2 ± 0.7	24.9 ± 0.9	15.6 ± 1.3	30.8 ± 1.2
CB ( <i>n</i> = 5)	28 ± 0.8	27.7 ± 0.4	15.4 ± 1.5	28.8 ± 1.3
AJ ( <i>n</i> = 4)	28.4 ± 0.4	26.0 ± 0.7	15.5 ± 0.8	30.1 ± 0.8

Mbuna (LF, MZ, and CA) have larger telencephala and smaller thalami than nonmbuna (CB, MC, and AJ). Control (DMSO) and treated (LiCl) LF embryos exhibit similar proportions to mbuna and nonmbuna cichlids, respectively. The area of each compartment is expressed as a percentage ( $\pm 1$  SD) of the total forebrain area.



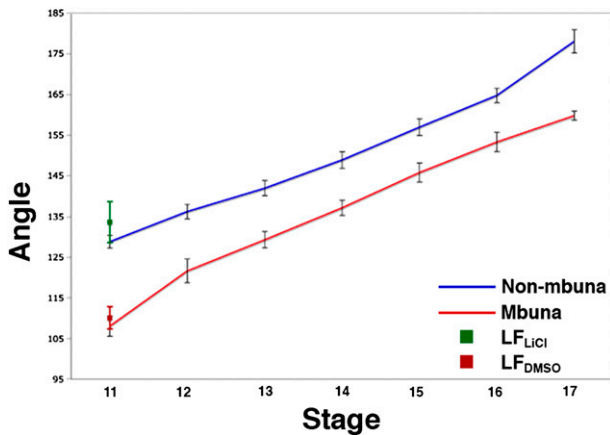
**Fig. 2.** The forebrain-patterning network differs between rock-dwellers and sand-dwellers. (A) Double in situ hybridization (ISH) of genes *shh* (blue) and *irx1b* (red). (B) Double ISH of *fezf2* (blue) and *irx1b* (red); the presumptive ZLI (pZLI) is shown by the dotted white line. (C) Double ISH of genes *shh* (blue) and *six3* (red). A and B are embryos of nonmbuna *M. conophorus* (MC); C is nonmbuna *C. borleyi* (CB); A–C are stage 10 embryos. (Scale bar: 100  $\mu$ m.) (D) From left to right, double ISH of *wnt1* (blue) and *six3* (red) in *A. jacobfreibergi* (AJ; nonmbuna), *M. zebra* (MZ, mbuna), and a schematic summarizing expression differences between nonmbuna and mbuna. Arrowheads mark the relative positions of *wnt1* and *six3* expression in mbuna (gray) and nonmbuna (black), respectively. (E) From left to right, double ISH of *wnt1* (blue) and *irx1b* (red) in MC (nonmbuna), *L. fuelleborni* (LF, mbuna), and LF treated for 5 h with 5 mM LiCl. For all panels in D and E, the line above the embryo represents the total length of the dorsal brain anterior to the MHB, and the gray portion of the line represents the rostral extent of *wnt1* expression (the *wnt1* percentage). The measured *wnt1* percentage for each embryo, in each panel, is given. Below row E, from left to right, are the average *wnt1* percentages for nonmbuna, mbuna, and LiCl-treated LF, respectively. All embryos in D and E are at stage 10; the scale bars represent 100  $\mu$ m. (F) ISH for the gene *shh* demonstrating the angle of the ZLI at stage 11. The ZLI is marked by the black arrowhead in nonmbuna (MC), the white arrowhead in mbuna (MZ), and the gray arrowhead in LiCl-treated LF. The dotted red and white lines show the ZLI angle (Methods). The values below row F show the average ZLI angles for nonmbuna, mbuna, and LiCl-treated LF, respectively. (Scale bar: 100  $\mu$ m.) All images from all panels are parasagittal sections, with anterior to the left.

a smaller thalamus, a posterior shift in the angle of the ZLI, and a larger telencephalon (23). *six3* and *wnt1* direct the activity of mutually repressive transcription factors *fezf2* and *irx1*, which in turn set the AP position of the *shh*-positive ZLI (23–25). Knockdown of *irx1* in zebrafish produces a posterior expansion of the ZLI at the expense of thalamus, along with a shortening of the *wnt1* forebrain domain (25, 26). WNT signaling might induce or be mediated by *Irx1* to specify posterior fate (26, 27). We hypothesized that differences between rock-embryonic and sand-embryonic forebrains are produced by temporal and/or spatial shifts in AP forebrain patterning.

Using two-color in situ hybridization (Methods), we observed expected gene expression patterns of *six3*, *wnt1*, *fezf2*, *irx1b*, and *shh* in the cichlid embryonic brain. The ZLI begins to form as an initial wedge of *shh* at stage 10; *fezf2* is expressed rostral to the wedge, and *irx1b* is positioned caudal to the wedge, in the presumptive thalamus (Fig. 2 A–C). The antagonists *six3* and *wnt1* are initially localized to the ANR and MHB, respectively, at the neurula stage. *wnt1* extends rostradorsally from the MHB as development proceeds, encompassing the presumptive midbrain and the caudal forebrain, where it is coexpressed with *irx1b*. At stage 10, *six3* and *wnt1* show contrasting distributions between mbuna and nonmbuna cichlids (Fig. 2D). Mbuna are characterized by a shortened *wnt1* rostradorsal domain and more caudodorsal expression of *six3*; nonmbuna exhibit the opposite pattern. Despite species-specific brain shapes, the *wnt1* rostral domain marks a greater proportion of the dorsal brain anterior to the MHB in stage 10 nonmbuna CB, MC, and AJ ( $47 \pm 2\%$ ) than in mbuna LF, MZ, and CA ( $37 \pm 2\%$ ; Student's *t* test, two-tailed;  $t = 11.78$ ;  $P < 0.0001$ ; between three and seven individuals of each species,  $n = 32$  embryos) (Fig. 2D and E).

By stage 11, the ZLI is a narrowing finger of *shh* expression within the diencephalon, forming a characteristically obtuse angle with the alar domain (Fig. 2F). We observed that the ZLI angle is greater in nonmbuna CB, MC, and AJ than in mbuna LF, MZ, and CA ( $129^\circ \pm 2^\circ$  vs.  $108^\circ \pm 3^\circ$ , Student's *t* test, two-tailed;  $t = 18.24$ ;  $P < 0.0001$ ; between two and four individuals of each species;  $n = 20$  embryos). The larger ZLI angle in nonmbuna, as measured at stage 11, matches the more rostradorsal expression of *wnt1-irx1b* and reduced *six3* domain at stage 10. We tracked the angle of the *shh*-positive ZLI from stage 11 to stage 17 (encompassing 3 days of development and the time point of forebrain compartment measurements; Table 1) in replicate embryos of the three mbuna and nonmbuna species (between two and five embryos per species per stage;  $n = 120$  embryos). In both mbuna and nonmbuna, the angle of the ZLI increases as thalamic and tectal structures grow and proliferate, yet the nonmbuna maintain a greater ZLI angle throughout (Fig. 3 and Fig. S4).

**Manipulation of WNT Signaling Mimics Natural Variation Among Cichlid Forebrains.** The brain and gene expression phenotypes that we observed to differentiate mbuna cichlids (reduced rostradorsal extent of *wnt1-irx1b*, more acute ZLI angle, smaller thalamus, larger telencephalon) from nonmbuna cichlids (greater rostradorsal extent of *wnt1-irx1b*, more obtuse ZLI angle, larger thalamus, smaller telencephalon) (Table 1 and Fig. 2) partially phenocopy zebrafish *irx1* knockdown versus control embryos (23) and *Wnt1* null versus control mice (26). Thus, we manipulated WNT signaling in vivo by treating cichlid embryos with nonlethal doses of the chemical agonist LiCl (Methods). This approach does not allow the genetic specificity of other methods, such as morpholinos, but does provide the temporal precision critical to our experiments. We bathed stage 9 embryos (during which *wnt1* expression “moves” rostradorsally from the MHB; Fig. S1) of the mbuna LF in a 5 mM solution of LiCl or a vehicle control (DMSO), for 3 or 5 h. We then washed the embryos, returned them to fish water, and cultured them until sacrifice (*i*) at stage 10 to measure the rostradorsal extent of the *wnt1-irx1b* expression domain, (*ii*) at stage 11 to measure the



**Fig. 3.** The angle of the signaling boundary ZLI impacts brain regionalization during neurogenesis and growth. The graph presents the average ZLI angle ( $\pm 1SD$ ; between two and five specimens per species per stage;  $n = 120$ ) over seven ontogenetic stages for nonmbuna CB, MC, and AJ (blue) and mbuna LF, MZ, and CA (red) (also see Fig. S4). The green and red data points at stage 11 represent LiCl-treated and DMSO control LF embryos, respectively.

angle of the *shh*-positive ZLI, or (iii) at stage 16 to measure the relative area of forebrain compartments. We predicted that up-regulation of WNT signaling would transform the treated mbuna brains of LF to resemble those of nonmbuna.

At stage 10, LiCl-treated LF ( $n = 6$ ; 3 and 5 h treatments combined) exhibited *wnt1-irx1b* expression domains that covered a greater proportion of the dorsal brain anterior to the MHB ( $51 \pm 3\%$ ) compared with DMSO controls ( $n = 4$ ,  $37 \pm 1\%$ ; Student's *t* test, two-tailed;  $t = 9.05$ ;  $P < 0.0001$ ) (Fig. 2E, Right). At stage 11, LiCl-treated LF ( $n = 4$ ; 3 and 5 h treatments combined) exhibited greater ZLI angles ( $133^\circ \pm 5^\circ$ ) than did DMSO controls ( $n = 2$ ;  $110^\circ \pm 3^\circ$ ; Student's *t* test, two-tailed;  $t = 5.96$ ;  $P = 0.004$ ) (Fig. 2F, Right). Finally, at stage 16, LiCl-treated LF ( $n = 5$ ; 3 and 5 h treatments combined) exhibited smaller telencephala ( $27.7 \pm 1.3\%$ ) and larger thalami ( $24.7 \pm 0.9\%$ ) compared with DMSO controls ( $n = 3$ ;  $31.7 \pm 0.6\%$  for telencephala and  $20.6 \pm 0.6\%$  for thalami; Table 1). The areas of prethalami and hypothalami did not differ between treatment and control embryos (Table 1). Our measurements for LF bathed in DMSO (controls) fell within the statistical distribution for untreated mbuna, and the LiCl-treated LF embryos had *wnt1-irx1b* gene expression patterns, ZLIs, and forebrains that strongly resembled those of nonmbuna (Table 1 and Figs. 2 and 3). These data demonstrate that manipulation of WNT signaling during early embryogenesis is sufficient to produce distinct cichlid forebrains, which nearly exactly mimic the natural developmental differences between rock-dwellers and sand-dwellers.

**An SNP in *irx1b* Is Alternately Fixed Between Rock-Dwellers and Sand-Dwellers.** We wanted to identify genetic differences between mbuna and nonmbuna that might contribute to variation in gene expression and brain phenotypes. We did not use the typical approach to map quantitative trait loci responsible for phenotypic variance (9, 28) because we could not effectively cross chosen rock-dweller with sand-dweller individuals. Therefore, we explored an alternative strategy. Because mbuna and nonmbuna evolutionary groups diverged recently, the genomes of individuals share polymorphism across the lineage boundary (17). Loci that are strongly genetically differentiated between lineages are statistical outliers. For instance, only 1 of 96 independent SNPs is alternately fixed in a large sample of Malawi mbuna versus nonmbuna. The SNP is a replacement change in the 3' coding se-

quence of the transcription factor *irx1b*, fixed between endemic Malawi mbuna (25 species, 140 alleles) and nonmbuna (52 species, 230 alleles) (Fig. S5 and SI Methods). This represents a signature of divergent selection against a background of shared polymorphism (17), and suggests that genetic variation in the *irx1b* cistron might play a role in the differentiation of Malawi cichlid forebrains. At this point, we are uncertain whether this substitution itself is causative or linked (physically and/or epistatically) to other causal mutations, but the central position of *irx1b* in the forebrain-patterning network (Fig. 2) implies that mbuna and nonmbuna alleles might interact differently with *fezf2*, *wnt1*, and/or *shh*. These data, coupled with the discovery that two amino acid changes between the human versus chimpanzee gene *Foxp2* can drive considerable transcriptional variation in the brain (29), make cichlid *irx1b* a prime target of future study.

**Brain Diversity by Patterning Differences.** We interpret our natural experiments in comparative neuroanatomy, development, and genomics, coupled with functional information from model organisms, to indicate that evolutionary modifications in a gene circuit composed of *six3*, *fezf2*, *irx1b*, *wnt1*, and *shh* establish two distinct modes of AP forebrain patterning in mbuna versus nonmbuna Malawi cichlids (Fig. 4). Nonmbuna embryonic forebrains are dominated by posterior signals (e.g., *wnt1*) and ultimately elaborate a posterior structure (thalamus), whereas their mbuna counterparts are under greater influence of the ANR (e.g., *six3*) and elaborate the telencephalon. Anterior and posterior signaling gradients converge on the transcription factors *fezf2* and *irx1b*, which integrate these cues to position the angle of the ZLI. This angle, once set, is important because it (i) apportions cells to the anterior versus the posterior forebrain and (ii) represents a boundary restricting further AP cross-talk between ANR and MHB. Our data highlight early patterning variation that initiates contrasting forebrain bauplans in rock-dwelling versus sand-dwelling Malawi cichlids. Species- and ecotype-specific adult brains (e.g., Fig. 1) are likely the result of this initial difference, modified throughout ontogeny by neurogenesis. Thus, the initial angle of the signaling boundary ZLI might impact subsequent forebrain patterning and neurogenesis in ecologically distinct Malawi cichlids.

We suggest a new interpretation of how organisms evolve brain diversity: Differences in the earliest signaling and patterning centers establish divergent blueprints for elaboration during growth and neurogenesis. Such comparisons are difficult to make in mammals, because deep evolutionary distances separate lineages and considerable size variation obscures brain adaptations (11, 16, 30). Our conclusion extends those from studies of cavefishes and birds (31, 32), and suggests that evolution might capture any point along the continuum of patterning and neurogenesis as neural systems diversify.

## Methods

**Cerebrotyping Analysis.** Mammalian brain data (multiple individuals of 75 species spanning seven taxonomic orders) are from the supplementary material of Clark et al. (16). The Malawi data (222 individuals from 113 species) represent a combination of published material (33) and new measurements made on preserved, wild-caught (from 2005) adult specimens for this study. The Tanganyika data (58 individuals, 53 species) are entirely from reference 33. Volumes for the Malawi and Tanganyika measurements were generated using the ellipsoid model,  $(L \times W \times H)/6$  (14,33). All cerebrotypes were calculated using the method of Clark et al. (16). Boxplots were generated using SPSS version 16.0.

**Embryo Staging.** Embryos were removed from the mouths of brooding females ~24 h after identification and, if required, maintained for further development in culture flasks at 28 °C. Embryos were observed every 8–12 h until they reached the desired stage. Stages were determined for Malawi cichlids by comparing embryonic morphology with zebrafish (34) and tilapia (35) descriptions. Identification of fish brain anatomy followed reference 36.



repeated twice, with two different LF broods. Treatment and control embryos were postprocessed (ISH, sectioning, measurements) identically to descriptions above for untreated embryos.

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