EVOLUTIONARY BIOLOGY

Divergence in problem-solving skills is associated with differential expression of glutamate receptors in wild finches

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Problem solving and innovation are key components of intelligence. We compare wild-caught individuals from two species that are close relatives of Darwin's finches, the innovative *Loxigilla barbadensis*, and its most closely related species in Barbados, the conservative *Tiaris bicolor*. We found an all-or-none difference in the problem-solving capacity of the two species. Brain RNA sequencing analyses revealed interspecific differences in genes related to neuronal and synaptic plasticity in the intrapallial neural populations (mesopallium and nidopallium), especially in the nidopallium caudolaterale, a structure functionally analogous to the mammalian prefrontal cortex. At a finer scale, we discovered robust differences in glutamate receptor expression between the species. In particular, the GRIN2B/GRIN2A ratio, known to correlate with synaptic plasticity, was higher in the innovative *L. barbadensis*. These findings suggest that divergence in avian intelligence is associated with similar neuronal mechanisms to that of mammals, including humans.

INTRODUCTION

Innovative problem solving is a key feature of intelligence and has played a major role in the evolution of both human and non-human animals. Growing evidence indicates that fundamental differences in innovative capacity and tool use are associated with the enlargement of certain brain structures, notably the cortex in primates and the intrapallial connected cell populations (also called associative pallium) in birds (*1*–*3*). Beyond these differences in brain structure, however, we know very little about the processes that control divergence in innovative problem solving at the neuronal level. In birds in particular, innovation has been well studied, but detailed neurobiological investigations explaining natural variation in innovative problem solving are still lacking (*4*, *5*). The outstanding cognitive capacities of birds, some species comparable to those of primates in terms of innovation and tool use (*6*), have been partly explained by higher neuronal numbers in the forebrain of both taxa (*7*).

Here, we compared two closely related species of birds that show extreme differences in foraging strategies despite being sympatric: the innovative Barbados bullfinch *Loxigilla barbadensis* and the conservative black-faced grassquit *Tiaris bicolor*. The two species are close relatives of Darwin's finches and belong to the family Thraupidae (Fig. 1A) (*8*), a neotropical clade that shows high rates of evolutionary diversification, colonization, and feeding innovations in the wild (*9*). In Barbados, the endemic *L. barbadensis* (Fig. 1B) has recently evolved from the Lesser Antillean bullfinch *Loxigilla noctis* (*10*) and frequently uses opportunistic, innovative feeding behaviors that take advantage of anthropogenic foods (*11*, *12*). By contrast, *T. bicolor* (Fig. 1C) is conservative, shy, and feeds on grass seed (*13*). The two species are each other's closest relative in Barbados (Fig. 1A), where they overlap in their habitat use and are both territorial.

We used a battery of cognitive tasks on wild-caught birds to show that differences in innovation in the field are matched by problem-solving abilities in captivity. We further found that the two species have different levels of expression of genes involved in synaptic plasticity in the cell population types that are the functional avian equivalent of intracortical cell layers of the mammalian cortex, more specifically the prefrontal cortex. By correlating marked behavioral differences between two closely related species with brain gene expression differences, we have identified brain regions and candidate genes potentially involved in the evolution of problem solving.

RESULTS

Laboratory tests confirm innovative differences between species

To experimentally validate that the two species studied have differences in innovative behavior, we captured adults of both species in mist nets in Barbados, housed them in aviaries, and presented them with a battery of problem-solving, learning, and boldness tests. The first test was an obstacle-removal problem designed to mimic technical innovations in the wild (fig. S1A) (*14*). Consistent with their divergence in innovativeness in the field, we found a nearly all-or-none difference in problem solving between the two species; 24 of the 29 *L. barbadensis* tested completed the obstacle-removal task in a mean of 4.4 ± 1.09 trials, but none of the 15 *T. bicolor* tested succeeded before the maximum number of 15 trials allowed (Fig. 1D). The poor performance of *T. bicolor* was not due to lack of motivation: All individuals contacted the apparatus, and the amount of time spent trying to solve it did not significantly differ between the two species (fig. S2A). To eliminate the possibility that *T. bicolor*, which is smaller than *L. barbadensis*, was physically incapable of solving the task, we trained all the unsuccessful birds of both species for up to 60 trials (for the shaping procedure, see the Supplementary Materials and fig. S2C). With our shaping, *T. bicolor* eventually solved the problem (fig. S2D), albeit in 12 times more trials than the five *L. barbadensis* that required shaping (fig. S2, B and E).

The problem-solving difference between *L. barbadensis* and *T. bicolor* was accompanied by a difference of performance in a detour-reaching task (fig. S1B). This test measures the ability to inhibit a behavior (direct

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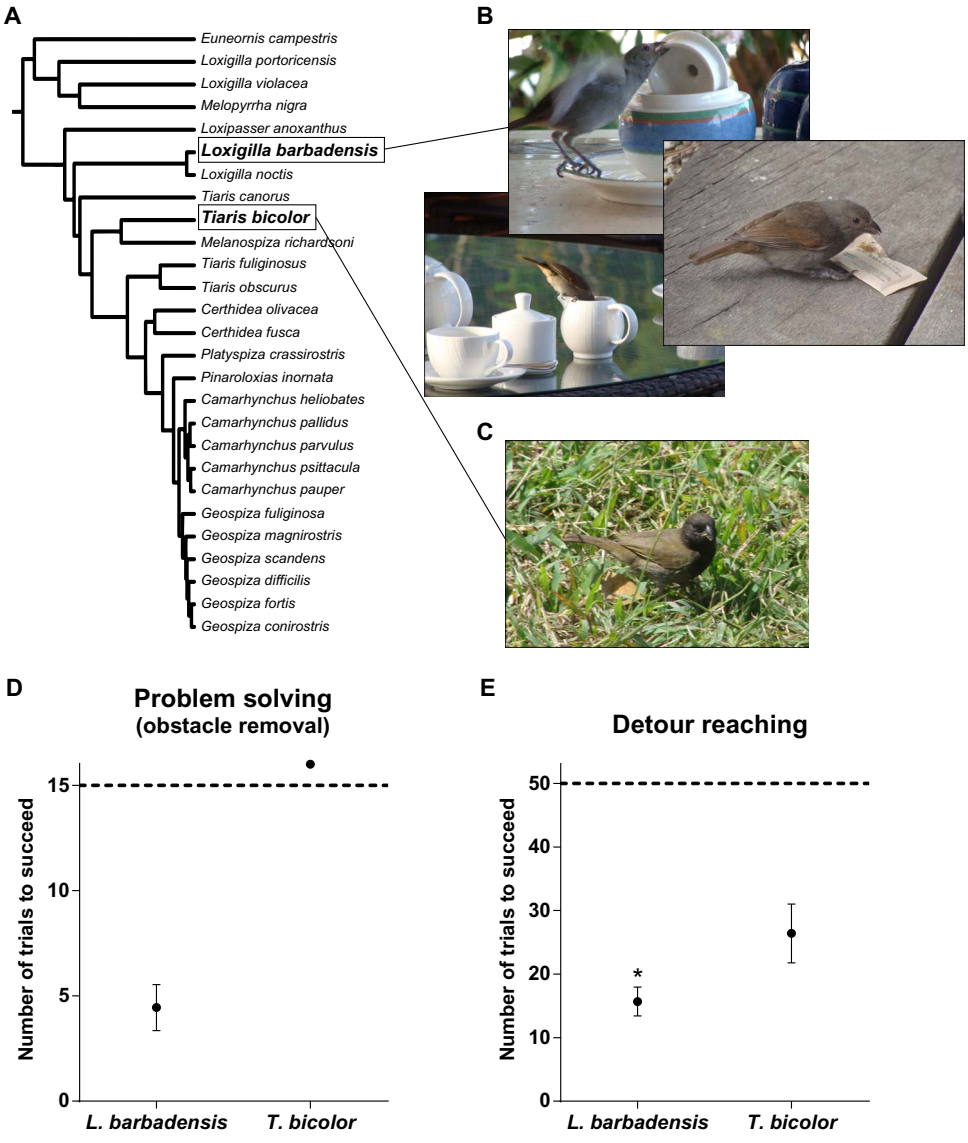

Fig. 1. Study species. (**A**) Portion of the phylogenetic tree of the Thraupidae family that includes *T. bicolor* and *L. barbadensis* (*38*). (**B**) In the wild, *L. barbadensis* is bold, opportunistic, and highly innovative, whereas (**C**) *T. bicolor* is shy, conservative, and noninnovative. (**D**) Number of trials needed to complete the obstacle removal problem. *L. barbadensis* completed the task in a mean of 4.4 ± 1.09 trials, but none of the tested *T. bicolor* solved it within the 15 allocated trials. (**E**) The number of trials to reach the success criterion in the detour reaching task was lower in *L. barbadensis* (15.7 ± 2.3 trials, $n = 29$) than in *T. bicolor* (26.4 ± 4.6 trials, $n = 15$; $^*P_{\text{Mann-Whitney}} = 0.0143$). Means ± SEM. Photo credit: S. Ducatez, McGill University.

reach) in the presence of an obstacle, forcing the individual to shift to an alternative strategy to obtain a reward (food, in our case). Again, *L. barbadensis* outperformed *T. bicolor*: They needed a lower number of trials to reach the success criterion (Fig. 1E). The two species also differed in two types of novelty responses linked to innovativeness, namely, neophobia and boldness. In independent tests, *L. barbadensis* tended to be bolder (fig. S3A) and less neophobic (fig. S3B) than *T. bicolor* (see table S1 for all significant linear models using behavioral variables).

By contrast, the two species did not differ in two tasks that involve stimulus learning (fig. S1C): color discrimination learning (fig. S3C) and reversal learning (fig. S3D). The two closely related thraupids are thus highly divergent for innovative problem solving, but they are similar in terms of habitat preference, territoriality, and stimulus learning, yielding a specific behavioral basis for brain and genetic comparisons.

The species differ the most in transcriptomes of higher forebrain pallial cell populations

In birds, species differences in innovativeness have been found to be positively associated with allometrically corrected differences in the size of specific brain subdivisions/cell populations. More specifically, variations in innovation have been associated with the size of the mesopallium and nidopallium (*1*–*3*), which have cell types analogous to layers 3 and 2, respectively, of the mammalian cortex (*2*, *15*–*19*). These two cell populations in both birds and mammals are responsible for the intrapallial and intracortical connections, respectively, and are sometimes referred to collectively as the "associative pallium" in birds (*3*, *19*). The differences in problem solving between *L. barbadensis* and *T. bicolor* were not reflected in differences in residual brain mass when plotted with other *Thraupidae* (fig. S4).

Therefore, we went to a deeper level, using molecular approaches, to examine in progressively finer detail the expression levels of genes in five forebrain cell populations: the mesopallium, the nidopallium, the motor arcopallium (tertiary pallium), the visual entopallium (a part of the primary pallium), and the hippocampus (Fig. 2A). Within the nidopallium, we separated out the nidopallium caudolaterale (NCL), a region of the nidopallium proposed to be functionally analogous to the prefrontal cortex (*20*). We dissected these pallial cell populations in ~400-µm-thick midsagittal sections throughout as much of the brain as possible, with fine dissection tools under a dissecting microscope. The mRNA was extracted, and cDNA libraries were prepared and sequenced on eight naïve (that is, different from the ones tested above) individuals per species (see the Supplementary Materials for details). The resultant ~19.2 billion RNA sequencing (RNA-seq) reads (~200 million paired-end reads per sample) were mapped to the high-resolution and well-annotated chicken (*Gallus gallus*) genome to obtain a nonbiased differential gene expression analysis. Principal components analysis (PCA) of the mean expression pattern for all genes in each region per species revealed larger differences between regions than between species (Fig. 2B). Consistent with previous findings that were obtained by analyzing the expression of

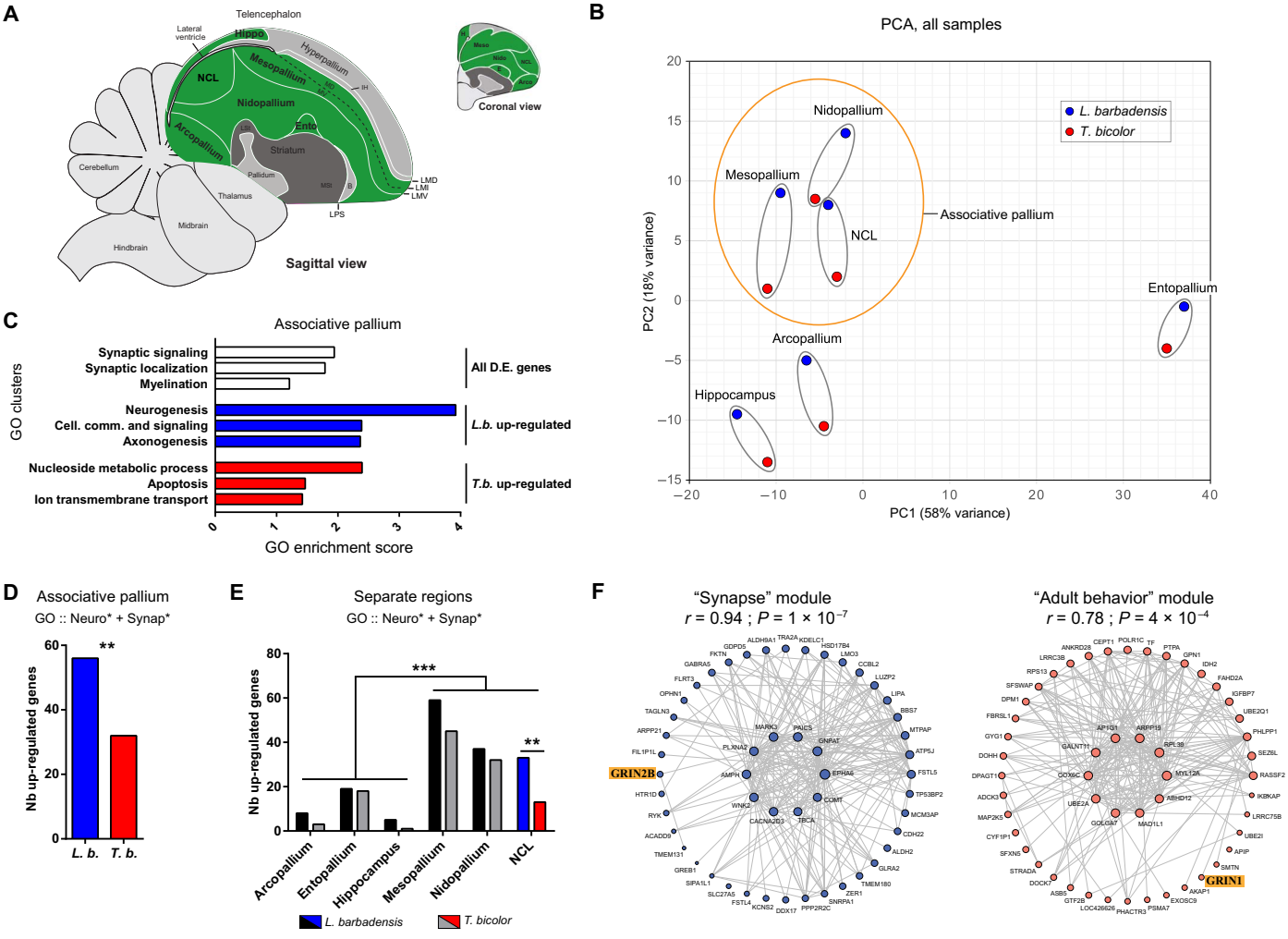

Fig. 2. RNA-seq analysis of *L. barbadensis* and *T. bicolor* transcriptomes. (**A**) Schematic view of the avian brain (*16*), with the regions that were examined in this study colored in green. (**B**) PCA of gene expression pattern per species and per region. Individual blue (*L. barbadensis*) and red (*T. bicolor*) circles include the mean of the reads from all individuals for a given species/region. The orange outline designates the regions that form the associative pallium (mesopallium and nidopallium, including NCL). (**C**) Gene ontology (GO) clustering analysis of differentially expressed genes, using, separately, the whole data set of differentially expressed genes, the genes that are up-regulated in *L. barbadensis* (*L.b.*), or the genes that are up-regulated in *T. bicolor* (*T.b.*). The three clusters with the highest enrichment scores are shown (all $P < 0.05$ except myelination $P = 0.0517$). Cell. comm., cellular communication. (**D**) Considering only the genes that are characterized by synaptic (Synap) and neuronal (Neuro) GO terms, the number of genes that are up-regulated in *L. barbadensis* is higher than the number of genes that are up-regulated in *T. bicolor* in the associative pallium. $^{**}P < 0.01$. (**E**) Using the same subset of genes, the number of genes that are up-regulated in *L. barbadensis* were compared to the number of genes that are up-regulated in *T. bicolor* in each of the regions. *L. barbadensis* had more up-regulated genes in the NCL. $^{**}P < 0.01$. The total number of differentially expressed genes is significantly higher in the associative pallium than in the three other regions. $^{***}P < 0.001$. (**F**) Two significant constructed network modules: "Synapse" and "Adult Behavior." See fig. S7 for all other modules. Both have a positive *r* value, indicating that the mean expression in the modules is higher in *L. barbadensis* compared to *T. bicolor*. Hippo, hippocampus; IH, intercalated hyperpallium; MD, dorsal mesopallium; MV, ventral mesopallium; Ento, entopallium; LSt, lateral striatum; MSt, medial striatum; B, basorostralis; LMD, lamina mesopallium dorsale; LMI, lamina mesopallium intermediate; LMV, lamina mesopallium ventrale; LPS, lamina pallio-subpallialis; Meso, mesopallium; Nido, nidopallium; Arco, arcopallium.

50 genes by in situ hybridization in the brains of a model songbird, the zebra finch (*16*), the entopallium clustered furthest away from all the other pallial populations and the intrapallial populations (associative pallium: mesopallium and nidopallium, including the NCL), clustered next to each other. Similarly, hierarchical cluster analysis revealed that the entopallium was the most distant region from the others, whereas the mesopallium and nidopallium were very similar to each other, and the arcopallium and hippocampus were clustered together (fig. S5A) (*16*). These findings indicate that our approach was successful at revealing known molecular relationships between brain cell populations.

We next asked whether there were differences between species and found in the principal component 2 (PC2) that the species differed the least in the entopallium but the most in the highest-order connected region, the mesopallium, followed by the nidopallium and the NCL (Fig. 2B). This was concordant with the total number of differentially expressed genes between species, with the mesopallium, nidopallium, and NCL showing the highest number of differentially expressed genes (fig. S5B). Normalizing with the total number of genes expressed in each region resulted in the same rankings, which means that the observed differences are not simply due to differences in the number of expressed genes per region (fig. S6A).

Because of the similarity in their expression and their potential cooperative role in problem solving, we combined data from the mesopallium and the nidopallium (including the NCL), hereafter referred to as the associative pallium, to perform GO analyses. GO clustering revealed an overrepresentation of differentially expressed genes related to synaptic signaling and localization and, to a lesser extent, myelination (Fig. 2C). Genes up-regulated in *L. barbadensis* were enriched for functions of neurogenesis and axonogenesis, as well as cellular communication and signaling (terms associated with neurotransmission; Fig. 2C). Genes up-regulated in *T. bicolor* were enriched in nucleoside metabolic processes, apoptosis, and ion transmembrane transport (Fig. 2C). Overall, genes related to specific neuronal and synaptic functions appear to be the most represented in differentially expressed genes between the species.

Focusing on the genes that contain "neuron" or "synap" in any of their GO terms, *L. barbadensis* had more up-regulated genes than *T. bicolor* in their associative pallium ($P\ \chi2 = 0.0075$; Fig. 2D). Focusing on the individual cell populations, NCL had the most up-regulated genes in *L. barbadensis* compared to *T. bicolor* ($P\ \chi2 = 0.0031$; Fig. 2E). Selecting other sets of genes instead, for example, genes that have "apopto" or "mitochondri" in their GO terms, yielded no difference or differences in favor of *T. bicolor* (fig. S6, B to E). Together, these results suggest that divergence in problem-solving skills is associated with up-regulation of genes in higher-order pallium populations and, to a greater extent, in the NCL within the nidopallium and that those differences are specifically related to neuronal and synaptic activity. This is in accordance with several lines of evidence that suggest that the nidopallium and mesopallium perform similar functions to upper layers of the mammalian cortex and that the NCL is involved in higher sensory processing and associative functions (*20*, *21*).

Network analyses and in situ hybridizations reveal glutamate receptors involved in neural plasticity

To further gain insight into which groups of genes covary between species, we performed a coexpression network analysis, based on hierarchical clustering, and discovered nine network modules that were significantly associated with species (fig. S7A). Two modules consisted of genes enriched in the GO terms synapse and adult behavior (also referred to as "adult behavioral response to stimulus"; Fig. 2F; for all other modules see fig. S7B). The *N*-methyl-D-aspartate (NMDA) glutamate receptor subunits glutamate receptor ionotropic NMDA 1 (GRIN1) and GRIN2B, known to promote synaptic plasticity (*22*, *23*), were found in these modules (Fig. 2F). Another metabotropic glutamate receptor (GRM2) also involved in cognition (*24*) was up-regulated in *L. barbadensis* in a cluster of genes enriched for organelle organization (fig. S7B).

This led us to analyze the expression of 18 glutamate receptors that was detectable by RNA-seq among the various brain regions. We found that four of five subunits of NMDA receptors and three of the five metabotropic receptors were differentially expressed in the associative pallium populations (Fig. 3A). The other receptors did not show significant differences.

To validate the RNA-seq analyses and determine the specificity of the anatomical profile, we performed in situ hybridization on the brains of the two species (Fig. 3B). We confirmed that GRIN1 and GRIN2B were broadly up-regulated in the mesopallium and nidopallium of *L. barbadensis*, whereas GRIN2A was up-regulated in *T. bicolor* (Fig. 3C). GRM2 was also significantly higher in *T. bicolor* compared to *L. barbadensis*. Consistent with the RNA-seq analyses, none of the AMPA or kainate glutamate receptors differed in expression between species (Fig. 3, B and C). GRIN3, GRM3, and GRM4 were differentially expressed in the RNA-seq analysis, whereas differences in GRIN3 and GRM4 expression in the in situ hybridization were not significant once Bonferroni corrections were applied. Nevertheless, both techniques showed very consistent results. Details on the expression of all glutamate receptors in individual regions by RNA-seq and in situ hybridization are given in table S2.

The finding of contrasting species differences in GRIN2A and GRIN2B expression is marked because these are the most acknowledged types of glutamate receptor subunits linked with learning, memory, and cognition, where they play opposite roles (*25*). In particular, the GRIN2B/GRIN2A ratio is positively associated with the intensity of long-term potentiation, long-term depression, dendritic spine density, and learning proficiency (*25*–*27*). We therefore investigated these two NMDA receptors more closely in each region of the pallium, using the mRNA quantification data obtained from in situ hybridization (fig. S8A) and RNA-seq, as well as protein levels quantified using immunohistochemistry performed with GRIN2A- and GRIN2B-specific antibodies (fig. S8B). There was a clear agreement in the results obtained from the three methods: The GRIN2B/GRIN2A ratio was reliably higher in *L. barbadensis* in all examined regions except the entopallium (Fig. 4, A to C), in line with behavioral differences between the two species.

DISCUSSION

The all-or-none difference in problem solving between *L. barbadensis* and *T. bicolor* we found here is likely the result of species-level evolutionary divergence in behavior. It is less likely due to differential experience with the novel conditions of the urban habitat where both species were captured: In a separate study (*28*), urban *L. barbadensis* were significantly faster than rural ones in solving two obstacle removal problems, but they still far outperformed the *T. bicolor* tested here. In our study, *L. barbadensis* did not differ from *T. bicolor* in reversal learning, a task that is presumed to require complex cognitive skills. This result is in line with the comparison of rural versus urban *L. barbadensis*, which differed in their problem-solving skills but not in their reversal learning ability (*28*), in addition to increasing evidence that reversal learning and problem-solving tasks measure different abilities (*29*).

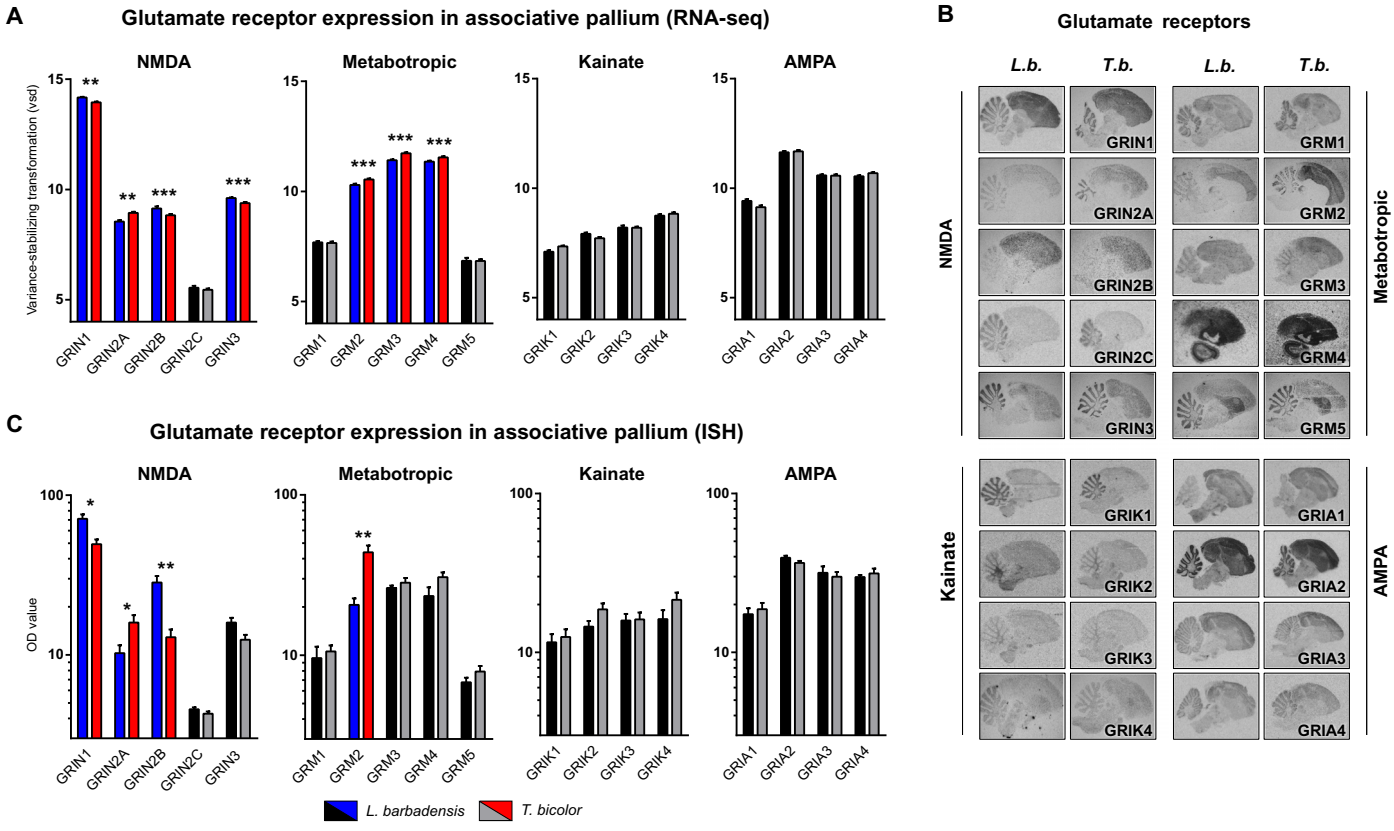

Fig. 3. Glutamate receptor expression analysis. (**A**) RNA-seq data (variance stabilizing transformation of reads) of all glutamate receptors. *P* values were obtained by differential expression analysis. (**B**) Representative autoradiography images of glutamate receptor in situ hybridizations (ISH). (**C**) Quantification of the signal obtained by in situ hybridization for all assessed glutamate receptors. Significantly different expression is indicated by colored bars. Means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

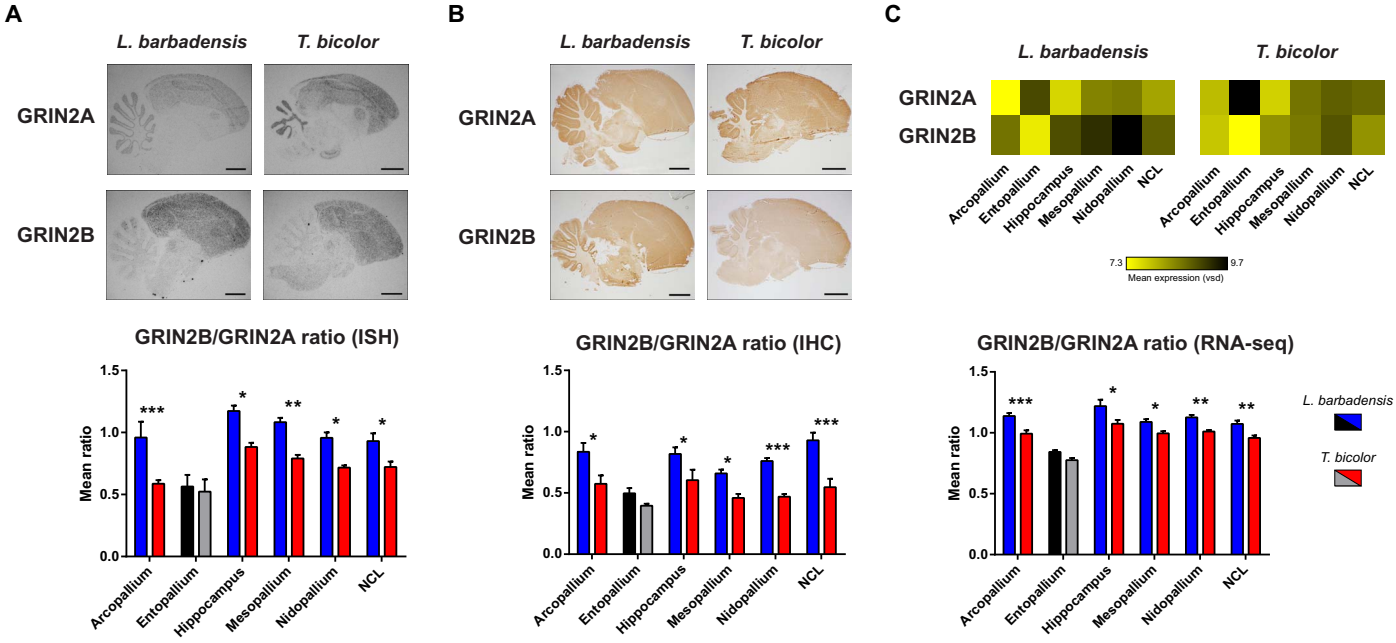

Fig. 4. GRIN2A and GRIN2B expression across all brain regions. (**A**) In situ hybridizations of GRIN2A and GRIN2B mRNA with their mean GRIN2B/GRIN2A ratios below, calculated with the quantifications of individual receptor expression in each region. (**B**) Immunohistochemistry (IHC) targeting proteins with GRIN2A- and GRIN2B-specific antibodies, with their mean GRIN2B/GRIN2A ratios below, calculated with the quantifications of individual receptor expression in each region. (**C**) Heatmaps of mean expression (vsd-transformed reads) for GRIN2A and GRIN2B from RNA-seq data. Bottom: Mean ratios calculated with individual receptors. Means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Scale bars, 500 μm.

Because relative brain size is associated with innovation (*1*–*3*) and similar neuron counts in primates and birds were hypothesized to be responsible for comparable cognitive skills in both clades (*7*), it would have been reasonable to predict that *L. barbadensis* would have a bigger brain than *T. bicolor*, assuming that they have matching relative neuron densities. However, our analysis revealed that their relative brain size did not differ. Increases in pallial volume, neuronal density, and receptor expression thus appear to be different candidate ways in which the information processing that underlies innovative problem solving could be increased.

Our study is the first to link divergence in behavioral innovation in the field, problem solving in captivity, and receptor expression levels in the brain. The receptors that are up-regulated in *L. barbadensis* are those that promote neuronal plasticity (GRIN2B, GRIN1, and GRIN3), whereas the ones that are up-regulated in *T. bicolor* are receptors that diminish it (GRIN2A, GRM2, GRM3, and GRM4). The association between the GRIN2B/GRIN2A ratio and behavioral divergence between *L. barbadensis* and *T. bicolor* is particularly appealing, considering that (i) this ratio is one of the most promising candidates to explain variation in mammalian intelligence (*27*); (ii) in songbirds, this ratio changes in the song-learning nuclei and is thought to contribute to changes in the critical period for vocal learning as the animals become adults (*30*, *31*); and (iii) glutamate receptors are highly conserved (*32*) and their functions are thought to be similar across species (*33*). Up to now, studies on GRIN2B/GRIN2A ratio variation have been based on experimental comparisons of transgenic or aging rodents and normal versus neurologically diseased humans (*27*). Our finding of natural variation in this ratio in wild, closely related species with divergent foraging strategies provides an excellent opportunity to study the convergent evolution of innovative problem solving, similar to that proposed for song learning in birds and speech in humans (*34*). A next obvious step would be to experimentally manipulate this ratio in the mesopallium and nidopallium and determine whether changes in innovation behavior follow. In addition, although the role of NMDA receptors has been broadly assessed in relation to some other behaviors in captive birds [for example, reversal learning (*35*)], the link between individual subunits and especially the GRIN2B/GRIN2A ratio and different behaviors have yet to be investigated. Finally, because our study initiates a new research approach using only two species, investigating the relationship between problem solving and the GRIN2B/GRIN2A ratio in more species from different lineages is important to determine the evolutionary importance of NMDA receptors for innovative skills.

METHODS

Animals

For behavioral analyses, 30 *L. barbadensis* of both sexes and 15 male *T. bicolor* were captured using mist nets between February 2012 and May 2012 in Holetown, Barbados. *T. bicolor* are sexually dichromatic; the monomorphic *L. barbadensis* were sexed molecularly from blood samples. Sex had no effect on our results. After capture, birds were brought to aviaries and housed in individual cages visually but not acoustically isolated from each other. After a 2-day habituation period during which the birds were left undisturbed and fed ad libitum, they were food-deprived overnight and tested on the next morning.

Behavioral tests

On the third day of captivity, we assessed boldness by presenting a petri dish (same as during habituation period) full of seeds, hiding behind a curtain, and measuring the latency to feed following withdrawal of the experimenter. Birds were given a capped value of 1201 s if they did not feed before the 20 min allotted. The obstacle-removal apparatus (see picture of the task in fig. S1A) was then presented open and full of seeds for the first time to measure avoidance of novel stimuli ("neophobia," latency to feed in the apparatus from which we subtracted the boldness latency). Once the bird had fed from the open apparatus, the problem-solving trial began, with the lid closed but loosely fitted. Birds were given a maximum of 15 trials of 5 min (each trial being separated by 20 min) to solve the problem, after which they were attributed a capped value of 16 trials. After this phase, unsuccessful birds were gradually trained (shaped) to solve the task (see the Supplementary Materials for details and fig. S2C) for up to 60 trials. Then, birds were given the detour-reaching task (see picture of the task in fig. S1B). They were first trained to reach a seed at the center of an opaque cylinder without pecking on the sides for seven trials in a row, after which they were given a transparent cylinder and had to perform with the same success criterion (see the Supplementary Materials for details). The discrimination learning task consisted of two colored platforms in which petri dishes were placed, one with the seeds available and the other with the seeds glued to the bottom of the dish so that there was no reward for choosing this dish, although glued versus nonglued seeds were impossible to distinguish from a distance. The success criterion was set choosing the correct (rewarded) color at seven trials in a row. On the following day, reversal learning was assessed using the same protocol but with the rewarded color switched with the previously unrewarded color.

RNA sequencing

A separate cohort of birds was captured to prevent the modulation of brain mRNA caused by the stress of captivity and the experience of the behavioral tests. A total of eight birds per species were used for the RNA-seq experiments. After capture, the birds were decapitated, and their brains were dissected and placed into RNAlater solution (catalog # AM7021, Thermo Fisher Scientific). Brains were then sliced in thick (400 μm) sections, and the regions of interest were dissected with small scissors and a scapel under an Olympus dissecting microscope. RNA from the six individual brain regions of the eight individuals for both species was then extracted separately (for a total of 96 samples) using a standard TRIzol extraction procedure. mRNA was purified using the MicroPoly(A)Purist Kit (catalog # AM1919, Thermo Fisher Scientific), which binds polyA regions on cellulose spin columns. Library preparations were then performed using a NextFlex Directional RNA-Seq Kit (deoxyuridine triphosphate–based; catalog # 5129-06, Bioo Scientific; see the Supplementary Materials for details). The samples were pooled at a concentration of 200 mM (quantified using quantitative real-time polymerase chain reaction) into six different lanes for high-output 2 × 100–base pair paired-end sequencing. Bioinformatics analyses were performed on the Harvard Faculty of Arts and Sciences Odyssey Cluster (see the Supplementary Materials for details). Briefly, the reads were trimmed and then mapped to the *G. gallus* genome. The number of aligned reads for each sample was compiled and used for differential expression analysis, which was performed using DESeq2 (*36*). For the associative pallium analyses, the model (reads ~ region + species) was run to obtain a measure of the associative pallium while taking into account variation caused by individual regions. For individual region analyses, a one-factor model (reads ~ species) was run. The principal components and hierarchical analyses were performed in DESeq as well. GO enrichment analysis was performed using the DAVID 6.8 functional annotation clustering tool (*37*). The network analysis was performed

using the Weighted Gene Co-expression Network Analysis package (*38*), using raw number of reads.

In situ hybridization

Another cohort of five naïve birds per species was captured for in situ hybridization. Brain tissue hybridizations were performed, as described in the study of Wada *et al.* (*39*). Briefly, ^{35}S-labeled riboprobes were made from T3, T7, or SP6 promoter sites of cDNA clones from the study of Wada *et al.* (*32*) using T3, T7, or SP6 RNA polymerases (Roche). Ten-micrometer fixed sections on slides were hybridized for 16 hours at 65°C. They were then washed, dehydrated, and exposed in an autoradiography cassette with a Kodak BioMax MR film in the dark at 4°C for 48 to 72 hours before developing the films. Optical densities were then measured with ImageJ2. To assess differences in individual regions (data presented in Fig. 4A, fig. S8, and table S2), we performed two-way analysis of variance (ANOVA) using data from all regions and report interspecific Bonferroni post-test comparisons for each region. To test for differences in the associative pallium (data presented in Fig. 3B), we performed two-way ANOVA and report differences for the species factor and then applied Bonferroni corrections for multiple comparisons.

Immunohistochemistry

The same animals from in situ hybridization were used for immunohistochemistry. Fixed sections were blocked using BLOXALL (catalog # SP-6000, Vector Laboratories), rinsed, and incubated in normal blocking serum. Sections were then incubated in 1:500 primary antibody (anti-GRIN2A, catalog # Ab118587; anti-GRIN2B, catalog # Ab65783, Abcam) overnight at 4°C. They were then incubated with appropriate secondary antibodies followed by avidin-biotin complex reagent (catalog # PK-6100, Vector Laboratories). Finally, they were washed and incubated in 3,3′-diaminobenzidine (DAB) peroxidase substrate (catalog # SK-4100, Vector Laboratories) and coverslipped. They were quantified the same way as in the in situ hybridization analysis.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/3/eaao6369/DC1

Supplementary Materials and Methods

fig. S1. Behavioral tasks.

fig. S2. Problem solving and shaping.

fig. S3. Risk-taking behavior and discrimination learning.

fig. S4. Residual brain mass of our two species versus other thraupids.

fig. S5. Cluster analysis of gene expression per region and MA plots showing the distribution in the two species of fold change in all genes as a function of expression, for each analyzed brain region.

fig. S6. Proportion of differentially expressed genes and absolute number of up-regulated genes for specific GO terms.

fig. S7. Network dendrogram and modules following weighted correlation network analysis.

fig. S8. GRIN2A and GRIN2B expression across all brain regions.

table S1. Linear model outputs for all behavioral variables.

table S2. Mean expression of each glutamate receptor in each region, using in situ hybridization and RNA-seq data.

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