Variation in Brain Regions Associated with Fear and Learning in Contrasting Climates

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Amygdala · Arcopallium · Avian brain · Fear · Lateral striatum · Learning · Nucleus taeniae of the amygdala

Abstract

In environments where resources are difficult to obtain and enhanced cognitive capabilities might be adaptive, brain structures associated with cognitive traits may also be enhanced. In our previous studies, we documented a clear and significant relationship among environmental conditions, memory and hippocampal structure using ten populations of black-capped chickadees (*Poecile atricapillus*) over a large geographic range. In addition, focusing on just the two populations from the geographical extremes of our large-scale comparison, Alaska and Kansas, we found enhanced problem-solving capabilities and reduced neophobia in a captive-raised population of black-capped chickadees originating from the energetically demanding environment (Alaska) relative to conspecifics from the milder environment (Kansas). Here, we focused on three brain regions, the arcopallium (AP), the nucleus taeniae of the amygdala and the lateral striatum (LSt), that have been implicated to some extent in aspects of these behaviors in order to investigate whether potential differences in these brain areas may be associated with our previously detected differences in cognition. We compared the variation in neuron number and volumes of these regions between these populations, in both wild-

caught birds and captive-raised individuals. Consistent with our behavioral observations, wild-caught birds from Kansas had a larger AP volume than their wild-caught conspecifics from Alaska, which possessed a higher density of neurons in the LSt. However, there were no other significant differences between populations in the wild-caught and captive-raised groups. Interestingly, individuals from the wild had larger LSt and AP volumes with more neurons than those raised in captivity. Overall, we provide some evidence that population-related differences in problem solving and neophobia may be associated with differences in volume and neuron numbers of our target brain regions. However, the relationship is not completely clear, and our study raises numerous questions about the relationship between the brain and behavior, especially in captive animals.

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Introduction

Behavioral flexibility allows for the expression of different behavioral outcomes based on previous experiences [Dukas, 1998; Reader, 2003], and such flexibility seems to be adaptive, especially in challenging environments [Dukas, 1998; Martin and Fitzgerald, 2005; Echeverria and Vassallo, 2008; Liker and Bokony, 2009]. Enhanced cognitive abilities (behaviors associated with advanced learning) are often associated with living in difficult, en-

ergetically demanding environments [Dukas, 1998; Shettleworth, 1998; Roth and Pravosudov, 2009; Roth et al., 2010a]. The use of advanced cognition may be adaptive, especially in regards to obtaining food and other resources in difficult environments. Indeed, differences in cognitive traits such as memory, problem solving and learning appear to exist in populations originating from different environments with different demands for resources.

According to the adaptive specialization hypothesis, the neural mechanisms that underlie these advanced behavioral traits should follow a similar pattern with environmental demands [Krebs et al., 1989]. In environments in which resources are more challenging to obtain and selection for enhanced cognitive capabilities might occur, brain structures associated with cognitive traits should also be enhanced. For example, some species use spatial memory to retrieve cached food. In harsh environments in which demands on memory use are high, individuals tends to possess better spatial memory and enhanced features of the hippocampus (Hp), a region of the brain involved in spatial memory [Pravosudov and Clayton, 2002; Roth and Pravosudov, 2009; Chancellor et al., 2011; Roth et al., 2011, 2012].

This work also suggests that there may be a strong inherited component to some features of the brain. For example, Hp neuron number and neurogenesis levels in black-capped chickadees (*Poecile atricapillus*) originating from populations with extremes in environmental conditions (Alaska and Kansas) but raised in captivity were not significantly different from those of their wild-caught counterparts. Moreover, the differences detected between wild populations remained even in birds raised in the captive laboratory environment. Oler et al. [2010] found a similar pattern in primates, with heritable differences in Hp-related behavior associated with changes in Hp morphology.

Individuals from different environments also show differences in nonspatial cognitive behaviors. For example, the aforementioned chickadees from Alaska (the harsher environment) had enhanced problem-solving skills and showed less of a neophobic response to a novel object, i.e. more rapid learning, than their Kansas counterparts, even when reared in an identical laboratory environment [Roth et al., 2010a]. These results suggest that many facets of cognition may be under selection in these birds and that enhanced cognition may be associated with the severity of the environment.

Given this variation in nonspatially related behaviors and the previously documented relationship between the

natural variation in spatial memory and its association with the brain, the main goal of this study was to explore the volume and neuron number of some brain regions that may be involved in problem solving and neophobia across different environments to determine if there is an association between these behavioral traits and the brain. We examined three brain regions that have been implicated to varying degrees in aspects of these behaviors: the arcopallium (AP), the nucleus taeniae of the amygdala (TnA) and the lateral striatum (LSt). We compared the morphology of these regions in the black-capped chickadee in the same two populations in which we had previously detected significant differences in neophobia and problem solving [Roth et al., 2010a, 2012]. To examine the natural variation in these brain regions as it may relate to living in different climates, we compared the brains of wild birds from these two populations. To understand the importance of experiences in different environments in explaining that potential variance in the brain, we also examined the three brain regions in birds from the two populations that had been hand-raised in captivity in the same laboratory environment [see also Roth et al., 2012].

The selection of our three target regions stems from avian functional data and mammalian homologies. We first focus on a region of the brain associated with fear and emotional learning. In mammals, increases in apprehension and fear levels have been associated with increased amygdala activity [Phan et al., 2002] and an enlarged amygdaloid complex [LeDoux, 2000; van der Plas et al., 2010]. However, long-term fear can produce reductions in the volume of this region in some cases [Yang et al., 2008], perhaps mediated via corticosterone [Brown et al., 2008]. In birds, the AP is in part homologous to the amygdala of mammals [Abellan et al., 2009; Medina et al., 2011] and seems to be responsible in part for the fear response [Zeier and Karten, 1971; Cohen, 1975]. As in mammals, this region seems to play an important role in learning and cognition in birds, especially in relation to fear [reviewed by Davis, 1992; LeDoux, 2000]. For example, ablation of the AP and subregions produces reductions in fear and fear learning in birds [Martin et al., 1979; Phillips and Youngren, 1986; Burns et al., 1996; Saint-Dizier et al., 2009].

In particular, the medial and posterior portions of the AP may be important for fear learning in birds [Zeier and Karten, 1971; Cohen, 1975]. The ablation of these regions has been associated with a reduced ability to learn about threatening stimuli [Cohen, 1975]. However, it is important to note that recent work in quail (*Coturnix japonica*) suggests that the anterior regions of the AP may also play

an important role in fear [Saint-Dizier et al., 2009]. Thus, in addition to measurement of the entire AP region, we measured a medial subregion, the TnA. This medial region of the AP was among those identified by Cohen [1975] to play a role in fear learning and has been implicated in social learning in birds [Thompson et al., 1998]. The mammalian homolog of the TnA, the medial amygdala, also seems to be important in social and fear learning as well as unconditioned fear responses [Li et al., 2004; Walker et al., 2005].

We also examined one region that may be associated in part with cognitive function, the LSt. Based on Schrott and Kabai [2008], the mammalian homolog of the LSt is the caudate nucleus and the putamen. These regions collectively play a role in goal-oriented and other types of learning [Yamada et al., 2004; reviewed by Graybiel, 2005; Ell et al., 2006]. The function of this region is less well studied in birds but may be involved in learning [Stewart et al., 1996; Kabai et al., 2004].

Based on our previous results [Roth et al., 2010a], birds from the harsher climate (Alaska) showed enhanced problem-solving abilities and reduced neophobia relative to those from the mild climate (Kansas). Thus, we expect differences in the brain regions between the two populations, specifically a larger AP and TnA with more neurons in the Kansas population (which showed increased neophobia), but a larger LSt with more neurons in birds from Alaska (which showed increased problem-solving capabilities). We also expect the differences in neurons between the two populations to be maintained in captivity, but volumetric differences to be lost [LaDage et al., 2009; Tarr et al., 2009; Roth et al., 2012].

Methods

Study Species

Black-capped chickadees were collected at the latitudinal extremes of their range (Anchorage, Alaska: 61° 10' N, 149° 53' W; Manhattan, Kans.: 39° 08' N, 96° 37' W). Wild birds (hereafter referred to as wild-caught) were collected between 21 and 23 October 2007 in Manhattan, Kans. (n = 12, 5 males and 7 females), and between 18 and 20 September 2008 in Anchorage, Alaska (n = 12, 5 males and 7 females), in the context of other studies [Roth and Pravosudov, 2009; Roth et al., 2011]. Captive-raised birds were collected in May and June 2009 from independent nests (n = 12, 6 males and 6 females, from each population). The individuals used in this study were also used in other studies by our group [Roth et al., 2010a, 2012].

Hand-Rearing and Housing in Captivity

A full description of collection and housing procedures can be found elsewhere [Roth et al., 2010a]. Briefly, captive-raised chicks

were approximately 10 days old at the time of collection and were hand-reared indoors on site until they were approximately 18 days old, when they were transported to the University of Nevada, Reno (Nev., USA). Temperature, lighting and all techniques were similar for animals from both populations, from the first day of collection.

All chicks raised in captivity were fed a diet of Orlux hand-rearing bird formula, insects and nuts throughout the daylight hours [Roth et al., 2010a]. Food and water were provided ad libitum after the birds reached independence (approx. 30–35 days after hatching).

During hand-rearing, chicks were housed in groups of 4–6 individuals in 17 × 17 × 24 cm wood boxes filled with sawdust to simulate nest cavities. At the fledgling stage (approx. 18–20 days after hatching), chicks were housed in pairs in 120 × 42 × 60 cm wire cages. To avoid aggressive interactions but to maintain birds in a seminatural social setting, at the dispersal stage (approx. 60 days after hatching), all birds were placed individually into 60 × 42 × 60-cm wire cages in a male/female/female/male arrangement within a row of 4 cages with visual contact. Sex was determined initially by wing cord length and later confirmed by postmortem inspection of the gonads. The populations were systematically partitioned as Alaska/Kansas/Alaska/Kansas within these rows.

The two populations were kept under the same light cycle. The summer cycle was 15 h of light/9 h of darkness beginning from the day of collection for both populations. Beginning in early August until mid-October, the light cycle gradually shifted (approx. 0.5 h/week) to a winter cycle of 9 h of light/15 h of darkness. All birds were maintained on the winter light cycle for the remainder of the study.

Brain Preparation

Wild-caught birds were sacrificed on site (following the protocol in Roth and Pravosudov [2009], Roth et al. [2011] and below) within 4 h after capture. All captive-raised birds were sacrificed on 2 February 2010. All birds were anesthetized (0.07 ml of 50 mg/ml Nembutal) and perfused transcardially with phosphate-buffered saline followed by 10% methanol-free formalin (from paraformaldehyde). Brains were postfixed for 7 days, cryoprotected and then frozen for storage at –80°C. Tissue was cut into 40-μm coronal sections on a Leica CM 3050S cryostat at –20°C.

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Nevada, Reno (protocol A05/06-35), and followed all federal and local guidelines for the use of animals in research.

Morphological Measures

All measurements of volume and neuron numbers were estimated on Nissl-stained sections with modern stereological methods using StereoInvestigator software (Microbrightfield Inc.) and a Leica microscope (M4000B). All regions as well as the remainder of the telencephalon (Te; for use as a covariate to control for the overall size of the brain) were measured in their entirety. The atlas of Izawa and Watanabe [2007] was used as a stereotaxic reference (fig. 1).

Due to the variation in size of the three regions measured, we used different sampling schemes to estimate volume and neuron numbers in the different regions. However, the sampling scheme was consistent within treatment groups. All sampling schemes were optimized by one of the authors (T.C.R.) according to strat-

Fig. 1. Three regions of the black-capped chickadee brain measured for investigation of an association with fear and learning: the AP, the TnA and the LSt. Shown are representative images both without (top) and with (bottom) region designations.

egies in StereoInvestigator version 8 [MBF Bioscience, 2008]. The LSt and AP were measured on every 8th section, while the TnA was measured on every 4th section. Volumes were estimated using the Cavalieri procedure [Gundersen and Jensen, 1987]. LSt and AP volumes were measured with a 200- μm grid, while TnA volume was measured with a 100- μm grid. Neuron counts were performed using an optical fractionator procedure [West et al., 1991] at $\times 1,000$. A 200- μm sampling grid was used for the TnA, and a 325- μm grid was used for the LSt and AP. All counting

frames were $30 \times 30 \mu\text{m}$, with a disector height of $5 \mu\text{m}$ and 2- μm guard zones. We calculated a coefficient of error (CE) to estimate precision with the nugget effect [West et al., 1991], as follows [mean CE (SE)]: TnA, 0.113 (0.002) for neurons and 0.029 (0.001) for volume; LSt, 0.089 (0.006) for neurons and 0.036 (0.002) for volume; AP, 0.089 (0.002) for neurons and 0.039 (0.001) for volume. The left and right hemispheres were summed to produce the reported total values. There were no significant differences between the sexes for measurements in any region (all p values > 0.251); thus, data for both sexes were pooled.

Statistical Analyses

We analyzed all morphological comparisons with general linear models. We report least-squares means $\pm$ SE using as covariates the remainder of the Te (for volume and neuron number) or the volume of the region (for neuron density) [Roth and Pravosudov, 2009; LaDage et al., 2010; Roth et al., 2011]. For the TnA, we performed two additional analyses that used the remainder of the AP volume (for TnA volume) and the number of neurons in the remainder of the AP (for TnA neuron number) as covariates. To control for interobserver variation in analysis among different studies (for a discussion of this problem, see Roth et al. [2010b]), all histological data were collected exclusively by a single author (C.M.G.) who was blinded to the geographic origin of the birds.

All data were log transformed to meet the assumptions of normality and homogeneity of variance as necessary.

Results

Morphological Comparisons between Populations Wild-Caught Birds

There were some differences in morphology between wild-caught birds from Kansas and Alaska. Although not significantly different, birds from Kansas had a marginally larger LSt than those from Alaska ($F_{1,21} = 3.130$; $p = 0.084$; Te covariate; table 1). However, birds from Alaska had significantly higher densities of neurons in the LSt ($F_{1,21} = 5.213$; $p = 0.033$) than those from Kansas, but there were no differences in absolute neuron numbers ($F_{1,21} = 0.296$; $p = 0.592$).

There were no significant differences in the volume, neuron density or neuron number in the TnA between the populations (volume: $F_{1,21} = 2.175$, $p = 0.155$, Te covariate; $F_{1,21} = 0.010$, $p = 0.920$, relative to the rest of the AP; neuron density: $F_{1,21} = 1.874$, $p = 0.185$; number relative to number in AP: $F_{1,21} = 0.911$, $p = 0.351$; table 1).

However, the remainder of the AP (not including the TnA) was significantly larger in the Kansas population ($F_{1,21} = 12.508$; $p = 0.002$; Te covariate). There were no differences in neuron density ($F_{1,21} = 0.225$; $p = 0.640$) or number ($F_{1,21} = 2.621$; $p = 0.120$) in the remainder of the AP. When the analysis was performed with the TnA and AP combined, the same pattern held. Kansas birds had

Table 1. Volumes and neuron estimates of the AP, the TnA and the LSt in wild-caught and captive-raised black-capped chickadees from Alaska and Kansas

Population		n	LSt volume mm ³	LSt neurons × 10 ⁵	TnA volume mm ³	TnA neurons × 10 ⁵	AP volume mm ³	AP neurons × 10 ⁵	Te volume mm ³
Wild-caught	Alaska	12	14.134 ± 0.396	14.161 ± 0.615	0.911 ± 0.045	0.741 ± 0.027	15.573 ± 0.369	9.546 ± 0.361	538.611 ± 18.135
	Kansas	12	14.845 ± 0.330	13.262 ± 0.721	0.954 ± 0.045	0.799 ± 0.030	16.290 ± 0.487	10.084 ± 0.338	488.918 ± 14.464
Captive-raised	Alaska	12	13.227 ± 0.404	12.005 ± 0.665	0.843 ± 0.044	0.715 ± 0.034	13.942 ± 0.461	8.401 ± 0.261	459.070 ± 12.932
	Kansas	12	12.733 ± 0.290	11.004 ± 0.068	0.803 ± 0.036	0.647 ± 0.033	13.288 ± 0.321	8.613 ± 0.298	422.658 ± 6.868

Values are shown as least-squares means ± SE.

a larger AP ($F_{1,21} = 11.870$; $p = 0.002$) with no differences in neuron density ($F_{1,21} = 2.731$; $p = 0.113$) or number ($F_{1,21} = 0.274$; $p = 0.606$). The consistency of the difference is likely driven by the large size of the AP relative to the TnA alone (fig. 2; table 1).

Captive-Raised Birds

There were no statistical differences between the captive-raised birds from Alaska and Kansas with regard to any of the above variables (all p values > 0.111 ; table 1).

Morphological Comparisons between Captive-Raised and Wild-Caught Birds

Overall, the wild-caught birds had significantly larger volumes and more neurons in many cases. Birds from the wild populations had larger LSt volumes ($F_{1,43} = 4.571$; $p = 0.038$) and marginally significantly greater LSt neuron density ($F_{1,43} = 3.889$; $p = 0.055$) and numbers ($F_{1,43} = 3.939$; $p = 0.054$) with no significant interaction effects (volume: $F_{1,43} = 3.625$; $p = 0.064$; density: $F_{1,43} = 0.098$; $p = 0.756$; number: $F_{1,43} = 0.023$; $p = 0.880$).

There were no significant differences between wild-caught and captive-raised birds with regard to absolute TnA volume ($F_{1,43} = 0.327$; $p = 0.570$), relative to the rest of AP ($F_{1,43} = 0.180$; $p = 0.673$), TnA neuron density ($F_{1,43} = 1.670$; $p = 0.130$) or neuron number ($F_{1,43} = 1.052$; $p = 0.311$). There were no significant interaction effects (volume: $F_{1,43} = 1.458$, $p = 0.234$; density: $F_{1,43} = 3.552$, $p = 0.066$; number: $F_{1,43} = 3.809$, $p = 0.058$), although the interaction with neuron density and number was nearly significant. This interaction is driven by a trend for increased neuron number in the wild-caught birds from Kansas (fig. 2).

Wild-caught birds had a larger AP complex when analyzed with and without the TnA (volume of AP minus TnA: $F_{1,43} = 4.371$, $p = 0.043$; total AP volume: $F_{1,43} = 4.158$, $p = 0.048$) with more neurons (AP minus TnA: $F_{1,43} = 5.014$, $p = 0.030$; total AP: $F_{1,43} = 5.555$, $p = 0.023$;

Fig. 2. Volume of the AP (with Te as a covariate) in wild-caught and captive-raised black-capped chickadees from Alaska and Kansas. There was a significant interaction effect between population and treatment group (i.e. captive or wild; $F_{1,43} = 7.554$; $p = 0.009$). Wild-caught birds from Kansas had larger AP volumes than those from Alaska or either of the captive groups.

table 1). There were also significant interaction effects in the analysis with volume (volume of AP minus TnA: $F_{1,43} = 7.664$, $p = 0.008$; total AP volume: $F_{1,43} = 7.554$, $p = 0.009$) driven by large volumes in the wild-caught birds from Kansas (fig. 2). There were no significant interaction effects in the analyses with neuron numbers (neurons in AP minus TnA: $F_{1,43} = 0.444$, $p = 0.509$; total AP neurons: $F_{1,43} = 0.680$, $p = 0.414$). There were no differences in neuron density (AP minus TnA: $F_{1,43} = 2.010$, $p = 0.163$; total AP: $F_{1,43} = 1.949$, $p = 0.170$), with no significant interaction effects (neuron density of AP minus TnA: $F_{1,43} = 0.050$, $p = 0.825$; total AP neuron density: $F_{1,43} = 0.011$, $p = 0.916$).

Discussion

To summarize, wild-caught birds from Kansas had a larger AP volume than their wild-caught conspecifics from Alaska. In addition, the Kansas population possessed a lower density of neurons in the LSt. These results are consistent with our predictions. However, there were no significant differences between the two wild-caught populations with regard to the volume or absolute number of neurons in the LSt, the volume or absolute number of neurons in the TnA or the absolute number of neurons in the AP. In addition, there were no significant differences in any of the measured target brain structures between populations in the captive-raised groups. However, there were large differences between wild-caught and captive-raised birds. Individuals from the wild had a larger LSt and AP with more neurons than those raised in captivity. There were no significant differences in the TnA between the wild-caught and captive-raised groups.

Our results for the AP are generally in line with other studies in birds that identify the AP as important for fear learning [Martin et al., 1979; Phillips and Youngren, 1986; Burns et al., 1996; Saint-Dizier et al., 2009]. In addition, this work is consistent with studies that show an increase in apprehension and fear levels associated with an enlarged AP. For example, using functional MRI technology, Phan et al. [2002] showed increased activity in the amygdala associated with fearful images. Similarly, van der Plas et al. [2010] found increased amygdala volumes in children who tended to be more fearful [see also van Elst et al., 2000; Fales et al., 2009]. However, Yang et al. [2008] suggested that a reduction in amygdala volume is associated with fear learning in strains of mice bred for various amygdala volumes (basolateral amygdala). That study suggests that natural variation in the amygdala may be related to variation in fear-related behavior, which is consistent with our results. However, the direction of the relationship is the opposite of that found in our study. One possible explanation for these inconsistencies in the literature may be that pathological behaviors (the topic of many studies that have found a reduction in AP volume with increased fear, e.g. posttraumatic stress disorder [Bonne et al., 2001]) may be the result of chronic stress and increased levels of corticosterone. The result of the increase in corticosterone may be a reduction in amygdala volume and incorrect function [Brown et al., 2008]. These issues have been documented in mammals and seem relevant to birds as well, although additional studies will be required to confirm this supposition.

However, it is our result of no difference in AP volume between the captive birds that is most striking. Captive-raised individuals from both populations had similar AP volumes and volumes similar to those of the wild Alaskan group. We speculate that the well-documented effect of captivity in reducing brain volume may have been particularly strong in the Kansas birds. However, it is just as possible given our design that the effect of captivity was lessened in the Alaskan birds. Either way, this is an interesting result. The variation in the response to captivity of different populations remains a topic for future examination. The maintenance of the behavioral response (as observed in these same individuals by Roth et al. [2010a]) in spite of the lack of difference between the APs of the two groups suggests that the expression of fear in the brain is more complex than volume alone (or the volume of a single region alone) can describe.

Additionally, the association between the neural density of the LSt and problem solving in this study is consistent with other studies suggesting a link between the basal ganglia and cognition. Stewart et al. [1996] and Kabai et al. [2004] found elevated dopaminergic activity in striatal regions as a result of avoidance learning in domestic chicks. In mammals, the homolog of the LSt (the caudate nucleus/putamen) is generally associated with various types of learning. For example, Yamada et al. [2004] found increased neuron activity in primates in this region, which is associated with motivational goal-oriented learning. Likewise, humans with putamen damage due to strokes perform poorly on rule-based cognitive tasks [Ell et al., 2006], suggesting that this region of the brain is involved in reasoning. In addition, a similar relationship has been observed by McDonald and Hong [2004], who showed in a lesioning study that the striatum is necessary for stimulus-response-based learning in rats. Thus, based on the results of our study and previous studies, there appears to be a relationship between basal ganglia structure and learning. We provide some preliminary evidence that the LSt may be important for cognition in birds, although additional work is necessary.

Interestingly, we did not find differences between the populations in any aspect of the TnA. Cohen [1975] identified this region (among several others) as important for fear conditioning. However, the failure to find such differences in this region may suggest that the relationship between cognition and the TnA may not be related to our target behaviors. Several studies have suggested that the medial amygdala (the equivalent to the TnA in birds) is involved in learning about fear as well as the social environment in mammals [Li et al., 2004; Walker et al., 2005].

However, Thompson et al. [1998] suggests that social learning is the main function of this region in Japanese quail. A possibility, then, is that at least in the avian system, the TnA may be associated more with learning about the social environment rather than learning about potential dangers. It is also possible that the other medial and posterior regions of the AP are more important for fear learning. More research is needed specifically in birds to understand the functions of the TnA.

Another potential reason for our failure to find the expected differences in the TnA and other regions, in spite of the behavioral differences previously reported, is that our neuroanatomical measures may not be good correlates of complex behaviors [Roth et al., 2010b]. The functional data suggesting that the TnA is important for fear learning are predominantly from ablation studies. However, the morphological variables used in this study are admittedly coarse and exploratory and are proxies for other aspects of brain function that may ultimately be more relevant for the production of behavior (e.g. neurotransmission and connectivity [Davis et al., 1994; Walker and Davis, 2002]). Although volume is a variable that has been used in numerous previous studies of this type [Clayton and Krebs, 1994; Garamszigi and Eens, 2004; Lefebvre et al., 2004], it can be quite plastic and variable [for a discussion, see Roth et al., 2010b]. Likewise, the estimated total number of neurons has been used in many of our recent studies [Pravosudov and Clayton, 2002; Roth and Pravosudov, 2009; LaDage et al., 2010; Roth et al., 2011], but this assumes that an increase in the number of neurons is associated with increased processing or enhanced cognitive abilities. Although this assumption may be correct in regions such as the Hp (based on our previous work), it is somewhat simplistic, given the complexity of the brain, to assume that the same relationship will hold across other regions. We nevertheless use these variables as ‘first steps’ along the way to establishing better correlates of brain morphology and function. Much more work remains to be done to relate natural variation in the brain to behavior, especially in birds.

The neuronal differences observed between the wild-caught and captive-raised birds were unexpected based on our previous comparisons of the Hp in these same individuals. Although we did find large differences in volumetric measures of the Hp between captive-raised and wild-caught birds, there were no significant differences in Hp neuron numbers [Roth et al., 2012]. Similarly, there were no significant differences in the number of hippocampal neurons between wild and captive mountain chickadees, *Poecile gambeli* [LaDage et al., 2010]. This is

interesting and puzzling in that the behavioral differences in problem solving and neophobia published in Roth et al. [2010a] were obtained specifically from these same captive-raised individuals, in which we detected no differences in the target brain structures. So, the captive-raised birds that exhibited strong behavioral differences in problem solving and neophobia showed no significant differences in any of the target brain areas, even though they did show reduced neuronal morphology relative to their wild-caught counterparts. The absence of differences in captive-raised birds makes our findings in wild-caught birds ambiguous, as it remains unknown if wild birds show the same (or more pronounced) problem solving and neophobia behaviors.

The differences in these brain regions between wild-caught and captive-raised birds suggest that perhaps the processes that affected Hp neurons (i.e. those that produced differences between populations but consistencies within populations between captive and wild groups) may affect other regions in a different manner. It is possible that the behaviors mediated by the AP and LSt (and other brain regions as well) may be expressed in a complex manner, perhaps based on specific experiences or some other factors. This possible explanation is consistent with another study that specifically examined the heritability of anxious behaviors. Oler et al. [2010] found that anxious temperaments in nonhuman primates are highly heritable and highly associated with the Hp and the AP. However, the neural circuitry of these regions showed differences in heritability, with the morphological variation in the Hp, but not that in the AP, following that observed in the behavior. Thus, even though the AP is associated with the response to novelty and learning about fear, the heritability of the neural features of that region may be more complex than the relationship between spatial memory and the Hp. This suggests that while variation in some behaviors (and the associated region of the brain) may be highly heritable (e.g. spatial memory as associated with Hp neurons), others may not. These results might explain in part why we did see significant variation in AP-associated behaviors in our previous study [Roth et al., 2010a] and an increase in that region in the wild-caught animals but did not see morphological differences in the brains of these same captive-raised birds. This remains an important topic for future studies.

Ultimately, this work suggests that the neural mechanisms of problem solving and the response to neophobia are complex and may involve multiple regions of the brain [Burns et al., 1996; Balaban, 1997; LeDoux, 2000].

Not only may specific brain regions be implicated in a given behavior, but a given area may be critical not for its neural processing per se, but in mediating neural processing in another region. For example, even though the LSt was implicated as important for fear conditioning in McDonald and Hong [2004], both the AP and the LSt were required for classical conditioned learning in rats.

Similarly, the Hp may be important for learning about fear. In mammals, the amygdaloid complex is well known to be associated to a large extent with Hp activity [LeDoux, 2000; Kjelstrup et al., 2002]. Similar relationships have been proposed in birds [Szekely and Krebs, 1996; Atoji and Wild, 2006], although the strength of the link in birds relative to mammals is not completely clear [reviewed by Rattenborg et al., 2011]. Nevertheless, context-dependent learning that occurs in the Hp may be critical for the fear response in both conditioned [for a review, see LeDoux, 2000; Wiltgen et al., 2006; Bissiere et al., 2011] and innate fear [Kjelstrup et al., 2002]. For example, lesions to the dorsal Hp can suppress fear for an unconditioned task [Kjelstrup et al., 2002] as well as reduce learning capabilities [Moser et al., 1993]. Oler et al. [2010] found a similar effect in the Hp of nonhuman primates. Thus, the differences reported here with the AP and the LSt along with previously reported significant differences in the Hp in this species [Roth and Pravosudov, 2009; Roth et al., 2012] may be related to and may also in part be associated with differences in the response to novelty and problem solving found in our previous study [Roth et al., 2010a]. One possible next step might be to examine the extent to which variation in the level of connectivity between the Hp, AP and LSt between populations explains these behavioral differences.

Overall, a great deal of work still needs to be done to understand how the different regions of the brain may interact to produce complex cognitive responses in birds. Although we have focused on a few regions of possible interest, other regions such as the neostriatum caudolaterale and area corticoidea dorsolateralis are certain to play important, and probably integrated, roles in cognition [Hartmann and Güntürkün, 1998; Waldmann and Güntürkün, 1993]. In addition, to better compare and contrast our knowledge collected from the mammalian brain to that from birds, more research is needed to understand the differences and similarities in brain structure, development, connectivity and function between these taxa. Although many homologies exist, in terms of both development and function, between the mammalian and avian brain [Reiner et al., 2002], differences remain [Rattenborg et al., 2011], which make our under-

standing of the evolution of cognition and the brain more complex and interesting.

In conclusion, in examining several regions of the brain possibly associated with fear, problem solving and learning, we have failed to provide conclusive evidence that population-related differences in problem solving and neophobia are associated with differences in volume and neuron numbers in captive animals. However, we found some differences in these structures between wild-caught chickadees from different populations, which raises questions about the effects of captivity on the expression of brain regions. In addition, our results raise several important questions about our understanding of and ability to examine the relationship between behavior and the brain: (1) How broad is the adaptive specialization hypothesis of the brain and can it be used to explain the differences in complex cognitive behaviors between populations? (2) Do our measures of brain morphology (e.g. volume, total neuron number) possess adequate accuracy and precision to detect subtle changes in brain structures should they exist? (3) How conserved are the proposed functions of the regions analyzed between mammals and birds, and how might evolution have produced similar behaviors from different regions of the brain? (4) To what extent are cognitive behaviors associated with the Hp (i.e. might it act as a mediator)? (5) Similarly, are other regions of the brain more important than those examined for the expression of advanced cognition and how might they be connected to those targeted in this study?

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