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Corticosterone and cortisol binding sites in plasma, immune organs and brain of developing zebra finches: Intracellular and membrane-associated receptors

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ABSTRACT

Glucocorticoids (GCs) affect the development of both the immune and nervous systems. To do so, GCs bind to intracellular receptors, mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). In addition, GCs bind to membrane-associated corticosteroid receptors (mCR). Two well-known GCs are corticosterone and cortisol. Whereas corticosterone is the primary GC in zebra finch plasma, cortisol is the primary GC in zebra finch lymphoid organs and is also present in the brain and plasma during development. Here, we characterized binding sites for corticosterone and cortisol in plasma, liver, lymphoid organs, and brain of developing zebra finches. In tissues, we examined both intracellular and membrane-associated binding sites. For intracellular receptors, there were MR-like sites and GR-like sites, which differentially bound corticosterone and cortisol in a tissue-specific manner. For mCR, we found little evidence for membrane-associated receptors in immune organs, but this could be due to the small size of immune organs. Interestingly, cortisol, but not corticosterone, showed a low amount of specific binding to bursa of Fabricius membranes. For neural membranes, corticosterone bound to one site with low affinity but a relatively high B_{max}, and in contrast, cortisol bound to one site with high affinity but a lower B_{max}. Our results indicate that intracellular and membrane-associated receptors differentially bind corticosterone and cortisol suggesting that corticosterone and cortisol might have different roles in immune and nervous system development.

1. Introduction

Glucocorticoids (GCs) have important effects on the development of many physiological systems, particularly the immune and nervous systems. For example, in the immune system, developmental GC treatment can suppress cell-mediated immune responses (Loiseau et al., 2008; Rubolini et al., 2005), and GCs may regulate positive and negative selection of $CD4^+$ $CD8^+$ thymocytes (Ashwell et al., 2000; Vacchio et al., 1999). In the brain, neonatal GC treatment can alter corticosteroid receptor levels in the brain of rats, affecting the hypothalamic–pituitary–adrenal (HPA) axis in adulthood (Welberg et al., 2001; Liu et al., 2001). Lastly, neonatal GC administration and stressors can cause long-term impairments in learning and cognition in a variety of species (Spencer and Verhulst, 2007; Aisa et al., 2007).

GCs affect the development of the immune system and brain by binding to receptors in target tissues. There are two intracellular receptors that bind GCs: the Type 1 or mineralocorticoid receptor (MR), which binds endogenous GCs with high affinity, and the Type 2 or glucocorticoid receptor (GR), which binds endogenous GCs with lower affinity (Funder, 1992; de Kloet et al., 1990). The intracellular corticosteroid receptors are part of the steroid receptor superfamily and act as ligand-dependent transcription factors (Carson-Jurica et al., 1990). GR is expressed in cells of the immune system and brain during development. In the immune system, GR

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is present in lymphocytes of embryonic mice and regulates thymocyte selection (Ashwell et al., 2000; Jondal et al., 2004). For example, in transgenic mice, thymocyte GR over-expression decreases thymocyte number (Pazirandeh et al., 2005). In addition, administering the mammalian GR antagonist RU 486 to cultured thymic cells increases apoptosis of immature thymocytes (Vacchio et al., 1994). In the brain, expression of GR and MR in the hippocampus of rats and mice increases across development and reaches adult-like levels between the second and third weeks (Pryce, 2008). Intracellular corticosteroid receptors are involved in neuronal migration (Fukumoto et al., 2009) and synapse maturation (Kumamaru et al., 2008) during development.

In addition to the cytosolic receptors, GCs can bind to membrane-associated receptors (Song and Buttgereit, 2006; Trueba et al., 1987). In the immune system, membrane-associated corticosteroid receptors (mCR) have been detected in human peripheral blood mononuclear cells (Tryc et al., 2006), T cells (Löwenberg et al., 2008), and murine lymphoma cells (Gametchu, 1987; Gametchu et al., 1999) and may mediate GC-induced apoptosis (Stahn and Buttgereit, 2008). In the brain, mCR have been well characterized in adult rough-skinned newts (*Taricha granulosa*; Orchinik et al., 1991) and tiger salamanders (*Ambystoma tigrinum*; Orchinik et al., 2000). In newts, GCs bind to mCR in medullary neurons to rapidly inhibit reproductive behavior (Rose et al., 1993; Moore and Orchinik, 1994). Brain mCR have also been examined in adult house sparrows (*Passer domesticus*; Breuner and Orchinik, 2001, 2009) and rats (Moutsatsou et al., 2001; Towle and Sze, 1983). Few studies have examined mCR in the immune system or brain during development (Sze and Towle, 1993).

The two well-known GCs that bind to corticosteroid receptors are corticosterone and cortisol. Corticosterone is considered the primary circulating GC in some species (e.g., rats, mice, birds), whereas cortisol is considered the primary circulating GC in other species (e.g., humans, fish). The prevailing belief is that a species has just one predominant GC, corticosterone or cortisol, and that these two GCs are interchangeable, in the sense that they act via similar mechanisms and have similar effects. However, there are species that have both hormones present in equal concentrations, for example, New Zealand white rabbits (Szeto et al., 2004). In addition, the ratio of these two GCs can vary depending on the site of sampling. In humans, the corticosterone to cortisol ratio is five to sixfold higher in cerebrospinal fluid (Raubenheimer et al., 2006) and post-mortem brain tissue (Karssen et al., 2001) than in plasma, which suggests that corticosterone might have more important effects on the human brain than previously thought (Raubenheimer et al., 2006). Furthermore, although corticosterone is the predominant circulating GC in adult birds (Wingfield et al., 1982; Nakamura and Tanabe, 1973), cortisol is the predominant GC in lymphoid organs of developing zebra finches (*Taeniopygia guttata*) and is also present in the plasma and brain (Schmidt and Soma, 2008; Schmidt et al., 2009). These findings suggest that the identity of the predominant GC can be organ-specific and age-specific (Schmidt and Soma, 2008) and raise the hypothesis that corticosterone and cortisol may exert different effects (Schmidt et al., 2008) by differentially binding to corticosteroid receptors. For example, in young chickens, the GR in the bursa of Fabricius (hereafter bursa; the primary avian immune organ that produces B cells; Glick and Olah, 1982; Cooper et al., 1966) have a higher affinity for cortisol than corticosterone (Sullivan and Wira, 1979).

There were two goals of the current study. First, we wanted to determine whether there are both intracellular and membrane-associated corticosteroid receptors in immune organs and brain during development. Second, we wanted to determine if corticosteroid receptors differentially bind corticosterone and cortisol. Cortisol is the more abundant GC in zebra finch lymphoid organs during development, so we predicted that there would be a receptor in lymphoid organs that preferentially binds cortisol. Also, locally-synthesized steroids are more likely to act via membrane-associated receptors (Schmidt et al., 2008). For example, in mice, 17β-estradiol is the principal estrogen in the blood, but 17α-estradiol may be more abundant in the brain and binds with higher affinity to a membrane-associated receptor (Toran-Allerand et al., 2005). Therefore, we predicted that cortisol may be the preferred ligand for a mCR in immune organs.

2. Methods

2.1. Subjects

Research was carried out under a University of British Columbia (UBC) permit, following the guidelines of the Canadian Council on Animal Care. Subjects were male and female juvenile zebra finches (n = 110 total). The zebra finch is an altricial songbird species that is commonly used for studies of avian endocrinology, neurobiology, and stress physiology (Goodson et al., 2005). Zebra finches serve as an excellent species for this study because plasma GC levels have been well characterized in adulthood (Wada et al., 2008) and development (Schmidt and Soma, 2008; Wada et al., 2009). Further, developmental changes in both corticosterone and cortisol levels in immune organs and brain have been determined (Schmidt and Soma, 2008; Schmidt et al., 2009).

Zebra finches were sampled at approximately 30 days post-hatch (P30) (mean ± SEM = 30.21 ± 0.18 d). Cortisol levels in immune tissues are elevated, relative to corticosterone levels, at P30 (Schmidt and Soma, 2008). Moreover, this is the youngest age at which we could collect a sufficient amount of tissue for the binding assays. Breeding pairs of adult zebra finches were given millet seeds, water, grit, and cuttlefish bone *ad libitum*. Breeding pairs were also given a daily supplement consisting of boiled chicken eggs, cornmeal, and bread. The light cycle was 14L:10D (lights on at 08:00). Temperature was held at ~23 °C and relative humidity at ~50%. At P30, zebra finches had fledged the nest but remained in the cage with their parents. Zebra finches reach nutritional independence at ~P30 and reproductive maturity at ~P90 (Zann, 1996).

2.2. Chemicals

[1,2,6,7-^{3}H]Corticosterone (specific activity = 70 Ci/mmol, NET399001MC) and [1,2,6,7-^{3}H]cortisol (specific activity = 72.4 Ci/mmol, NET396001MC) were purchased from Perkin-Elmer Life Sciences (Waltham, MA, USA). Unlabeled steroids were purchased from Steraloids (Newport, RI, USA). Mitotane, RU 486, activated charcoal, Trisma base, HEPES, sucrose, EDTA, polyethylenimine, molybdic acid, glycerol, bovine serum albumin, and dithiothreitol were all purchased from Sigma–Aldrich (Oakville, ON, Canada).

2.3. Sample collection and preparation

2.3.1. Plasma

All subjects were sampled between 08:00 and 12:00. Plasma was collected to characterize the corticosteroid-binding globulin (CBG). Whole blood was collected by puncturing the brachial vein with a 26-gauge needle and collecting blood into heparinized micro-hematocrit tubes. All blood samples were collected within 3 min of opening the cage door. Blood was centrifuged at 10,000 rpm for 10 min. Plasma was collected and stored at −20 °C. For equilibrium saturation binding and competition assays, plasma from multiple subjects was pooled. Prior to the binding assays, plasma was charcoal stripped (1% Norit A charcoal and 0.1% dextran in 50 mM Tris buffer) to remove endogenous steroids.

Plasma and charcoal were incubated at room temperature for 20 min and then centrifuged at 1400 rpm for 10 min at 4 °C. Supernatant was collected and stored at −20 °C. Plasma was diluted 1:999 (final dilution) for use in binding assays. To verify that the charcoal stripping procedure removed endogenous GCs, plasma corticosterone levels were measured in three adult zebra finches. Half of the plasma from each individual was used without charcoal stripping, and half of the plasma was used after charcoal stripping. In all three individuals, corticosterone levels were non-detectable in charcoal-stripped plasma but detectable in non-charcoal-stripped plasma (range = 2.36–16.93 ng/mL).

2.3.2. Cytosol

To decrease endogenous GCs that might interfere with the ability to detect GC binding sites in cytosol, birds used for cytosolic receptor assays were given one intramuscular injection of 1.2 mg/g mitotane (ortho, para, dichlorodiphenyl dichloroethane) between 20:00 and 21:00 and then sacrificed 36–39 h post-injection between 08:00 and 12:00 (Breuner et al., 2000; Breuner and Orchinik, 2009). There are no significant variations in baseline plasma corticosterone levels throughout this 4 h time period in white-crowned sparrows (Breuner et al., 1999). Mitotane decreases circulating corticosterone levels in songbirds (Breuner et al., 2000), and surgical adrenalectomy is very difficult in small songbirds. Prior to sacrifice, a blood sample was collected within 3 min of disturbance (see above) for CBG characterization and corticosterone measurement. Next, to remove circulating CBG that interferes with cytosolic receptor assays, zebra finches were anesthetized with isoflurane and transcardially perfused with 10 mL ice-cold heparinized avian saline (0.75% NaCl). After saline perfusion, the brain and body were chilled on wet ice for 2 min. The brain was dissected first, and brain regions collected include the cerebellum, telencephalon, and diencephalon. The cerebellum was dissected first. Next, the telencephalon was bisected into left and right hemispheres and collected. Lastly, the diencephalon was collected to the depth of the anterior commissure (Schmidt et al., 2009). Peripheral tissues collected include the bursa, thymus, spleen, liver, and breast muscle. As each tissue was dissected, it was immediately frozen on dry ice. Tissues were stored at −80 °C.

On the day of the assay, tissues were homogenized over ice with ice-cold TEGMD buffer (10 mM Trisma base, 1 mM EDTA, 10% glycerol, 20 mM molybdic acid, and 5 mM dithiothreitol, pH 7.40) using a glass-Teflon homogenizer (10 strokes). Then an equal volume of charcoal was added (1% Norit A charcoal and 0.1% dextran in TEGM buffer) and samples were centrifuged at 104,000*g* for 1 h at 4 °C (Breuner and Orchinik, 2009). The supernatant was then collected and used in the radioligand binding assays on the same day. The pellet containing cell nuclei and membranes was discarded. Protein concentration in cytosol samples was determined using Bradford reagent and a standard curve of bovine serum albumin (BSA).

The effect of mitotane treatment on circulating corticosterone levels was determined with a sensitive and specific ^{125}I-radioimmunoassay (MP Biomedicals, 07-120103) that has been validated for songbird plasma (Newman et al., 2008; Charlier et al., 2009). The detection limit was 3.12 pg corticosterone per tube (defined as the lowest standard). Non-detectable samples were set to zero (Schmidt and Soma, 2008; Schmidt et al., 2009). Plasma was assayed without an extraction step (2 μL per assay tube). Positive and negative controls were analyzed in each corticosterone assay ($n = 2$). Plasma corticosterone levels were lower in birds injected with mitotane (0.76 ± 0.12 ng/mL, range = 0–2.96 ng/mL) compared to birds that were not injected with mitotane (4.24 ± 0.80 ng/mL, range = 0–21.69 ng/mL; $t = 5.53$, df = 93, $P < 0.0001$). Plasma corticosterone levels were detectable in 42% of mitotane-treated birds and 91% of untreated birds. These data verify that mitotane treatment effectively decreased circulating corticosterone levels in zebra finches.

2.3.3. Membranes

For membrane assays, birds were sacrificed without mitotane injection or saline perfusion. Previous studies in house sparrows indicate that mitotane treatment, saline perfusion, or charcoal stripping do not affect specific binding of GCs to membranes (Breuner and Orchinik, 2001, 2009). A blood sample was taken from the brachial vein and birds were sacrificed by rapid decapitation within 3 min of disturbance. The brain and body were then chilled on wet ice for 2 min, and tissues were dissected as described above and stored at −80 °C. To prepare membranes, tissue was homogenized over ice in ice-cold buffer containing 5 mM Hepes and 0.32 M sucrose (pH 7.40) using a glass-Teflon homogenizer (8 strokes) and then brought up to 25× tissue weight with buffer containing 5 mM Hepes and 0.32 M sucrose and centrifuged at 1000*g* for 12 min at 4 °C. The resulting pellet containing cell nuclei was discarded, and the supernatant was collected and centrifuged at 30,000*g* for 30 min at 4 °C. The supernatant was then discarded and the pellet was resuspended in hypoosmotic buffer (5 mM Hepes, 1 mM EDTA, and bacitracin 60 μg/mL, pH 7.4) using trituration and dounce homogenization. The supernatant and hypoosmotic buffer mixture was incubated for 2 h at 4 °C to dissociate endogenous ligand. After the 2 h incubation, homogenate was centrifuged at 30,000*g* for 30 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 25 mM Hepes (pH 7.40) using trituration and dounce homogenization. The homogenate was then spun a final time at 30,000*g* for 30 min at 4 °C. The resulting pellet was stored at −20 °C overnight and used in radioligand binding assays the following day. This differential centrifugation procedure results in a well-washed membranous pellet that is enriched with synaptosomes and mitochondria for brain tissue and enriched with mitochondria for peripheral tissues. Protein concentration in membrane samples was determined using a Bradford assay with NaOH treatment to remove proteins from membranes (Breuner and Orchinik, 2009).

2.4. Radioligand binding assays

Procedures were similar to Breuner and Orchinik (2001, 2009). Optimal incubation time was determined for cytosol and membrane binding assays by incubating pools of cytosol or membranes for 2, 3, 4, or 5 h to determine when the percentage of specific binding was greatest. For cytosol, brain and liver tissue were incubated at room temperature for 2 h, and immune tissues were incubated at room temperature for 4 h. All membrane assays were incubated at 10 °C for 2 h. Plasma was incubated at 4 °C for 2 h (Breuner and Orchinik, 2009). All incubations included 25 μL of [^{3}H]corticosterone or [^{3}H]cortisol, 25 μL of prepared cytosol, membranes, or plasma, and 25 μL of assay buffer (for total binding tubes) or 25 μL of competitor. Non-specific binding was determined using 1 μM unlabeled corticosterone or cortisol. The assay buffer was 50 mM Trisma base (pH 7.40) for plasma, TEGM (10 mM Trisma base, 1 mM EDTA, 10% glycerol, 20 mM molybdic acid, pH 7.40) for cytosol, and 25 mM Hepes (pH 7.40) for membranes. The rinse buffer was 25 mM Trisma base (pH 7.40) for plasma and membranes and TEM (5 mM Trisma base, 1 mM EDTA, 10 mM molybdic acid, pH 7.4) for cytosol.

Equilibrium saturation binding studies were performed to determine the dissociation constant (K_d, an estimate of affinity), the binding maximum (B_{max}), and the number of types of binding sites. We used both [^{3}H]corticosterone and [^{3}H]cortisol as radioligands. To facilitate direct comparisons of the affinity of the receptor/binding globulin to corticosterone versus cortisol, the same pool of cytosol, membranes, or plasma was used for both [^{3}H]corticosterone and [^{3}H]cortisol saturation binding assays. For cytosol, 12–16 concentrations of radioligand were used (0.08–63 nM); for membranes, nine concentrations of radioligand were used (0.86–24 nM); and for plasma, nine concentrations of radioligand were

used (0.45–12 nM). Because tissues in juvenile zebra finches are small, generally only one equilibrium saturation binding assay using [^{3}H]corticosterone and one using [^{3}H]cortisol as ligand was done per tissue. However, we were able to do two saturation assays per ligand for liver cytosol, as well as three saturation assays for plasma using [^{3}H]corticosterone, and the results were highly reproducible (see below). Since some tissues were small, for cytosol saturation analysis, we pooled 19 bursa, 21 thymi, 32 spleens, 12 cerebellums, or 15 diencephalons. For membrane saturation analysis, we pooled 35 each of bursa, diencephalon, thymi, or spleen, 34 cerebellums, or 18 pieces of liver.

Competition binding studies were also performed with cytosol from select tissues (bursa, thymus, telencephalon, and diencephalon) and plasma to determine the specificity of the binding sites. Competition studies were not done for membranes because of limitations in tissue amounts. Competition studies were done using 2 nM [^{3}H]corticosterone and 6–12 concentrations of unlabeled competitor (0.1 nM–1 μM).

For all assays, incubations were terminated using rapid vacuum filtration (Brandel Harvester, Gaithersburg, MD, USA) over Whatman glass-fiber filters. For studies using cytosol and plasma, GF/B filters were soaked in rinse buffer with 0.3% polyethylenimine for 60 min prior to use. For assays using membranes, GF/C filters were soaked in rinse buffer for 60 min prior to use. Vacuum filtration was followed by 3 × 3 mL (plasma and cytosol) or 2 × 3 mL (membranes) rinse with ice-cold rinse buffer. Filters were then placed into 7 mL scintillation vials, incubated with 100 μL of ethanol for 10 min, and then 5 mL of scintillation fluid was added and samples were mixed using a vortex. Samples were allowed to equilibrate at room temperature for 12 h and then counted by standard liquid scintillation counting. All points within assays were run in triplicate. For saturation and competition studies, pools of tissue or plasma from multiple individuals were used, and tissue or plasma was pooled regardless of sex, as no sex differences in GC binding has been detected in cytosol, membranes, or plasma in adult house sparrows (Breuner and Orchinik, 2001) and no sex differences have been detected in corticosterone or cortisol levels in developing zebra finches (Schmidt and Soma, 2008; Schmidt et al., 2009). Since comparisons between [^{3}H]corticosterone and [^{3}H]cortisol binding were performed using the same pool of plasma, cytosol, or membranes, differences between corticosterone and cortisol binding cannot be attributed to potential sex differences in glucocorticoid binding.

2.5. Statistics

Radioligand binding data were analyzed using non-linear regression analyses using GraphPad Prism (San Diego, CA). Binding parameter estimates (K_d and B_{max} values) with approximate SEMs were calculated by fitting data to appropriate equations using iterative, least-squares curve-fitting techniques. Standard errors were calculated from the goodness of fit of the data to the equation describing the relationship. K_d and B_{max} estimates were compared for [^{3}H]cortisol and [^{3}H]corticosterone assays using *t*-tests (GraphPad Prism). Tests were done using the calculated K_d or B_{max} and its SEM and the number of concentrations of ligand used in the saturation analyses as the mean, SEM, and sample size, respectively. Tests were two-tailed. Test results were considered significant for $p \leqslant 0.05$. Data are presented as means ± SEM.

3. Results

3.1. Plasma binding globulin

Plasma assays were optimized to >80% specific binding by incubating plasma at 1:999 final dilution for 2 h at 4 °C. Three equilibrium saturation binding assays were done for plasma using [^{3}H]corticosterone as ligand to verify that results were reproducible. In all three assays, data were best fit by a one-site binding model, with binding parameters consistent with CBG. The K_d ranged from 1.37 to 4.82 nM, with an average K_d of 3.58 ± 1.11 nM, which is similar to what has been reported for nestling zebra finches (K_d = 5.12; Wada et al., 2009).

To compare K_d and B_{max} estimates between [^{3}H]cortisol and [^{3}H]corticosterone, one pool of charcoal-stripped plasma was used for saturation analyses (Fig. 1A). Equilibrium saturation binding of both [^{3}H]corticosterone (K_d = 1.37 ± 0.14 nM, B_{max} = 69.01 ± 2.09 fmol/mg protein) and [^{3}H]cortisol (K_d = 1.29 ± 0.09 nM, B_{max} = 144.60 ± 2.81 fmol/mg protein) was best fit by a one-site model. There was no significant difference between the K_d estimates for [^{3}H]corticosterone and [^{3}H]cortisol binding (t = 0.48, df = 16, P = 0.64). The B_{max} estimate for [^{3}H]cortisol binding was significantly higher than for [^{3}H]corticosterone binding (t = 21.58, df = 16, P < 0.0001). For competition studies (Fig. 1B), the potencies for inhibition of [^{3}H]corticosterone binding are shown in Table 1. The plasma binding site had high affinity for progesterone, corticosterone, cortisol, and testosterone, but not 17β-estradiol (E_2), consistent with what has been found in house sparrows (Breuner and

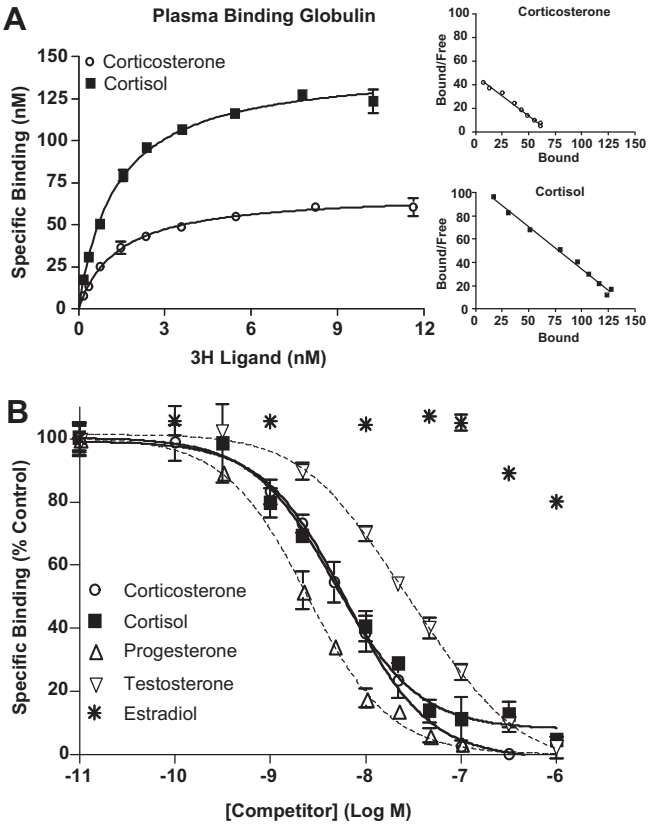

Fig. 1. Plasma. (A) Equilibrium saturation binding of [^{3}H]corticosterone and [^{3}H]cortisol to charcoal-stripped plasma from 30-day-old zebra finches. Shown are specific binding data (mean ± SEM for each concentration) done in triplicate, plus a line that best describes the fit of the data. Data were best fit by a one-site model for both [^{3}H]corticosterone (K_d = 1.37 ± 0.14 nM, B_{max} = 69.01 ± 2.09 fmol/mg protein) and [^{3}H]cortisol (K_d = 1.29 ± 0.09 nM, B_{max} = 144.60 ± 2.81 fmol/mg protein). Insets are Scatchard–Rosenthal replots of the data. (B) Inhibition of [^{3}H]corticosterone binding by various ligands in charcoal-stripped plasma from 30-day-old zebra finches. Shown is specific binding, expressed as the percentage of specific [^{3}H]corticosterone binding in the absence of competitor. The displacement of [^{3}H]corticosterone by both unlabeled corticosterone and cortisol was best fit by a one-site binding model. Inhibition constant (K_i) for [^{3}H]corticosterone = 2.52 nM, K_i for [^{3}H]cortisol = 1.84 nM. IC_{50} values are given in Table 1.

Table 1
Inhibition of [^{3}H]corticosterone specific binding by competitors in plasma and cytosol.

Tissue	IC_{50} Corticosterone	IC_{50} Cortisol	IC_{50} RU 486	IC_{50} PROG	IC_{50} T	IC_{50} E_2
Plasma	6.19	4.70	N/A	2.41	25.28	>200
Bursa	22.73	24.39	5.91	>200	>200	>200
Thymus	9.12	32.33	13.08	>200	>200	>200
Telencephalon Type 1	0.73	2.26	>200	>200	>200	>200
Telencephalon Type 2	6.98	26.40	>200	>200	>200	>200
Diencephalon	8.53	16.93	>200	>200	>200	>200

Note. N/A, data are not available because assay was not performed. IC_{50} values are in nM. PROG, progesterone; T, testosterone; E_2, 17β-estradiol.

Orchinik, 2009) and white-crowned sparrows (*Zonotrichia leucophrys*; Charlier et al., 2009).

3.2. Intracellular receptors

3.2.1. Liver: cytosol

Binding parameters estimates and results from *t*-tests comparing [^{3}H]corticosterone and [^{3}H]cortisol binding are summarized in Table 2. Cytosol assays for liver were optimized to >80% specific binding using a 3–5 mg/mL protein concentration in a 2 h incubation at room temperature. For liver cytosol (Fig. 2A), two equilibrium saturation binding assays were performed. The first assay was done using 12 concentrations (0.08–20 nM) of [^{3}H]corticosterone and [^{3}H]cortisol, and the second assay was done using 16 concentrations (0.08–60 nM). Both assays indicated that equilibrium saturation binding of [^{3}H]corticosterone to liver cytosol was best fit by a two-site model, with a high affinity site similar to a MR (MR-like), and a lower affinity site more similar to a GR (GR-like) (Table 2). The MR-like and GR-like sites had similar B_{max} estimates, indicating that the two receptors were present at similar densities. In contrast, both assays indicated that equilibrium saturation binding of [^{3}H]cortisol was best fit by a one-site model, with an affinity similar to a GR (Table 2). The K_d and B_{max} estimates were similar between the two assays, for both [^{3}H]corticosterone and [^{3}H]cortisol (Table 2), indicating that the method produced consistent results. There were no significant differences between the K_d estimates or the B_{max} estimates for [^{3}H]corticosterone and [^{3}H]cortisol binding to the GR-like site for either assay (Table 2).

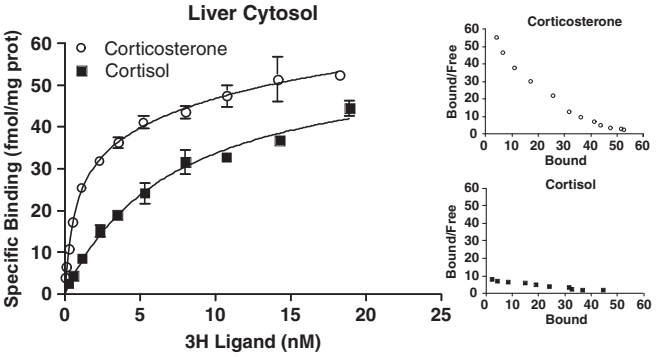

Fig. 2. Liver cytosol. Equilibrium saturation binding of [^{3}H]corticosterone and [^{3}H]cortisol in a pool of liver cytosol from 30-day-old zebra finches (n = 6 subjects for one pool). Shown are specific binding data (mean ± SEM for each concentration) done in triplicate, plus a line that best describes the fit of the data. Estimates of binding parameters (K_d and B_{max}) are shown in Table 2 "First assay". Insets are Scatchard–Rosenthal replots of the data.

3.2.2. Immune organs: cytosol

Cytosol assays for immune tissues were optimized to >70% specific binding using a 2–4 mg/mL protein concentration in a 4 h incubation at room temperature. A higher concentration of protein might have further increased the percentage of specific binding, but because immune tissues were so small it was not possible to increase the protein concentration in assays.

For both bursa (Fig. 3A) and thymus (Fig. 3B) cytosol, equilibrium saturation binding of [^{3}H]corticosterone was best fit by a two-site model, with a MR-like site that had a low B_{max} and a lower affinity GR-like site that had a high B_{max}. Equilibrium saturation binding of [^{3}H]cortisol to both bursa and thymus cytosol was best fit by a one-site model with an affinity more similar to a GR and a high B_{max}. In contrast, in spleen cytosol (Fig. 3C), [^{3}H]corticosterone was best fit by a one-site model, with a K_d characteristic of a MR, whereas [^{3}H]cortisol binding was best fit by a two-site model, with both a MR-like site that had a low B_{max}, and a GR-like site that had a higher B_{max} (Table 2). The B_{max} estimate for [^{3}H]corticosterone was significantly higher than the B_{max} estimate for [^{3}H]cortisol for the GR-like site in thymus and the MR-like site in spleen (Table 2).

Table 2
Cytosol: binding parameters for liver, immune organs, and brain.

Tissue	K_d Corticosterone	K_d Cortisol	*t*	*P*	B_{max} Corticosterone	B_{max} Cortisol	*t*	*P*
Liver Type 1 (1st assay)	**0.69 ± 0.21**	**ND**	–	–	**36.76 ± 7.92**	**ND**	–	–
Liver Type 1 (2nd assay)	**0.92 ± 0.52**	**ND**	–	–	**35.03 ± 13.30**	**ND**	–	–
Liver Type 2 (1st assay)	19.99 ± 33.55	7.22 ± 0.93	0.38	0.71	37.22 ± 22.36	58.03 ± 3.36	0.92	0.37
Liver Type 2 (2nd assay)	17.25 ± 14.57	7.18 ± 1.11	0.69	0.50	40.64 ± 9.23	45.91 ± 2.35	0.55	0.58
Bursa Type 1	**0.47 ± 0.27**	**ND**	–	–	**35.27 ± 11.44**	**ND**	–	–
Bursa Type 2	26.28 ± 10.51	12.79 ± 1.99	1.26	0.22	386.50 ± 75.87	242.90 ± 20.67	1.60	0.13
Thymus Type 1	**0.10 ± 0.06**	**ND**	–	–	**25.97 ± 5.68**	**ND**	–	–
Thymus Type 2	11.62 ± 2.70	9.19 ± 1.70	0.76	0.45	**226.60 ± 18.95**	**77.71 ± 7.52**	7.30	**<0.0001**
Spleen Type 1	0.39 ± 0.04	0.63 ± 0.44	0.54	0.60	**47.21 ± 0.99**	**14.39 ± 7.81**	4.17	**0.0004**
Spleen Type 2	**ND**	**13.28 ± 6.91**	–	–	**ND**	**79.16 ± 9.53**	–	–
Telencephalon Type 1	**0.22 ± 0.02**	**0.99 ± 0.16**	4.78	**<0.0001**	98.89 ± 2.84	101.20 ± 9.74	0.23	0.82
Telencephalon Type 2	389 ± 4256	243 ± 3239	–	–	471 ± 4902	203 ± 2429	–	–
Diencephalon Type 1	**0.15 ± 0.02**	**0.65 ± 0.05**	9.27	**<0.0001**	**68.53 ± 5.32**	**81.04 ± 1.41**	2.27	**0.03**
Diencephalon Type 2	**7.10 ± 5.59**	**ND**	–	–	**32.82 ± 5.23**	**ND**	–	–
Cerebellum Type 1	0.17 ± 0.05	0.36 ± 0.55	0.34	0.74	62.33 ± 9.04	20.24 ± 27.76	1.44	0.16
Cerebellum Type 2	**7.98 ± 1.52**	**3.36 ± 1.08**	2.48	**0.02**	**248.4 ± 10.51**	**146.4 ± 23.68**	3.94	**0.0007**

Note. Type 1 represents the binding site with mineralocorticoid receptor-like affinity, whereas Type 2 represents the binding site with glucocorticoid receptor-like affinity. K_d values ± standard errors are in nM, and B_{max} values ± standard errors are in fmol/mg protein. ND = specific binding was best fit by a one-site model and this binding site was not detected. Values in bold represent significant differences between corticosterone and cortisol binding, or cases when a binding site was detected for one ligand but not the other. Tissues were pooled from 30-day-old zebra finches (n = 6–32 individuals per pool, see Section 2).

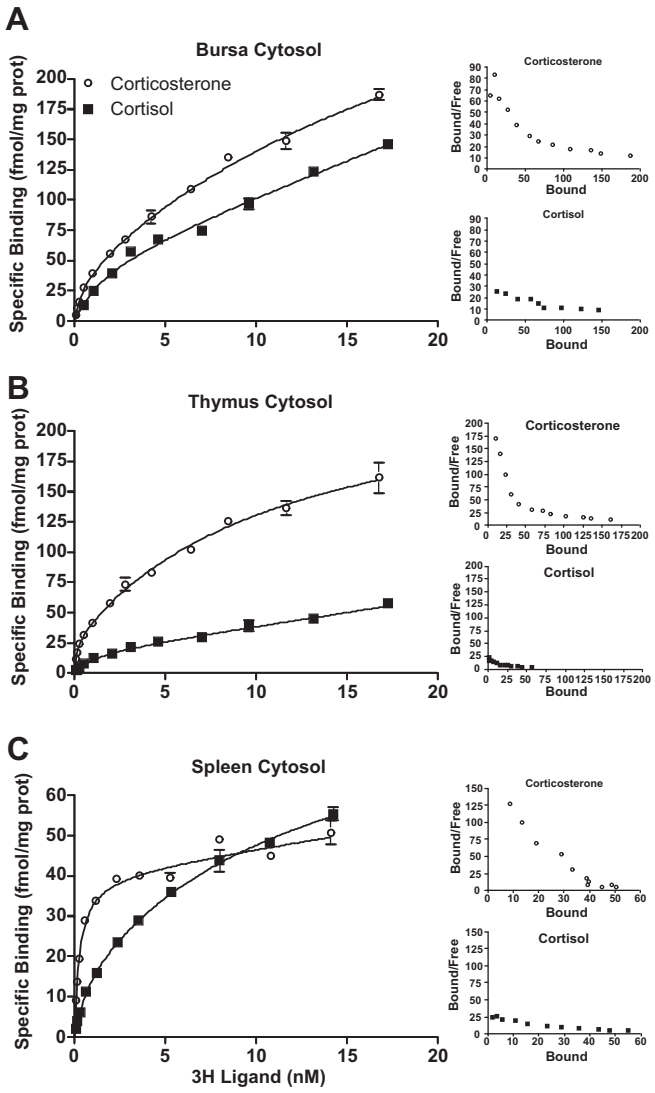

Fig. 3. Immune organ cytosol. Equilibrium saturation binding of [^{3}H]corticosterone and [^{3}H]cortisol in pools of (A) bursa (n = 19 subjects for one pool), (B) thymus (n = 21 subjects for one pool), and (C) spleen (n = 32 subjects for one pool) cytosol from 30-day-old zebra finches. Shown are specific binding data (mean ± SEM for each concentration) done in triplicate, plus a line that best describes the fit of the data. Estimates of binding parameters (K_d and B_{max}) are shown in Table 2. Insets are Scatchard–Rosenthal replots of the data.

For competition studies, the potencies for inhibition of [^{3}H]corticosterone binding are shown in Table 1. In bursa cytosol (Fig. 4A), the mammalian GR antagonist RU 486 displaced [^{3}H]corticosterone with the highest affinity, corticosterone and cortisol displaced [^{3}H]corticosterone with similar affinity, and progesterone, testosterone, and E_2 did not compete for [^{3}H]corticosterone binding sites. For thymus cytosol (Fig. 4B), corticosterone displaced [^{3}H]corticosterone with highest affinity, followed by RU 486 and then cortisol, whereas progesterone, testosterone and E_2 did not displace [^{3}H]corticosterone binding.

3.2.3. Brain: cytosol

Assays for brain cytosol were optimized to >80% specific binding using a 3–5 mg/mL protein concentration in a 4 h incubation at room temperature.

For both telencephalon (Fig. 5A) and diencephalon (Fig. 5B) cytosol, equilibrium saturation binding of [^{3}H]corticosterone was best fit by a two-site model, with a MR-like site and a lower affinity site (Table 2). [^{3}H]cortisol binding was best fit by a two-site model in telencephalon cytosol, but bound to only an MR-like site in diencephalon cytosol (Table 2). The K_d estimates for the second binding site in telencephalon cytosol for both [^{3}H]corticosterone and [^{3}H]cortisol binding were much higher than what is typically observed for GR. Furthermore, there were very large SEMs associated with the binding parameters for this second site, possibly because this second receptor represents only a small fraction of the total receptor number, making it difficult to get precise estimates of binding parameters. GR levels also appear low in diencephalon cytosol as suggested by the low B_{max} estimate for [^{3}H]corticosterone binding. In cerebellum cytosol (Fig. 5C), equilibrium saturation binding of both [^{3}H]corticosterone and [^{3}H]cortisol was best fit by a two-site model, with both ligands binding to a MR-like and a GR-like site (Table 2). Unlike telencephalon and diencephalon cytosol, the B_{max} estimates for the GR-like site in cerebellum cytosol were higher than the B_{max} estimates for the MR-like site, which appears to constitute a small fraction of the total receptor number (Table 2). In telencephalon and diencephalon cytosol, the K_d estimates for the MR-like site were significantly lower for [^{3}H]corticosterone than [^{3}H]cortisol. In contrast, in cerebellum cytosol the K_d estimate for the GR-like site was significantly higher for [^{3}H]corticosterone than [^{3}H]cortisol indicating that the presumed GR in cerebellum cytosol bound [^{3}H]cortisol with higher affinity than [^{3}H]corticosterone (Table 2).

For competition studies, the potencies for inhibition of [^{3}H]corticosterone binding are shown in Table 1. For both telencephalon (Fig. 6A) and diencephalon cytosol (Fig. 6B), corticosterone inhibited [^{3}H]corticosterone with the highest affinity, followed by cortisol. RU 486 did not compete for binding to [^{3}H]corticosterone binding sites, consistent with results from the saturation analyses indicating that GR levels in telencephalon and diencephalon cytosol are very low in P30 zebra finches.

3.3. Membrane-associated receptors

3.3.1. Liver and breast muscle: membranes

Binding parameters estimates and results from t-tests comparing [^{3}H]corticosterone and [^{3}H]cortisol binding are summarized in Table 3. Assays for liver membranes were optimized to >70% specific binding using a 4–5 mg/mL protein concentration in a 2 h incubation at 10 °C. For liver membranes (Fig. 7), equilibrium saturation binding of both [^{3}H]corticosterone and [^{3}H]cortisol was best fit by a one-site model (Table 3). The K_d estimate was significantly higher for [^{3}H]corticosterone than [^{3}H]cortisol indicating that the liver mCR bound [^{3}H]cortisol with higher affinity than [^{3}H]corticosterone. The B_{max} estimate was high for both [^{3}H]corticosterone and [^{3}H]cortisol and not different (Table 3).

[^{3}H]Corticosterone and [^{3}H]cortisol binding to breast muscle membranes (data not shown) did not converge to either a one-site or a two-site binding model and specific binding was close to 0 fmol/mg protein.

3.3.2. Immune organs: membranes

Despite pooling tissues from several subjects, protein concentration in immune organ membrane assays was low (<2 mg/mL, the lowest standard in the protein assay). This made it difficult to detect specific binding, if present. [^{3}H]Corticosterone binding to bursa membranes did not converge to either a one-site or two-site binding model, and specific binding was very low. [^{3}H]Cortisol binding to bursa membranes was best fit by a one-site model; however the B_{max} estimate was low (Table 3). [^{3}H]Corticosterone and [^{3}H]cortisol binding to thymus and spleen membranes did not converge to either a one-site or a two-site binding model and specific binding was very low (close to 0 fmol/mg protein) (Table 3).

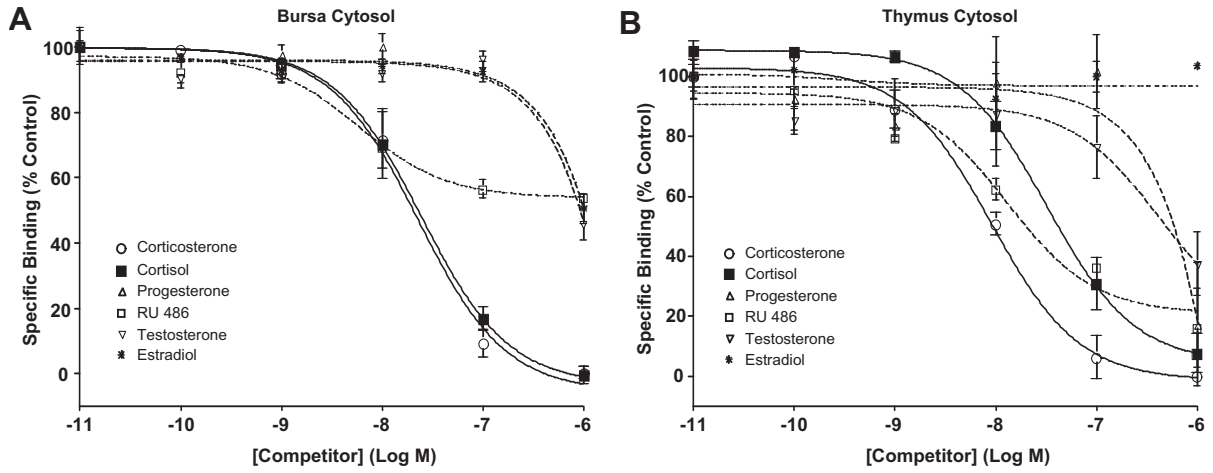

Fig. 4. Immune organ cytosol. Inhibition of [^{3}H]corticosterone binding by various ligands in pools of (A) bursa (n = 24 subjects for one pool) and (B) thymus (n = 20 subjects for one pool) cytosol from 30-day-old zebra finches. Shown is specific binding, expressed as the percentage of specific [^{3}H]corticosterone binding in the absence of competitor. The displacement of [^{3}H]corticosterone by unlabeled corticosterone and cortisol in bursa and thymus cytosol was best fit by a one-site binding model. IC_{50} values are given in Table 1.

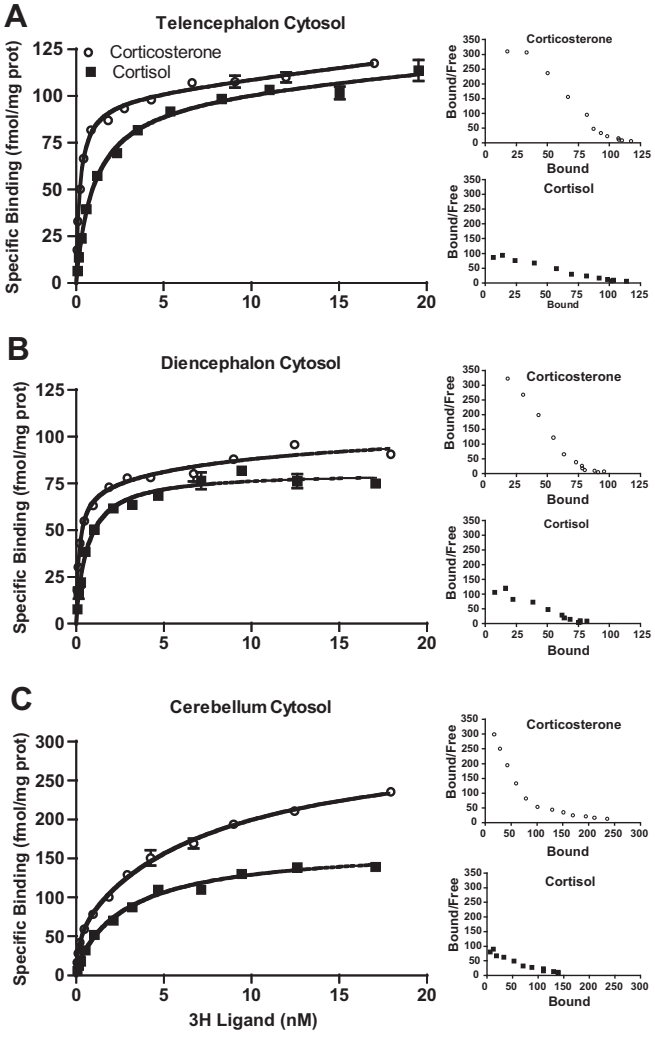

Fig. 5. Brain cytosol. Equilibrium saturation binding of [^{3}H]corticosterone and [^{3}H]cortisol in pools of (A) telencephalon (n = 6 subjects for one pool), (B) diencephalon (n = 15 subjects for one pool), and (C) cerebellum (n = 12 subjects for one pool) cytosol from 30-day-old zebra finches. Shown are specific binding data (mean ± SEM for each concentration) done in triplicate, plus a line that best describes the fit of the data. Estimates of binding parameters (K_d and B_{max}) are shown in Table 2. Insets are Scatchard–Rosenthal replots of the data.

3.3.3. Brain: membranes

Specific binding of [^{3}H]corticosterone to neuronal membranes in all three brain regions was ~50% of total binding. Membrane assays were performed using a 4–5 mg/mL protein concentration in a 2 h incubation at 10 °C.

For telencephalon (Fig. 8A), diencephalon (Fig. 8B), and cerebellum (Fig. 8C) membranes, equilibrium saturation binding of [^{3}H]corticosterone was best fit by a one-site model, and the K_d estimates were similar in all three regions (Table 3) and were similar to what has been reported for neural membranes in house sparrows (Breuner and Orchinik, 2001). Equilibrium saturation binding of [^{3}H]cortisol was also best fit by a one-site model for all three brain regions, however B_{max} estimates for [^{3}H]cortisol binding were low. Interestingly, although the B_{max} estimate for [^{3}H]cortisol binding was low in diencephalon and cerebellum membranes, the K_d estimate for [^{3}H]cortisol was also low, indicating that [^{3}H]cortisol bound to mCR with high affinity in these regions. For diencephalon and cerebellum membranes, the K_d and B_{max} estimates for [^{3}H]corticosterone binding were significantly higher than [^{3}H]cortisol binding (Table 3).

4. Discussion

We partially characterized GC binding sites in juvenile zebra finch plasma, liver, immune organs, and brain. For tissues, we examined intracellular as well as membrane-associated receptors. In addition, we used both [^{3}H]cortisol and [^{3}H]corticosterone as ligands. This is one of few studies to directly compare [^{3}H]corticosterone and [^{3}H]cortisol binding in tissues in any species (see Sutanto et al., 1988; Jones et al., 1979). Furthermore, we compared [^{3}H]corticosterone and [^{3}H]cortisol binding in zebra finches, a species in which we have previously shown that the identity of the predominant GC is organ-specific. In addition, this is one of the first studies to examine mCR in a developing animal (Sze and Towle, 1993). We found that both intracellular and membrane-associated receptors differentially bound [^{3}H]corticosterone and [^{3}H]cortisol. First, for brain cytosol, the MR-like site in forebrain bound [^{3}H]corticosterone with higher affinity, but the GR-like site in cerebellum bound [^{3}H]cortisol with higher affinity. Second, in thymus, bursa, and liver cytosol, [^{3}H]cortisol only bound to a GR-like site, whereas [^{3}H]corticosterone bound to both a MR-like and a GR-like site. Third, bursa membranes showed specific binding to [^{3}H]cortisol but not [^{3}H]corticosterone. Lastly, brain mCR bound [^{3}H]corticoste-

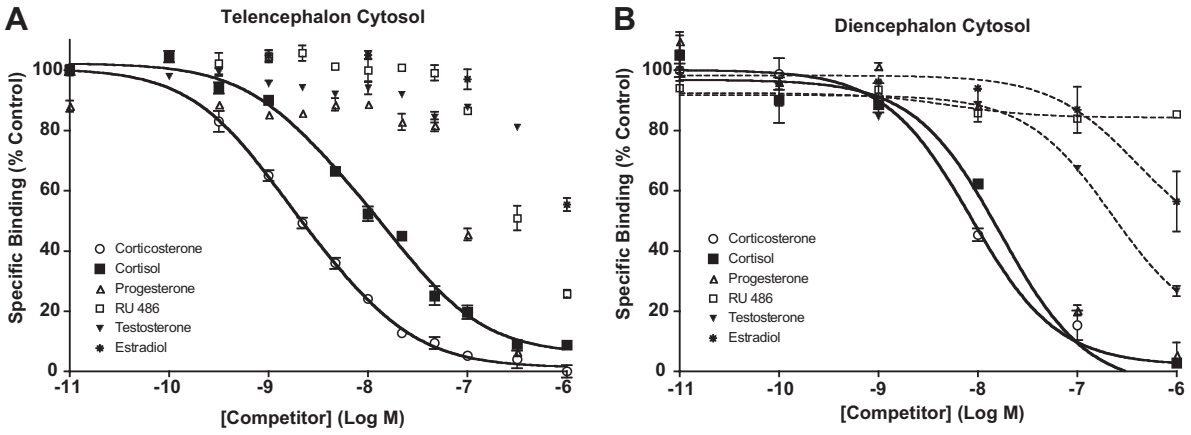

Fig. 6. Brain cytosol. Inhibition of [^{3}H]corticosterone binding by various ligands in pools of (A) telencephalon (n = 13 subjects for one pool) and (B) diencephalon (n = 19 subjects for one pool) cytosol from 30-day-old zebra finches. Shown is specific binding expressed as the percentage of specific [^{3}H]corticosterone binding in the absence of competitor. The displacement of [^{3}H]corticosterone by both unlabeled corticosterone and cortisol was best fit by a two-site binding model in telencephalon cytosol and by a one-site binding model in diencephalon cytosol. IC_{50} values are given in Table 1.

Table 3
Membranes: binding parameters for liver, breast muscle, immune organs, and brain.

Tissue	K_d Corticosterone	K_d Cortisol	t	P	B_{max} Corticosterone	B_{max} Cortisol	t	P
Liver	**3.60 ± 0.26**	**2.62 ± 0.14**	3.38	**0.004**	151.20 ± 3.96	151.50 ± 2.43	0.06	0.95
Breast muscle	ND	ND	–	–	ND	ND	–	–
Bursa	**ND**	**5.39 ± 2.38**	–	–	**ND**	**31.67 ± 5.46**	–	–
Thymus	ND	ND	–	–	ND	ND	–	–
Spleen	ND	ND	–	–	ND	ND	–	–
Telencephalon	15.51 ± 6.21	31.45 ± 28.90	0.54	0.60	54.47 ± 14.14	23.41 ± 15.66	1.47	0.16
Diencephalon	**14.60 ± 1.14**	**3.20 ± 1.30**	6.59	**<0.0001**	**89.03 ± 3.91**	**10.83 ± 1.53**	18.62	**<0.0001**
Cerebellum	**15.72 ± 2.32**	**2.02 ± 0.76**	5.66	**<0.0001**	**96.56 ± 8.20**	**12.54 ± 1.34**	10.11	**<0.0001**

Note. In no case were data best fit by a two-site binding model. ND = nondetectable, specific binding did not converge to either a one-site or a two-site binding model. K_d values ± standard errors are in nM, and B_{max} values ± standard errors are in fmol/mg protein. Values in bold represent significant differences between corticosterone and cortisol binding, or cases when a binding site was detected for one ligand but not the other. Tissues were pooled from 30-day-old zebra finches (n = 7–35 subjects per pool, see Section 2).

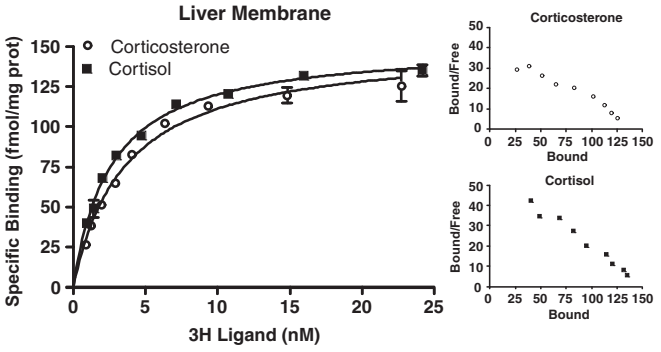

Fig. 7. Liver membranes. Equilibrium saturation binding of [^{3}H]corticosterone and [^{3}H]cortisol in a pool of liver membranes from 30-day-old zebra finches (n = 18 subjects for one pool). Shown are specific binding data (mean ± SEM for each concentration) done in triplicate, plus a line that best describes the fit of the data. Estimates of binding parameters (K_d and B_{max}) are shown in Table 3. Insets are Scatchard–Rosenthal replots of the data.

rone with low affinity and a high B_{max}, and in contrast bound [^{3}H]cortisol with high affinity and a low B_{max}. This suggests that there is one mCR that differentially binds these two ligands or that there is more than one mCR in brain.

4.1. Reproducibility of results

One limitation of the present study is that due to the small amount of tissue available from juvenile zebra finches, in most cases only one equilibrium saturation binding analysis using [^{3}H]corticosterone and one using [^{3}H]cortisol was done per tissue. However, several lines of evidence show that the radioligand binding assays produced reproducible results and that our estimates of binding parameters are reliable. First, for CBG, we conducted three separate equilibrium saturation analyses using pools of plasma from different individuals, and the K_d and B_{max} values obtained were similar across these three assays. Second, for liver cytosol, we conducted two separate equilibrium saturation analyses using pools of cytosol from different subjects, and in both assays [^{3}H]corticosterone bound to both a MR-like site and a GR-like site whereas [^{3}H]cortisol bound to only a GR-like site. In addition, the K_d and B_{max} values obtained for both [^{3}H]corticosterone and [^{3}H]cortisol were very similar in the two assays (Table 2). Third, points in all assays were run in triplicate, and variability among triplicates was very low, as indicated by the small error bars in the figures. Together, these data suggest that the methods used for binding studies produced reproducible and consistent results, as in previous investigations (Breuner and Orchinik, 2001, 2009).

4.2. Tissue differences in glucocorticoid binding

Our results indicate that the abundance of MR, GR, and mCR varies across tissues, suggesting that some tissues may be more sensitive to the effects of GCs during development. In bursa and thymus cytosol, the MR constituted a small fraction of the total receptor number, whereas GR was abundant, similar to the thymus of rats (Lowy, 1989; Reul et al., 1989; Spencer et al., 1993). In con-

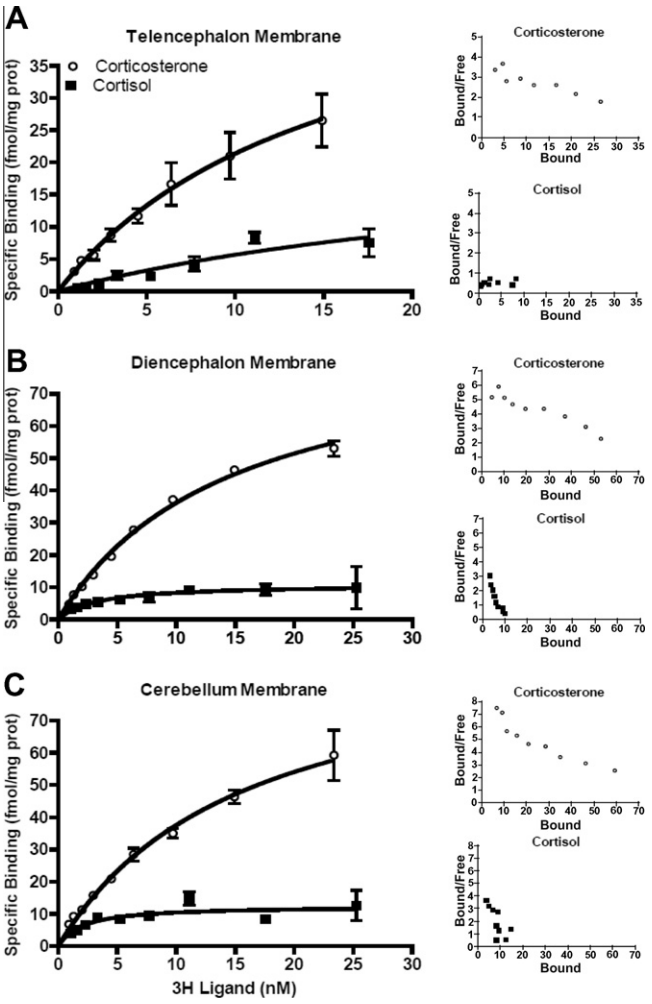

Fig. 8. Brain membranes. Equilibrium saturation binding of [^{3}H]corticosterone and [^{3}H]cortisol in pools of (A) telencephalon ($n = 7$ subjects for one pool), (B) diencephalon ($n = 35$ subjects for one pool), and (C) cerebellum ($n = 34$ subjects for one pool) well-washed membranes from 30-day-old zebra finches. Shown are specific binding data (mean ± SEM for each concentration) done in triplicate, plus a line that best describes the fit of the data. Estimates of binding parameters (K_d and B_{max}) are shown in Table 3. Insets are Scatchard–Rosenthal replots of the data.

trast, in telencephalon and diencephalon cytosol, MR was abundant whereas GR was present at very low densities. Further, there were regional differences within the brain, with GR levels being low in the forebrain but high in the cerebellum. Thus, the relative abundance of cytosolic MR and GR varies in a tissue-specific manner.

We found evidence for a mCR in all three brain regions and liver, but not in thymus, spleen, or breast muscle. A low amount of specific binding to [^{3}H]cortisol, but not [^{3}H]corticosterone, was also detected in bursa membranes. This suggests that GCs may bind to mCR in brain and liver to influence development, but not immune organs or muscle. However, immune organs from zebra finches are very small, and pooling tissues from several subjects (35 animals) still resulted in a very low concentration of protein per tube. Thus it is possible that specific binding of GCs to immune organ membranes would be more clearly detected if the protein concentration were increased (e.g., by using a larger species, such as the chicken).

Tissue differences in GC binding sites may be related to the roles that GCs play in development of specific organs. For example, in mice, GR regulate positive and negative selection of CD4$^+$ CD8$^+$ thymocytes through mutual antagonism with the T-cell receptor (Jondal et al., 2004). It is unclear whether GR has a similar role in regulating lymphocyte selection in birds. Results of the present study, along with evidence that immune cortisol levels are high in developing zebra finches (Schmidt and Soma, 2008), suggest that the immunosteroid cortisol could regulate lymphocyte selection in zebra finches via the GR. In the forebrain, GR levels were low which may be beneficial since high GC levels can impair brain development in rodents (Aisa et al., 2009) and songbirds (Buchanan et al., 2004).

4.3. Intracellular versus membrane-associated receptors

Our results show that intracellular and membrane-associated corticosteroid receptors differentially bind GCs. For example, for brain, mCR bound [^{3}H]corticosterone with lower affinity than the MR-like and GR-like site in brain cytosol, consistent with what has been shown for adult house sparrows (Breuner and Orchinik, 2009). For liver, mCR bound [^{3}H]corticosterone and [^{3}H]cortisol with higher affinity than the presumed GR and lower affinity than the presumed MR in liver cytosol. In addition, the B_{max} estimate for liver mCR was very high and ~4× higher than it was for the intracellular corticosteroid receptors. Thus, in juvenile zebra finches, the effects of GCs on the liver may be largely mediated through mCR.

The time required for intracellular and membrane-associated corticosteroid receptors to exert physiological and behavioral effects also differs. Intracellular corticosteroid receptors act as ligand-dependent transcription factors, with effects taking hours or days to occur. In contrast, the mCR can act non-genomically, with effects in seconds to minutes (Watson and Gametchu, 1999). In addition, GCs can be synthesized in extra-adrenal tissues, including immune and nervous tissues (Schmidt et al., 2008; Mellon and Deschepper, 1993; Lechner et al., 2001). Local GC synthesis can rapidly increase GC levels to very high levels within a small area. Very high GC concentrations could act via low affinity mCR to rapidly regulate immune and neural cells. In juvenile chickens, the bursa and thymus appear to synthesize cortisol *in vitro* (Lechner et al., 2001), in contrast to the adrenal glands, which synthesize corticosterone. Consistent with this, in young zebra finches, cortisol is the predominant GC in bursa, thymus, and spleen, and cortisol levels in these tissues are higher than cortisol levels in plasma (Schmidt and Soma, 2008). Thus, one intriguing hypothesis is that locally synthesized cortisol acts via immune mCR. In support of this, we detected specific binding of cortisol, but not corticosterone, to bursa membranes.

To date, it is unclear whether intracellular and membrane-associated corticosteroid receptors have different effects on physiology and behavior. The role of intracellular corticosteroid receptors has been well-studied across species. They have important effects on the immune system, both during development and adulthood, including regulation of lymphocyte selection (Jondal et al., 2004) and suppression of inflammation and cell-mediated immune responses (Stahn and Buttgereit, 2008). In the brain, they regulate negative feedback of the HPA axis and have numerous effects on neural development (Welberg et al., 2001). However, the roles of mCR in the immune and nervous systems are not clear, especially during development. In the immune system, GCs can rapidly induce apoptosis of lymphocytes via mCR and also non-genomic actions of intracellular receptors (Stahn and Buttgereit, 2008). There is substantial evidence that the intracellular GR regulates thymocyte selection (Vacchio et al., 1999), but it is unclear if mCR also regulates lymphocyte selection. The role of mCR in the brain during development is unknown, but mCR could mediate the rapid effects of corticosterone on behavior. For example, in white-crowned sparrow nestlings, corticosterone treatment rapidly decreases begging behavior within 25 min (Wada and Breuner, 2008).

4.4. Corticosterone and cortisol are not interchangeable

Although corticosterone and cortisol are often believed to be interchangeable, the evidence suggests that this is not the case. First, the predominant GC can be organ-specific. For example, the primary circulating GC in birds is corticosterone, but in juvenile chickens the bursa and thymus synthesize cortisol *in vitro* (Lechner et al., 2001) and in developing zebra finches cortisol is the predominant GC in lymphoid tissues and is present at similar concentrations to corticosterone in the brain (Schmidt and Soma, 2008). There is also evidence that the primary GC synthesized can be context specific. For example, a study of rabbits found that corticosterone was the predominant adrenal GC under baseline conditions, but cortisol became the predominant GC after chronic adrenocorticotropin hormone treatment (Kass et al., 1954).

Second, here we found evidence that GC binding sites in plasma, cytosol, and membranes differentially bind corticosterone and cortisol. For the plasma binding globulin, the B_{max} estimate was twice as high for [^{3}H]cortisol as [^{3}H]corticosterone, even though the K_d estimates for [^{3}H]cortisol and [^{3}H]corticosterone were similar. One possible explanation is that there are two GC binding sites in zebra finch plasma that bind cortisol with similar affinity, and one of these sites does not bind corticosterone. This would be similar to what has been found for plasma sex hormone-binding globulins (SHBG) in salmon. In salmon, there are two SHBGs, both of which bind testosterone, but SHBGα binds androstenedione with high affinity, while SHBGβ binds E_2 with high affinity (Miguel-Queralt et al., 2009). To determine if there are two CBGs in zebra finches, both of which bind cortisol but only one that binds corticosterone, future experiments that determine the ability of radioinert corticosterone to compete for [^{3}H]cortisol binding sites are needed. For cytosol, the MR-like site in forebrain bound corticosterone with higher affinity than cortisol, yet the GR-like site in the cerebellum bound cortisol with higher affinity than corticosterone. In bursa, thymus, and liver cytosol, cortisol bound to only a GR-like site, whereas corticosterone bound to both a MR-like and a GR-like site. For membranes, in brain, a mCR bound corticosterone with a low affinity and a moderately high B_{max}, but bound cortisol with a high affinity and a very low B_{max}. For bursa membranes, we detected a low amount of specific binding of cortisol, but not corticosterone. Lastly, the mCR in liver bound cortisol with a higher affinity than corticosterone. Previous studies have also shown differential binding of corticosterone and cortisol. In young chickens, bursa GR have a higher affinity for cortisol than corticosterone (Sullivan and Wira, 1979). In rats, CBG has a higher affinity for corticosterone than cortisol (Louw et al., 2000). In mice and humans, P-glycoprotein transports cortisol, but not corticosterone, out of the brain (Karssen et al., 2001).

Third, experimental studies suggest that corticosterone and cortisol can have different physiological effects, which could occur if these two GCs bind to different receptors, or if they induce different conformational changes in the same receptor. For example, corticosterone and cortisol decrease bursa weight in chickens, but cortisol causes more vacuolar spaces within the lymph follicle of the bursa, suggesting that cortisol induces more cell death (De and Guha, 1987). In juvenile chickens, corticosterone and cortisol induce apoptosis in the thymus, but cortisol is more effective (Compton et al., 1990). The mechanisms by which these two GCs exert different effects have been unclear, largely due to the lack of studies examining whether they differentially bind to receptors. Our results, along with past studies, suggest that corticosterone and cortisol may be synthesized in a tissue-specific manner (Lechner et al., 2001; Schmidt and Soma, 2008) and that they can differentially bind to receptors, which could explain how these prominent glucocorticoids may have different effects.

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