Spatial ability is impaired and hippocampal mineralocorticoid receptor mRNA expression reduced in zebra finches (*Taeniopygia guttata*) selected for acute high corticosterone response to stress

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In mammals, stress hormones have profound influences on spatial learning and memory. Here, we investigated whether glucocorticoids influence cognitive abilities in birds by testing a line of zebra finches selectively bred to respond to an acute stressor with high plasma corticosterone (CORT) levels. Cognitive performance was assessed by spatial and visual one-trial associative memory tasks. Task performance in the high CORT birds was compared with that of the random-bred birds from a control breeding line. The birds selected for high CORT in response to an acute stressor performed less well than the controls in the spatial task, but there were no significant differences between the lines in performance during the visual task. The birds from the two lines did not differ in their plasma CORT levels immediately after the performance of the memory tasks; nevertheless, there were significant differences in peak plasma CORT between the lines. The high CORT birds also had significantly lower mineralocorticoid receptor mRNA expression in the hippocampus than the control birds. There was no measurable difference between the lines in glucocorticoid receptor mRNA density in either the hippocampus or the paraventricular nucleus. Together, these findings provide evidence to suggest that stress hormones have important regulatory roles in avian spatial cognition.

Keywords: corticosterone; artificial selection; spatial learning; mineralocorticoid receptor; glucocorticoid receptor; zebra finch

1. INTRODUCTION

Glucocorticoids, released from the adrenal cortex following stressful events, have important effects on spatial learning and memory processes in vertebrates. Glucocorticoids enhance spatial learning and memory at intermediate levels (e.g. Shors *et al.* 1992), but low or high levels adversely affect cognition (Kirschbaum *et al.* 1996; Lupien & McEwen 1997). They influence the activity of the hypothalamic–pituitary–adrenal (HPA) axis through their action on mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) in the brain (e.g. Reul & de Kloet 1985; McEwen *et al.* 1986; Van Eekelen *et al.* 1988). GRs are suggested to play a role following stress and during the circadian rhythm in corticosterone (CORT) secretion and MR is thought to be important in the control of basal HPA activity. In mammals, GRs are found throughout the brain, but at the highest density in the paraventricular nucleus and hippocampus. MRs, which have higher affinity than GRs for glucocorticoids (they are occupied at lower concentrations), are mainly concentrated in the hippocampus and lateral septum (Reul & de Kloet 1985).

Songbirds provide useful models with which spatial memory and the structure and function of the hippocampus can be examined (e.g. Krebs *et al.* 1989; Healy & Krebs 1992). However, although hormonal effects on avian song learning have received a great deal of attention, there is still rather little known of the hormonal effects on spatial cognition and the avian hippocampus. There is some evidence to suggest that glucocorticoids affect avian cognition. For example, following CORT administration, food-storing mountain chickadees (*Parus gambeli*) show superior cache retrieval and spatial memory performance compared with control birds (Saldanha *et al.* 2000; Pravosudov 2003). Moreover, small but chronic elevations

in CORT triggered by unpredictable food also correlate with enhanced cache retrieval and performance by mountain chickadees on a one-trial associative spatial memory task (Pravosudov & Clayton 2001). Taken together, it appears that an elevation in circulating CORT levels is associated with enhanced spatial abilities.

In wild birds, selection pressures on the CORT response to stress occur when they must modulate their neuroendocrine and behavioural systems to successfully reproduce (Breuner *et al.* 2003; Meddle *et al.* 2003; Wingfield & Sapolsky 2003).

Given that it is likely to be selection on the CORT response to stress in the wild, and CORT can affect spatial cognition, we examined whether selection on the CORT response affected avian cognition. The performance of zebra finches selected to respond to mild stress with high plasma CORT levels (Evans *et al.* 2006) on spatial and visual one-trial associative memory tasks was compared with that of zebra finches from a random-bred control line. We predicted that performance specifically on the spatial task would correlate with plasma CORT levels. Furthermore, as spatial memory is dependent on hippocampal function, we examined the sensitivity of the avian hippocampus to glucocorticoids by examining MR and GR mRNA expression and comparing the corticosteroid receptor density in birds from both the lines.

2. MATERIAL AND METHODS

(a) Subjects

Birds were sexually mature, captive-bred adult zebra finches (13–20 g body weight) from either the high CORT ($n=20$, 10 females, 10 males) or the control ($n=16$, 8 females, 8 males) line, the selection process for which is described in detail by Evans *et al.* (2006). The lines differed in their peak CORT response after one generation of selection and all the birds were drawn from the F_3 generation, which also significantly differed in peak CORT between the lines (Evans *et al.* 2006).

All the birds received a unique combination of plastic coloured leg bands for identification and were housed in single sex groups of six or fewer birds in wire-mesh cages (77 cm long × 44 cm wide × 44 cm high) in a windowless room. All the birds were in full visual and auditory contact with each other and fed daily with a seed mixture supplemented by fresh vegetables, millet spray and dried cuttlefish bone; water was *ad libitum*. Birds were maintained on a 16 : 8 h light : dark cycle at a temperature range of 19–21°C. For both training and experiments, birds were deprived of food at 08.00 and provided with fresh food as soon as their session was complete. Training and testing began at 14.00.

One week prior to the beginning of behavioural training, the CORT response to stress was examined in all the birds. The stress response was elicited by a standardized capture/restraint technique (Wingfield *et al.* 1992), whereby a 100 µl blood sample was taken for the CORT measurement 20 min following capture and placement into a cloth drawstring bag. This technique was identical to the method used during the selection process. Blood samples were obtained by puncturing the alar wing vein with a 25 G needle and collecting the blood into heparinized microhaematocrit tubes. The blood sample was kept on ice until it was centrifuged (14 000 *g* for

10 min) within 1 h of collection. Plasma was collected and stored at −20°C until radioimmunoassay was performed for CORT (see §2*e*). All the procedures were carried out under licence from the UK Home Office and under local ethical review. All efforts were made to minimize the number of birds used.

(b) Behavioural training

An experimental tray (Perspex board 29 cm × 22 cm) with 48 circular wells (1 cm diameter, 1 cm deep and 2.5 cm apart from each other) arranged in a 6 by 8 grid was used to assess memory performance. The wells were surrounded by Velcro to which square felt flaps, measuring 2.5 cm × 2.5 cm, could be attached. The birds were trained by placing them each alone in the test cage for 30 min each day and gradually covering the wells containing seeds, so that the birds learned to pull the flaps off the wells to get to the seed. The test phase of the experiment began when the bird uncovered at least three wells (when seven wells were covered) in a 5 min period.

(c) One-trial associative memory task

Each task consisted of two phases (sample and choice) separated by a retention interval of 5 min. In both the phases, seven wells were covered with only one containing bird seed, chosen at random. In the spatial task, all the flaps were red and differed only in position. In the visual task, the rewarded well was covered with a piece of felt that differed in colour from the flaps covering the other six wells. There were six different flap colours used, with the flap colour for each trial chosen such that the unrewarded well cover colour was not used to cover the reward in the next trial.

In the sample phase, the tray was placed into the centre of the test cage and the bird was allowed to remove as many flaps as necessary to find the food and eat for 30 s (consuming some, but not all, of the seed). The tray was then removed for a 5 min retention interval.

In the choice phase of the spatial task, the tray was returned with the flaps in the same locations as in the sample phase. The seed remained in the rewarded well. In the visual task, the locations of the covered wells were all different from the sample phase when the tray was returned, although the flap colours designating the rewarded/unrewarded wells were unchanged. In both the versions of the task, the test ended when the bird had eaten all of the remaining food or after 5 min, whichever occurred sooner. The number of flaps the bird removed to find the food in the choice phase was recorded and used as the measure of performance. Revisits to the same compartment were not recorded, as once the flap had been removed the contents of the well were visible. As performance on cognitive tasks can vary across trials owing to variables extraneous to the experiment, we attempted to reduce the variation within each group by testing the birds once a day for 5 days for both the spatial and visual tasks. Half of the birds were tested on the spatial task followed by the visual task and the remaining half were tested in the reverse order. All the birds completed both the tasks. The bird was tested on each task as soon as it had reached the test criterion. The same test criterion was satisfied before it was tested on the second version of the task.

(d) Memory tests immediately following restraint stress

To investigate whether memory performance was affected in response to stress, and thereby increased the CORT levels,

memory performance was compared between the breeding lines following restraint stress. On completion of either a visual or a spatial task, each bird was restrained for 20 min in a cloth bag and then retested on that task. The following day, the procedure was repeated, but this time performance on the other task was tested before and after restraint.

(e) Plasma corticosterone

Following the second memory test, each bird was immediately captured, weighed, scored for fat deposits (the average of furcular and abdominal fat measured on a semi-quantitative scale of 1–5, with 5 being the fattest; see Helms & Drury 1960) and killed by decapitation. Blood sample (approx. 100 µl) was collected from the neck using heparinized capillary tubes. Brains were quickly removed and frozen immediately on dry ice and stored at −70°C until processed for *in situ* hybridization (see §2*f*). As plasma CORT concentrations increase rapidly following exposure to stressful or adverse conditions (e.g. Wingfield *et al.* 1992), all blood samples were taken within 3 min of capture to obtain circulating levels of CORT at the time of behavioural testing. Blood samples were refrigerated and then centrifuged (14 000 *g* for 10 min) within 1 h of collection. Plasma was collected and stored at -20°C until radioimmunoassay was performed for CORT. The plasma CORT concentrations after extraction were measured using anti-CORT antiserum (code B3-163, Endocrine Sciences, Tarzana, CA, USA) and [1,2,6,7-3H]-CORT label (Amersham, UK). The extraction efficiency was 75–100%. All samples were run in a single assay. Fifty per cent binding was at 8.9 ng ml^{-1}, the detection limit (for 7.3 µl aliquots of extracted plasma) was 0.73 ng ml^{-1} and the intra-assay coefficient of variation was 3.6%.

(f) In situ hybridization histochemistry for glucocorticoid receptor and mineralocorticoid receptor mRNA expression

Brain GR and MR mRNA expression was compared between the adult zebra finches from the high CORT ($n=9$) or the control ($n=7$) line. Levels of expression were compared in the hippocampal formation for both GR and MR and in the paraventricular nucleus for GR.

Fragments of zebra finch GR (479 bp; Genbank DQ864494) and MR (587 bp; nt 2239–nt 2825 Genbank DQ539433) were cloned into the vector pGEM-7zf. GR sense and antisense riboprobes were generated by *in vitro* transcription, in the presence of 35S-UTP, with SP6- and T7-RNA polymerase after plasmid linearization with EcoRI or HindIII, respectively. MR sense and antisense riboprobes were generated by *in vitro* transcription, in the presence of 35S-UTP, with T7- and SP6-RNA polymerase after plasmid linearization with HindIII or ApaI, respectively.

Whole brains were sectioned sagitally on a cryostat and thaw mounted onto polysine pretreated glass microscope slides and stored at −70°C. Every fifth section was separately mounted and stained with cresyl violet (Sigma, Poole, UK) and coverslipped with DPX mountant (Merck-BDH, Lutterworth, UK) to serve as a marker to locate the region of interest. Selected slide-mounted sections from each bird were thawed to room temperature and immersed in 4% paraformaldehyde solution for 10 min, then rinsed in PBS before treatment with 0.3% triethanolamine/acetic anhydride. The slides were then rinsed in PBS and dehydrated through a series of graded ethanols. Sections were incubated at 50°C with prehybridization solution for

2 h and hybridized with the 35S-labelled antisense or sense riboprobe directed against the zebra finch GR or MR in a solution mixed with 50% formamide. Each probe was applied to each section at a concentration of 10^6 cpm per slide in 200 µl hybridization solution for 18 h at 55°C in a humidified chamber. Post-hybridization washes consisted of three 5 min washes in 2× saline–sodium citrate (SSC). Sections were then incubated in a 30 µg ml^{-1} ribonuclease A (RNase-A) solution for 1 h at 37°C, followed by a 30 min rinse in 2× SSC at room temperature followed by stringency washes in 0.1× SSC at 50°C for 90 min, followed by two 60 min rinses in 0.1× SSC at room temperature. Test assays were used to determine the optimal wash temperature for each probe. Finally, the tissue was dehydrated in a graded series of ethanol containing 300 mM ammonium acetate. The hybridization signal was visualized at the cellular level by dipping the slides in autoradiographic emulsion (Ilford 135 5053). Slides were air dried and stored with desiccant at 4°C for four to five weeks before being developed (Kodak D19), counterstained with haematoxylin and eosin, dehydrated through ethanol to xylene and finally cover slipped with DPX mountant (Merck-BDH, Lutterworth, UK). Slides were examined with a light microscope, under brightfield illumination.

Control procedures for the antisense GR and MR probes included hybridization of sections with the sense riboprobe, or pretreatment with RNase-A prior to hybridization with the antisense riboprobe, conducted under identical conditions to those for the antisense probe. There was no detectable hybridization signal with the sense probe or following RNase-A pretreatment.

(g) Quantification of autoradiographs

Anatomical identification of brain structures was based on the stereotaxic canary brain atlas of Stokes *et al.* (1974) and www.avianbrain.org. The slides were coded so that during the quantitative analysis, the experimenter was unaware of the treatment group each slide belonged to. Autoradiographs were evaluated by measuring silver grain density over individual neurons within the region of interest (×40 objective) using a computer-aided image analysis system (Open Lab, Improvision). Neurons were considered labelled, if the mean number of overlying silver grains was three times greater than that of the equivalent area of background. Background measurements were made in the cerebellum. Silver grain counts were made over 20 randomly chosen labelled neurons per region of interest per section and in four sections per bird. Means were calculated for each variable in each animal; these values were used to calculate group means.

(h) Statistical analyses

The data were analysed with SAS (SAS Institute) using a general linear model with score (number of flaps lifted in choice phase) as the dependent variable and sex, line, group, task, bird (nested within sex, breeding line and group) and day as the independent variables. To account for repeated sampling, *F*-values and associated *p*-values were calculated using the bird mean sum of squares term as the denominator to calculate the *F*-statistic for sex, line, group and the line by task interaction. To compare between breeding lines, Student's *t*-test was employed with significance considered at $p<0.05$.
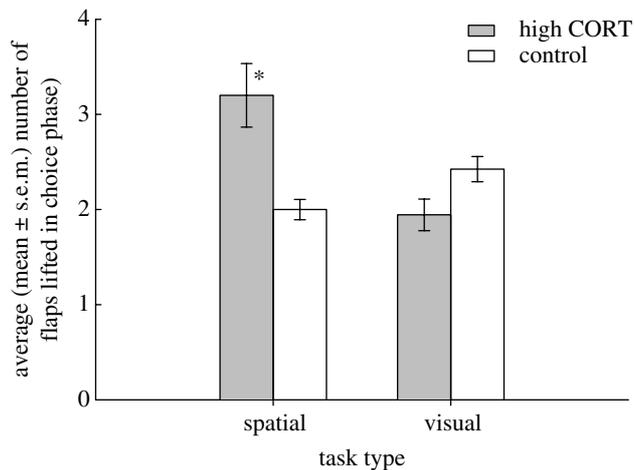
Figure 1. Mean ($\pm$s.e.m.) number of flaps lifted in the choice phase of the visual and spatial tasks ($n=19$). All the birds tended to perform better on the visual task than on the spatial task. The high CORT birds performed significantly worse than the random-bred controls on the spatial task, whereas there was no difference in performance between the lines on the visual task. Asterisk indicates $p<0.05$.

3. RESULTS

(a) *One-trial associative memory task*

Only 19 out of the 36 birds (11 high CORT line, 8 control line; 9 females, 10 males, equally divided across the lines) reached test criterion. Performance (measured as the number of flaps raised) showed no significant effect of bird ($F_{16,169}=0.87$, $p=0.60$), sex ($F_{1,16}=0.02$, $p=0.90$), task ($F_{1,16}=3.55$, $p=0.08$) or line ($F_{1,16}=2.54$, $p=0.13$). However, there was a significant interaction between task and line ($F_{1,16}=14.57$, $p=0.002$). The data from the two tasks were then analysed separately.

On the spatial task, the high CORT birds performed significantly less well than did the control birds ($F_{1,16}=7.80$, $p=0.01$). There was no effect of bird (figure 1; $F_{16,76}=1.37$, $p=0.18$) or sex ($F_{1,16}=0.10$, $p=0.76$) on performance. On the visual task, there was no significant effect of bird (figure 1; $F_{16,76}=0.58$, $p=0.89$), sex ($F_{1,16}=0.10$, $p=0.75$) or line, although the high CORT birds tended to perform better than the control birds ($F_{1,16}=3.90$, $p=0.07$).

Following restraint, all the birds, regardless of breeding line, failed to perform either the spatial or the visual task (i.e. they did not lift any flaps).

(b) *Body measurements and plasma corticosterone*

The high CORT birds had significantly lower body mass than the random-bred control birds (high CORT, $n=9$: 16.2 ± 0.5 g versus control, $n=8$: 18.3 ± 0.5 g; $t=2.83$, $p=0.013$). However, there was no significant difference between the lines in fat score (high CORT, $n=9$: 2.5 ± 0.25 g versus control, $n=8$: 3.4 ± 0.5 g; $t=85.5$, $p=0.21$).

The plasma data were analysed using a general linear model, with CORT level as the dependent variable and sex, line and, where relevant, bird (nested within sex and line) as the independent variables. The level of circulating CORT levels at the time of testing did not explain the performance discrepancy between lines, as there was no significant difference in circulating CORT levels ($F_{1,12}=3.32$, $p=0.10$; $n=14$); the amount of plasma collected from five birds was insufficient for radioimmunoassay.
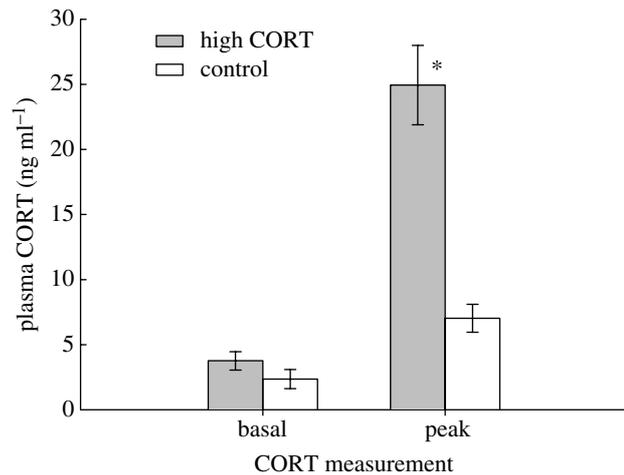

Figure 2. Average ($\pm$s.e.m.) stress-induced plasma CORT levels and those immediately after memory task performance (basal) in the high CORT and random-bred control line zebra finches. The difference between the two CORT measurements (taken during memory testing and following 20 min of restraint stress) was significantly greater in the high CORT line (*$p<0.05$).

Although circulating levels of CORT did not differ between the lines, peak CORT levels (taken following an acute stressor, prior to behavioural training) did differ: the high CORT line birds had significantly higher peak levels of CORT than did the controls ($F_{1,16}=15.82$, $p=0.001$; figure 2). There were no other significant effects found among the variables assessed.

(c) *Glucocorticoid receptor and mineralocorticoid receptor expression*

GR mRNA expression in the zebra finch brain was found to be most dense in the hippocampus, paraventricular nucleus, nidopallium and cerebellum. The distribution of MR mRNA expression was more restricted than that of GR mRNA expression, with the highest expression in the hippocampus. MR mRNA expression was significantly higher in the hippocampus of birds from the control line ($n=7$) compared with those from the high CORT line ($n=8$, $t=2.22$, $p=0.045$; figure 3). There was no significant difference in GR mRNA expression in the hippocampus ($t=0.37$, $p=0.72$; figure 3) or the paraventricular nucleus between the lines of zebra finches (high CORT line: 897 ± 78.4 versus control line: 1128 ± 217.4, $t=1.26$, $p=0.25$).

4. DISCUSSION

Zebra finches selectively bred for high peak plasma CORT levels in response to an acute stressor performed significantly less well than did the random-bred control birds on a one-trial associative spatial memory task. This difference in performance cannot be explained by the variation in levels of CORT circulating immediately after testing, as these did not differ, although among the same birds peak CORT levels were significantly different between the lines. Birds from the two lines also differed in expression levels of MR mRNA specifically in the hippocampus, but not in GR mRNA expression. MR mRNA was more restricted to the hippocampus than was the expression of GR mRNA.
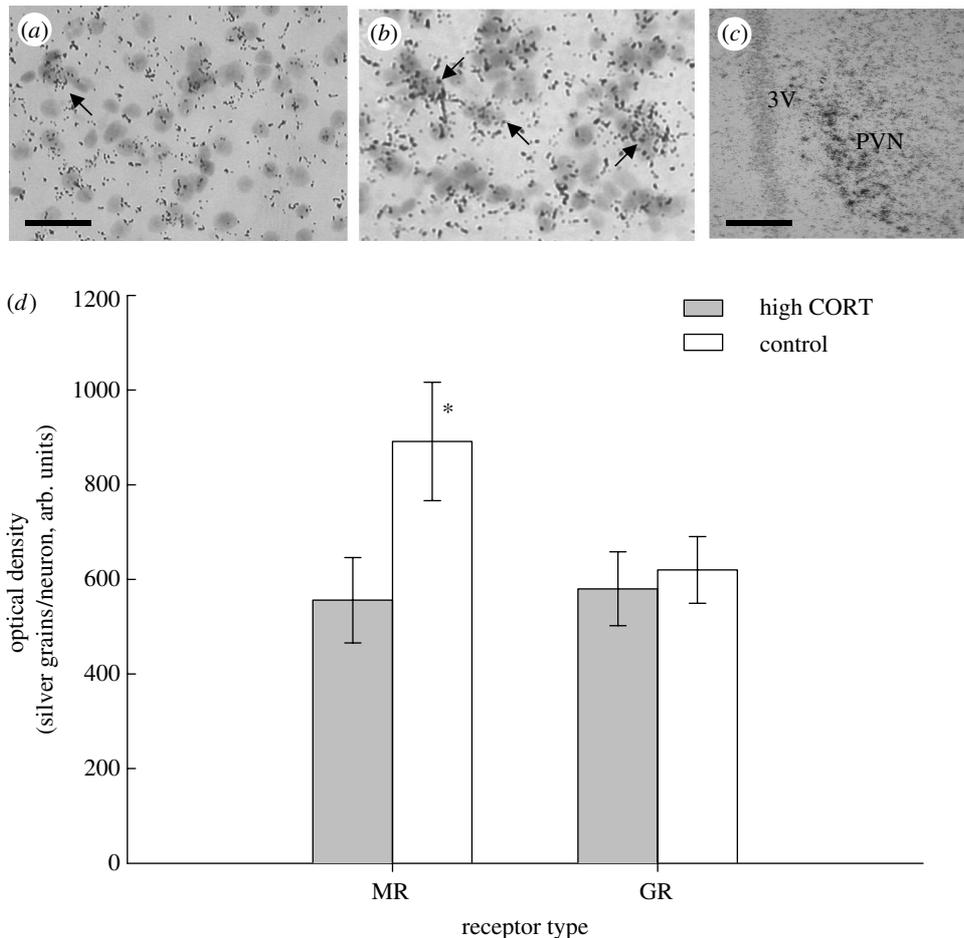
Figure 3. Representative photomicrographs of autoradiographs in brightfield showing cells in the hippocampus expressing MR mRNA in (*a*) high CORT and (*b*) random-bred control line zebra finches. Arrows indicate examples of a MR mRNA expressing cell. Scale bar, 50 μm. (*c*) Representative photomicrograph of autoradiograph from a sagital section showing cells expressing GR mRNA expression in the paraventricular nucleus (PVN). 3V, third ventricle; scale bar, 1.75 mm. (*d*) Mean MR and GR mRNA expression ($\pm$ s.e.m.) in the hippocampus of zebra finches bred for high stress levels of corticosterone (high CORT) compared with a random-bred control line (control). MR mRNA expression is significantly lower in the high CORT line compared with the control line (*$p < 0.05$). There was no significant difference in GR mRNA expression between the lines ($p > 0.05$).

As there was no relationship between the circulating levels of CORT and task performance, it seems unlikely that the impairment on the spatial task can be attributed to activational steroid effects. Instead, we suggest that selection for a high peak stress CORT level exerts an organizational effect in the brain (particularly in the hippocampus) during early development and that the resulting modification to the hippocampus has a specific effect on spatial learning and memory. This explanation is consistent with the finding that spatial ability in black-legged kittiwakes (*Rissa tridactyla*) was compromised by experimentally elevated CORT levels during early development, even though CORT levels were similar at the time of testing (Kitaysky *et al.* 2003). Although the mechanism underlying the performance impairment of the high CORT line is not yet clear, the fact that we have found differences in hippocampal MR mRNA expression leads us to suggest that the spatial memory difference may be attributable to the differences in adrenal steroid receptor densities in the brain.

We do not have an explanation for the tendency of the high CORT birds to perform better than the controls at the visual task. It is interesting to note that their learning and memory abilities are not generally impaired, supporting the interpretation that the variation in hippocampal MR mRNA expression impacts specifically on spatial cognition. It is possible that slightly better performance on the visual task is a result of differences in attention between the groups or in learning and memory for visual features that comes about because the spatial abilities are impaired. This would be consistent with the data from experiments comparing memory for visual and spatial features in mammals, in which females have poorer spatial abilities than do males but tend to outperform males when the task can be solved using visual cues (Jones *et al.* 2003; Jones & Healy 2006).

Our mapping of GR and MR mRNA distribution in the zebra finch brain is comparable with *in situ* hybridization data from rats, in which MR mRNA expression is higher in the hippocampus relative to other brain regions, including the hypothalamus (Chao *et al.* 1989). In the rat brain, as we have found in the zebra finch, GR mRNA expression is widespread, with strong labelling in the amygdala, hypothalamus, including the paraventricular nucleus (Aronsson *et al.* 1988; Morimoto *et al.* 1996). The similarity in the distribution of GR mRNA expression between rat and zebra finch implies it is probably that, as in rats, neuronal populations in zebra finches are sensitive to the regulatory action of glucocorticoids.

The reduction in hippocampal MR mRNA expression in the high CORT birds is also consistent with rat data. Chronic stress in, or glucocorticoid administration to, rats results in the decreases in MR and GR binding of mRNA expression (Chao *et al.* 1989 Herman *et al.* 1995; Kitraki *et al.*1999). Moreover, increases in hippocampal MR mRNA occur after adrenalectomy (Reul *et al.* 1989; Herman & Spencer 1998), all of which support the suggestion that these receptors are regulated by gluco-corticoids. Receptor downregulation may protect neurons from the detrimental effects of stress, especially since hippocampal neurons are particularly vulnerable to damage after prolonged CORT exposure (McEwen & Sapolsky 1995). This notion is supported by the finding that white-crowned sparrows (*Zonotrichia leucophrys gambelii*) breeding in extreme habitats have lower levels of GR-like receptors than their temperate conspecifics *Zonotrichia leucophrys oriantha* (Breuner *et al.* 2003). It has been hypothesized that such differences are a consequence of having a less sensitive response to stressors.

Unlike the sparrows, the zebra finch lines differed in hippocampal MR densities rather than in GR densities. In rats, blockage of GRs, but not MRs, disrupts the consolidation of spatial information in the Morris water maze performance (Oitzl & de Kloet 1992; Conrad *et al.* 1999). However, chronic central blockade of MR in rats does impair spatial memory in the water maze (Yau *et al.* 1999). Moreover, our data are consistent with the previously published studies, whereby hippo-campal MR expression is increased and memory improved, following chronic antidepressant treatment (Casolini *et al.* 1997) or CORT treatment during lactation (Yau *et al.* 1995).

In conclusion, selection for a high CORT response to an acute stressor affects spatial learning and memory. Unlike mountain chickadees, which respond with increased spatial ability to an increase in circulating CORT (Pravosudov 2003), zebra finches selected for high peak CORT levels in response to an acute stressor performed significantly less well than did the random-bred control birds on a spatial memory task. As the lines of birds did not differ in the circulating levels of CORT immediately after task performance, it appears that the selection on these lines for variation in peak CORT levels is having its most significant neural effect during development. The coupling of poor spatial performance with fewer hippocampal MRs leads us to suggest that these receptors underpin spatial ability in these birds. This is the first demonstration, to our knowledge, that the distri-bution of MR and GR mRNA in the avian brain is correlated with cognitive performance.

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