17β-ESTRADIOL LEVELS IN MALE ZEBRA FINCH BRAIN: COMBINING PALKOVITS PUNCH AND AN ULTRASENSITIVE RADIOIMMUNOASSAY

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Abstract

Local aromatization of testosterone into 17β-estradiol (E_2) is often required for the physiological and behavioral actions of testosterone. In most vertebrates, aromatase is expressed in a few discrete brain regions. While many studies have measured brain aromatase mRNA or activity, very few studies have measured brain E_2 levels, particularly in discrete brain regions, because of technical challenges. Here, we used the Palkovits punch technique to isolate 13 discrete brain nuclei from adult male zebra finches. Steroids were extracted via solid phase extraction. E_2 was then measured with an ultrasensitive, specific and precise radioimmunoassay. Our protocol leads to high recovery of E_2 (84%) and effectively removes interfering brain lipids. E_2 levels were high in aromatase-rich regions such as caudal medial nidopallium and hippocampus. E_2 levels were intermediate in the medial preoptic area, ventromedial nucleus of the hypothalamus, lateral and medial magnocellular nuclei of anterior nidopallium, nucleus taeniae of the amygdala, and Area X. E_2 levels were largely non-detectable in the cerebellum, HVC, lateral nidopallium and optic lobes. Importantly, E_2 levels were significantly lower in plasma than in the caudal medial nidopallium. This protocol allows one to measure E_2 in discrete brain regions and potentially relate local E_2 concentrations to aromatase activity and behavior.

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INTRODUCTION

Aromatization of testosterone into 17β–estradiol (E_2) within the brain is critical for many behavioral actions of testosterone, such as regulation of aggressive and reproductive behavior (Naftolin et al., 1975; Schlinger and Callard, 1990; Balthazart and Foidart, 1993; Soma et al., 2003; Soma et al., 2008) as well as cognition and learning (Oberlander et al., 2004; Garcia-Segura, 2008; Roselli et al., 2009). Classically, E_2 has been thought to act via specific interactions with its cognate nuclear receptors. The binding of E_2 to nuclear receptors regulates transcription of target genes, and this process usually takes hours to days (Mangelsdorf et al., 1995). Recent evidence suggests that E_2, as well as other steroids, also affects brain and behavior very rapidly. For example, E_2 can within a few seconds or minutes modulate neuronal firing rate or activate a variety of intracellular signaling pathways (Kelly and Ronnekleiv, 2008; Micevych and Mermelstein, 2008) and can rapidly modulate sexual behavior (Cornil et al., 2003) and agonistic behavior (Remage-Healey and Bass, 2004; Trainor et al., 2008).

E_2 concentrations are unlikely to be homogenous throughout the brain but rather vary across regions. Indeed, the enzyme responsible for the transformation of testosterone into E_2, P450 aromatase, is present only in discrete brain regions, such as the preoptic area and hippocampus, in many vertebrates (Selmanoff et al., 1977; Schumacher et al., 1983; Roselli et al., 1985; Shen et al., 1995). In songbirds, there is more widespread expression of aromatase in the telencephalon (Balthazart et al., 1996; Silverin et al., 2000; Saldanha et al., 2000). While certain songbird brain regions such as the medial preoptic nucleus and caudal medial nidopallium (NCM) contain aromatase-immunoreactive cell bodies and fibers (Balthazart et al., 1996; Saldanha et al., 2000), other regions such as HVC (abbreviation used as proper name) and the shell of the robust nucleus of the arcopallium (RA) contain only aromatase-immunoreactive fibers and terminals (Saldanha et al., 2000; Peterson et al., 2005). The localized presence of aromatase suggests that the transformation of testosterone into E_2 occurs in discrete brain regions and could lead to high local concentrations of E_2 without affecting the periphery (Schmidt et al., 2008) Aromatase activity shows long-term, region-specific changes induced by chronic testosterone treatment and season (Schumacher and Balthazart, 1986; Soma et al., 2003). In parallel, more rapid changes in aromatase activity are also observed in certain brain regions (Cornil et al., 2005; Remage-Healey et al., 2009). These data on aromatase suggest that regional E_2 levels also vary, but the quantification of E_2 in discrete brain regions remains a challenge. A few studies have measured E_2 in relatively large pieces of brain following manual dissection (Amateau et al., 2004; Newman et al., 2008; Barker and Galea, 2009).

Here, we describe a method to quantify E_2 in microdissected brain regions from the zebra finch (*Taeniopygia guttata*). We used the Palkovits punch technique to collect 13 discrete regions from adult male zebra finch brain. We then developed an ultrasensitive and highly specific radioimmunoassay to measure E_2. The first major challenge is the presence in the brain of high concentrations of lipids, which interfere with immunoassays (Rash et al., 1980; Jawad et al., 1981). Although organic solvents have been traditionally used to extract steroids, solid phase extraction offers higher and more consistent analyte recoveries and a more complete removal of interfering substances. The second major challenge is the very small amount of tissue obtained from the microdissection technique, which reduces the probability of obtaining a detectable amount of E_2. We show here that the combination of Palkovits punch technique, solid phase extraction, and ultrasensitive radioimmunoassay allows for the measurement of E_2 in discrete brain regions.

MATERIALS AND METHODS

Experimental animals

Animal use protocols were approved by the Animal Care Committee at the University of British Columbia and conform to the guidelines of the Canadian Council on Animal Care. Adult sexually-naïve male zebra finches (*Taeniopygia guttata*) were single-sex, group-housed and given millet seeds, water, grit, and cuttlefish bone *ad libitum*. The very high level of brain aromatase in zebra finch (Silverin at al. 2000) suggests that E_2 will be important and more readily detectable to develop our protocol. The light cycle provided 14 h of light and 10 h of dark (lights on at 8:00 A.M.). Temperature was held constant at ~23°C and relative humidity at ~50%. Animals were killed between 8:30 and 10:00 AM within 3 min of disturbance to avoid potential effects of time of day or stress on steroid concentrations.

Palkovits punch technique

The technique originally developed by Palkovits in rats (Palkovits, 1973) was previously validated in quail (Schumacher and Balthazart, 1987) and zebra finch brain (Vockel et al., 1990) and was used here with only minor modifications. After rapid decapitation, brains were quickly removed out of the skull, frozen on dry ice, and stored at −80°C. Brains were embedded in Shandon Cryomatrix (ThermoFisher), with the forebrain facing the specimen holder of the cryostat (Microm HM505E). Coronal sections (300 micrometers) were made in the cryostat at −12°C and were collected starting from the caudal part of the brain. The plane of the sections was adjusted to match as closely as possible the plane of the zebra finch brain atlas (Nixdorf-bergweiler and Bischof, 2007). From these sections, individual nuclei were isolated from 2 to 5 adjacent sections, depending on the region of interest, by punching them out with stainless steel cannula (Brain Punch Set, #57401, Stoelting Co, IL). The diameter of cannula was adapted to the nucleus of interest: 0.74mm-diameter punches were obtained from RA, nucleus taeniae of the amygdala (TnA), and HVC; and 0.94 mm-diameter punches were obtained from lateral nidopallium (LatNP), hippocampus (Hp), caudal medial nidopallium (NCM), optic lobes (OL), ventromedial nucleus of the hypothalamus (VMN), medial preoptic area (mPOA), lateral magnocellular nucleus of anterior nidopallium (LMAN), medial magnocellular nucleus of anterior nidopallium (MMAN), and Area X (see Figure 1 for the location and Table 1 for the number of punches and mass of tissue obtained). It should be noted that some of the punches were only partial due to the proximity of ventricles (e.g. HVC and TnA) Punches were collected into pre-weighed "Pellet Pestle" microcentrifuge tubes (0.5 ml volume; #749510-0501, Kimble/Kontes, NJ, USA) and weighed as quickly as possible. The mass of tissue (to the nearest 0.1 mg) was obtained by subtracting the mass of the empty microcentrifuge tube from the mass of the microcentrifuge tube containing the tissue. The samples were stored at −80°C. Trunk blood was collected at sacrifice and centrifuged for 10 min at 10,000g. Plasma was stored at −80°C.

Solid phase extraction

Brain punches were homogenized in the microcentrifuge tubes with a CTFE/stainless steel "Pellet Pestle" (#749516-0500, Kimble/Kontes) with 50 μl ice-cold deionized water, and then 250 μl ice-cold methanol (HPLC-grade) was quickly added. Samples were left overnight at 4°C. Steroids were then extracted with solid phase extraction (Newman et al., 2008). We used a 24-place vacuum manifold and non-endcapped C18 columns (500 mg C18 material, 6 ml column volume, United Chemical Technologies). Columns were primed with absolute ethanol (3 ml) and equilibrated with deionized water (2×5 ml). 10 ml of deionized water was added to the brain samples and to plasma samples (52 μl), before loading onto the C18 columns. After sample loading, the C18 columns were washed with deionized water (2×5 ml), and the steroids were slowly eluted with 90% HPLC-grade methanol (5 ml). The eluates were then dried (Speedvac) prior to resuspension.

Several resuspension conditions were tested to maximize E_2 recovery. We analyzed the effect of the volume of resuspension buffer (400 or 800 μl) as well as sonication (0, 5, 30 or 60 min) on the recovery of a known amount of tritiated E_2 (Perkin Elmer). In addition, we previously showed that adding a small amount of ethanol increases the recovery of steroids (Newman et al., 2008). However, ethanol can affect immunoassays, and therefore we determined whether increasing concentrations of ethanol (0, 0.5, 1, 1.5, 3 and 5%) had an effect on known concentrations of E_2 (0.7125 and 1.425 pg per tube). Absolute ethanol was added directly to the bottom of the glass scintillation vials containing the dried eluates, the vials were vortexed for 2 min, and 0.1M phosphate-buffered saline containing 0.1% gelatin (PBSG) was then added. Based on initial results, we resuspended our samples in 650 μl PBSG and 0.7% ethanol (see details in the "Results" section below).

Estradiol radioimmunoassay

Resuspended samples were then run in duplicate using a commercially available double-antibody ^{125}I-E_2 radioimmunoassay (DSL-4800, Ultra-sensitive Estradiol RIA, Diagnostic System Laboratories, Webster, TX) that we modified and validated, similar to our previous studies with a different E_2 assay (DSL-39100) (Newman et al., 2008; Shirtcliff et al., 2000). The DSL-39100 assay is no longer available, and thus we validated the DSL-4800 E_2 assay. We tested different dilutions of the tracer and the antibody to increase the sensitivity of the assay. The protocol presented below offered the most sensitive and precise assay. Briefly, 100 μl of diluted anti-estradiol antiserum (dilution: 1 ml stock antibody + 2.5 ml PBSG) was added to 300 μl of sample, the tubes were quickly vortexed and incubated at room temperature for 4 hr. Then 100 μl of diluted ^{125}I-E_2 (dilution: 1 ml stock tracer + 2 ml PBSG) was added, and the tubes were vortexed and incubated for 24 hr at 4°C. Then 500 μl of precipitating reagent was added, and tubes were vortexed and incubated for 20 min at room temperature. The tubes were centrifuged at 1500g for 15 min at 4°C, the supernatant was decanted, and tubes were counted. The E_2 antibody has a low cross-reactivity with estrone (2.4%), estriol (0.64%), 17β-estradiol (0.21%), 17β-estradiol-3-glucuronide (2.56%), 17β-estradiol-17-glucuronide (<0.01%), estradiol-3-SO_4 (0.17%), testosterone (<0.01%) and DHEA (<0.01%), as per the manufacturer.

We also examined the recovery of a known quantity of radioinert E_2 (0.9 pg per assay tube) added to brain tissue (1.59 mg of dorsal telencephalon containing NCM per assay tube) before solid phase extraction. We calculated the recovery by comparing the quantity of E_2 in spiked (n=7) and unspiked samples (n=6).

Moreover, to further evaluate whether solid phase extraction effectively removed interfering substances, we serially diluted brain samples (from 11.8 to 0.18 mg per tube). If the substances that interfere with the RIA are effectively removed, the percentage of tracer bound from serially diluted samples should be parallel to the standard curve.

Testosterone radioimmunoassay

Total testosterone levels in plasma were measured using radioimmunoassay. Testosterone was extracted from plasma with HPLC-grade dichloromethane prior to radioimmunoassay. Briefly, steroids from 11.5 μl of plasma were extracted twice with 3 ml dichloromethane. Dichloromethane was then evaporated under nitrogen at 37°C. Steroids were resuspended in 900 μl PBSG.

Resuspended samples were then run in duplicate using a commercially available testosterone radioimmunoassay (DSL-4100) that was modified to increase sensitivity (Granger et al., 1999) and validated for songbirds (Charlier et al., 2009). Briefly, 20 μl anti-testosterone antiserum was added to 400 μl of sample, and the tubes were incubated at 37°C in a shaking

water bath for 30 min. Then 50 μl of ^{125}I-testosterone was added, and the tubes were vortexed and incubated for 3 hr at 37°C. 500 μl of precipitating reagent was then added, and tubes were vortexed and incubated for 20 min at room temperature. The tubes were then centrifuged at 1000g for 15 min at 4°C, the supernatant was decanted, and tubes were counted. The testosterone antibody has a low cross-reactivity with 5α-dihydrotestosterone (6.6%), androstenedione (0.9%), E_2 (0.4%), dehydroepiandrosterone (<0.1%), corticosterone (<0.1%) and progesterone (<0.1%). The lowest point on the standard curve was 0.4 pg testosterone/tube, and the detection limit for our assays with 5 μL plasma/tube was 80 pg/ml. Intra-assay variation was 6.2%, and inter-assay variation was 4.3%.

Fadrozole treatment

We tested the effects of fadrozole, a potent and specific non-steroidal inhibitor of aromatase, on E_2 levels in zebra finches. Fadrozole is known to reduce brain aromatase activity in this species (Wade et al., 1994). We injected adult male zebra finches intramuscularly in the breast muscle with 200 μg of fadrozole in saline (generous gift from Novartis, n=8) or saline vehicle (20 μl) (n=8). 24 hr after injection, the birds were rapidly decapitated, and we collected trunk blood to measure plasma E_2 and dorsal telencephalon, containing the NCM, to measure brain E_2. The mass of the dorsal telencephalon obtained from manual dissection was 14.00 ± 1.08 mg. Two values, one in each group, were over 2 standard deviations from the mean of their group and were therefore excluded.

Nissl staining

After the punch collection, sections were mounted on glass slides, dried overnight and Nissl-stained to confirm the location of the punches. Briefly, sections were allowed to return to room temperature and were postfixed in 4% paraformaldehyde for 15 min. The sections were rinsed 3 times in PBS (0.1M) and stained in 0.2% toluidine blue in Walpole solution (0.3M sodium acetate, 0.12% acetic acid) for 2 min. The sections were then quickly rinsed in deionized water, destained 2×15 min in Walpole solution, and the stain fixed by a 2.5 min incubation in 0.04M ammonium molybdate tetrahydrate. The sections were then dehydrated with increasing concentration of ethanol (20, 70, and 90% for 1 min), acetone (2×1 min) then xylene (2 min) before coverslipping with Permount® mounting medium (Fisher).

Statistical analysis

All data are presented as their mean ± SEM. Data analysis included Student's t-test and one- and two-way ANOVAs and were performed using Statview and SuperAnova (MacIntosh version 5.0.1, Abacus Concept Inc., Berkeley, CA, USA). When appropriate, ANOVA tests were followed by *post hoc* Tukey's HSD tests. To compare E_2 concentrations in brain tissue and plasma, it was assumed that 1 mL of plasma weighs 1 g, as previously shown (Schmidt and Soma, 2008). To determine parallelism between serial dilution of brain tissue and standard curve, we tested equality of slopes from linear regressions of log-transformed concentration (standard curve) or tissue amount (telencephalon) and percent binding using Prism 4 (v1.0, 2002, GraphPad, CA). Differences were considered significant for $p \le 0.05$.

RESULTS

Modifications of E_2 radioimmunoassay

After modifications, the lowest point on the standard curve was 0.1875 pg E_2 per tube. In contrast, the lowest point on the standard curve for the unmodified assay was 1.66 pg E_2 per tube, indicating an approximate 10-fold increase in sensitivity. The values for the water blanks (n=9) were below the lowest standard (0.120 ± 0.018 pg E_2 per tube). Samples within 2 standard deviations of the water blanks were considered non-detectable and set to zero. Intra-assay

variation was 1.81%, and inter-assay variation was 6.10% for a low control (0.375 pg E_2 per tube, n=6 assays) and 4.42% for a high control (1.125 pg E_2 per tube, n=6 assays).

Recovery of radiolabeled E_2

We first tested different conditions for the resuspension of E_2 after solid phase extraction. We added a known amount of tritiated E_2 to 5 ml of 90% methanol, dried the samples, and tested the effects of the volume of resuspension and of sonication. There was no significant effect of the volume of resuspension buffer (df=8, t=1.915, p=0.10) or sonication ($F_{3,15}$=1.158, p=0.35). Overall, the recovery of tritiated E_2 was 78.94 ± 1.44% (n=29 samples).

Recovery of radioinert E_2 after solid phase extraction

Ethanol in the resuspension buffer can improve the recovery of E_2 and other steroids (Newman et al., 2008; Charlier et al., 2009). However, ethanol can potentially affect the radioimmunoassay, so we tested the effects of different concentrations of ethanol on the measurement of 2 known concentrations of E_2 (0.7125 and 1.425 pg per tube; all data were expressed as percentage of controls without ethanol). The concentration of ethanol significantly affected the measured E_2 values (2-way ANOVA: $F_{4,50}$=133.5, p=0.0001, Figure 2A), but there was no difference between E_2 concentrations ($F_{1,50}$=0.88, p=0.35) and no interaction between ethanol and E_2 concentration ($F_{4,50}$=1.62, p=0.18). The absence of an interaction indicates that the effect of ethanol is similar across different concentrations of E_2. Importantly, the magnitude of the ethanol effect is extremely small at concentrations of ethanol below 1% (i.e., less than a 5% decrease in measured E_2 values). Based on these data, we used a relatively low concentration of ethanol (0.7%) in the resuspension to increase the recovery of E_2, without greatly affecting the E_2 radioimmunoassay.

Next, we determined the recovery of a known amount of E_2 (0.9 pg per tube) that was added to a pool of telencephalic tissue (n=7, 1.59 mg of tissue per tube) prior to solid phase extraction. We compared spiked and unspiked samples from the same pool of tissue, and the recovery of radioinert E_2 was 84.19% (Figure 2B).

Removal of interfering substances

To test whether the solid phase extraction protocol effectively removed interfering substances such as lipids from brain samples, we compared serial diluted brain samples and the standard curve. The slope of the curve obtained from serially diluted telencephalon was not significantly different from the slope of the E_2 standard curve, for samples containing between 1.48 and 11.8 mg of tissue per tube ($F_{1,4}$= 1.47, p=0.29, Figure 2C). At lower quantities of tissue per tube, there was a trend for the slopes to differ ($F_{1,10}$=4.57, p=0.06). Thus, we aimed for our samples to contain the appropriate amount of tissue per assay tube. Overall, the solid phase extraction protocol effectively removed interfering brain lipids.

Effect of fadrozole

Further validations of the quantification of E_2 were obtained via the *in vivo* inhibition of E_2 synthesis using fadrozole. As can be seen in Figure 2D, E_2 content in the telencephalon and in the plasma was significantly reduced 24 hr after a single injection of fadrozole. A 2-way ANOVA showed a significant effect of the fadrozole treatment ($F_{1,25}$= 6.283, p=0.019) as well as a significant effect of sample type (telencephalon vs plasma) ($F_{1,25}$= 17.80, p<0.001) where E_2 concentration in the telencephalon was higher than in plasma. There was no interaction between sample type and fadrozole treatment ($F_{1,25}$= 0.89, p=0.35).

E_2 concentration in brain punches

The validations described above confirm that E_2 can be quantified from very small brain samples using our modified RIA after SPE. The weight of the brain samples ranged between 3.70 and 5.86 mg of tissue, obtained from 4 to 10 punches (Table 1). Several nuclei from most individuals showed detectable levels of E_2. A significant regional difference in E_2 concentrations was observed ($F_{13,91}= 2.457$, $p=0.007$) and NCM showed significantly higher concentrations compared to plasma, TnA, Cb, HVC, LatNP, OL, VMN, Area X (*post hoc* Tukey's tests, $p<0.05$, see Figure 3). It should also be noted that the heterogeneity of E_2 content was reflected by the percentage of detectable samples for each region. This percentage for each brain nucleus is presented in Figure 3. The percentage of detectable samples was highest in NCM and HP, intermediate in MMAN, mPOA and VMN, and low in HVC, LatNP, OL and CB. In addition, brain levels of E_2 are in general higher than plasma E_2 levels, when considering that 1 ml of plasma weighs 1 g (Schmidt and Soma, 2008).

Circulating testosterone levels were ~100× higher than circulating levels of E_2 (Wilcoxon signed rank test: $p=0.008$, Figure 4). The levels of plasma testosterone and plasma E_2 were not correlated although there was a tendency ($p=0.09$). In addition, circulating levels of testosterone were not correlated with the level of E_2 in any brain nuclei (data not shown, all $p>0.1$). It should, however, be noted that 2 subjects had very low levels of plasma testosterone compared to the other birds (< 1 ng/ml), and very interestingly had less E_2 in their brain regions.

DISCUSSION

We describe here the use of solid phase extraction prior to radioimmunoassay of E_2 on microdissected brain samples. The solid phase extraction procedure leads to relatively high recovery of E_2 and effectively removes interfering lipids. Moreover, the development of an ultrasensitive assay to measure E_2 allowed us to detect small amounts of E_2 in punches weighing a few milligrams. Previous studies have examined the concentration of E_2 in various regions in rat brain (Bixo et al., 1986; Amateau et al., 2004; Pei et al., 2006; Barker and Galea, 2009) and human brain (Bixo et al., 1995). Recent studies have also used liquid or gas chromatography coupled to mass spectrometry to measure E_2 (Shimada et al., 1999; Labombarda et al., 2006; Caruso et al., 2008; Higo et al., 2009). However, all of these studies examined E_2 content in large brain regions, such as the cortex or hypothalamus. We describe here a sensitive and specific method to quantify E_2 in small, discrete brain regions using the Palkovits punch technique and analyzed 13 different regions within individuals.

Estradiol radioimmunoassay

We evaluated 4 aspects of our E_2 assay (Midgley et al., 1969; Abraham, 1975).

Sensitivity—While the lowest point on the standard curve of the unmodified assay was 1.66 pg E_2 per tube, we modified this assay to increase the sensitivity nearly 10-fold and were able to detect as little as 0.1875 pg E_2 per tube.

Precision—The precision of our assay was confirmed by the relatively low intra- and inter-assay coefficients of variability (~2 and 6%, respectively).

Accuracy—Using controls containing known amounts of E_2, we confirmed the high accuracy of the assay (e.g., Fig. 2A). Moreover, when comparing spiked and unspiked brain samples, the difference was close to the expected value (i.e., the added amount of estradiol).

Specificity—The E_2 antibody has very weak cross-reactivities for other steroids, including estrone, estriol, 17β-estradiol, testosterone and DHEA. In addition, the "interference elution"

with deionized water in the solid phase extraction procedure should eliminate most water-soluble metabolites, such as E_2 glucuronides and sulfates. Moreover, an aromatase inhibitor (fadrozole) significantly reduced both circulating and brain E_2 levels. Fadrozole treatment did not reduce E_2 values to non-detectable levels, and it is possible that the dose of fadrozole or the duration of treatment were not optimal. Several fadrozole doses and time points were previously tested by Wade and colleagues (1994), but aromatase activity was never fully inhibited, similar to the present results.

Solid phase extraction

The solid phase extraction protocol yielded consistent and high recovery (~80%) and removed major interfering substances (as in Newman et al., 2008). We examined recovery using radiolabeled E_2, and in addition spiked brain samples with a known amount of radioinert E_2. The results of these studies were highly consistent (recovery was ~80% in both cases). In addition, a serial dilution of telencephalon is parallel to the standard curve, demonstrating the effective removal of interfering lipids. The protocol might be improved even further through the use of a different washing protocol, such as 40% methanol instead of water (Belanger et al., 1990).

Regional differences in E_2 concentrations

The concentration of E_2 in the plasma was lower than in most brain regions, as in rats (Bixo et al., 1986; Higo et al., 2009). The high percentage of plasma samples with detectable amounts of E_2 was probably due to the larger quantity of plasma than brain tissue in punches. Future work could pool brain tissue from a few individuals to increase the probability of detecting E_2 in areas such as RA or HVC. Alternatively, samples could be run as singletons rather than in duplicate, given the very low intra-assay variability and close correspondence between duplicates.

The concentrations of E_2 in the brain appear highest in NCM and hippocampus. High local concentrations of E_2 are likely to originate from local synthesis (Schlinger and Arnold, 1992; Schlinger and Arnold, 1993). The enzyme responsible for the metabolism of testosterone into E_2, aromatase or CYP19, is expressed in several nuclei of the hypothalamus and telencephalon. Not surprisingly, the highest levels of E_2 were found in 2 regions expressing high levels of aromatase, NCM and hippocampus (Balthazart et al., 1996; Saldanha et al., 2000). Aromatase-immunoreactive cells are also present in MMAN, mPOA and VMN, where E_2 was detectable in the majority of samples, albeit at a lower level. There is little aromatase activity in the LatNP, OL and CB, and very few samples from these regions had detectable E_2. Interestingly, HVC and RA receive aromatase-immunoreactive fibers and could be expected to synthesize E_2 locally (Peterson et al., 2005). However, most HVC and RA samples had non-detectable amounts of E_2. Our measures are probably an underestimate of E_2 concentrations at the subcellular level. Aromatase is present in pre-synaptic terminals in HVC and RA and could lead to high levels of E_2 near the synapse. The high synaptic E_2 concentration would however be diluted by the surrounding tissue in the punches. Perhaps E_2 levels in HVC and RA might be higher in subjects that were known to be singing.

Brain E_2 could result from local *de novo* synthesis from cholesterol. All the enzymes in the neurosteroidogenesis pathway are expressed in the songbird telencephalon (London et al., 2006), and brain slices from developing zebra finches secrete E_2 *in vitro* (Holloway and Clayton, 2001). However, note that brain E_2 was almost completely non-detectable in two subjects with low levels of circulating T, suggesting that the brain E_2 results mainly from the aromatization of circulating T rather than from complete *de novo* synthesis. The absence of strong correlations between circulating T and brain E_2 levels might be related to individual variation of aromatase activity. In regions lacking aromatase, such as Area X, some E_2 might

be present because of passive diffusion away from sites of synthesis or because of sequestration (specific or non-specific). The peripheral or neural origin of the substrate needed for localized E_2 synthesis remains to be investigated in the distinct brain regions.

Linking local aromatase activity and E_2 concentrations

Local concentrations of brain E_2 are unlikely to be static but could vary according to the behavior and environment. The transcription of aromatase in several brain regions is strongly regulated by season and steroids (Balthazart et al., 1990; Soma et al., 2003; Silverin et al., 2004). These long-term changes in aromatase expression and activity very likely lead to changes in E_2 concentrations. In addition, rapid modulation in aromatase activity is also described in specific brain regions after performance of male sexual behavior in quail (Cornil et al., 2005) or singing behavior in zebra finches (Remage-Healey et al., 2009). Remage-Healey and colleagues (Remage-Healey et al., 2008) elegantly used *in vivo* microdialysis to measure E_2 concentrations in the zebra finch NCM. This exciting approach allows repeated measurement of E_2 from the same animal. However, the low recovery of steroid across the dialysis membrane requires sampling to last for 30 min, making potential rapid modulations of E_2 concentrations difficult to observe.

The methods described here offer several useful features. First, our procedure allows measurement of E_2 in plasma and brain. This permits direct comparison of circulating and local E_2 levels. Second, our method allows measurement of very rapid changes in brain E_2 levels (e.g., within 5 min). Third, our procedure permits the examination of multiple brain regions. Given the spatial specificity of brain aromatase expression and also the region-specific regulation of aromatase, this is an important point. Fourth, our method avoids the effects of injury-induced aromatase. Insertion of the guide cannula for the microdialysis probe induces aromatase around the site of injury, and therefore potentially increases E_2 concentrations at the site of dialysis (Garcia-Segura et al., 1999; Peterson et al., 2001; Peterson et al., 2004; Wynne and Saldanha, 2004; Wynne et al., 2008). Thus, we view the present technique and *in vivo* microdialysis as complementary approaches to understanding local E_2 levels in the brain and their regulation and function.

CONCLUSIONS

The present technique offers a method to investigate the concentrations of E_2 in small brain samples. The E_2 assay is precise, sensitive, specific and accurate. While many studies have measured aromatase activity in brain, far fewer studies have measured E_2 levels in brain. The present procedure will allow experiments aimed at determining whether E_2 can act rapidly under specific conditions. For example, it is possible that E_2 remains elevated for a substantial amount of time when acting at the genomic level, while a more rapid change of E_2 may be required when E_2 acts at the non-genomic level (Balthazart and Ball, 2006; Cornil et al., 2006). This present procedure will allow one to determine if E_2 is rapidly produced in specific region(s) and also whether E_2 can be rapidly eliminated from these areas.

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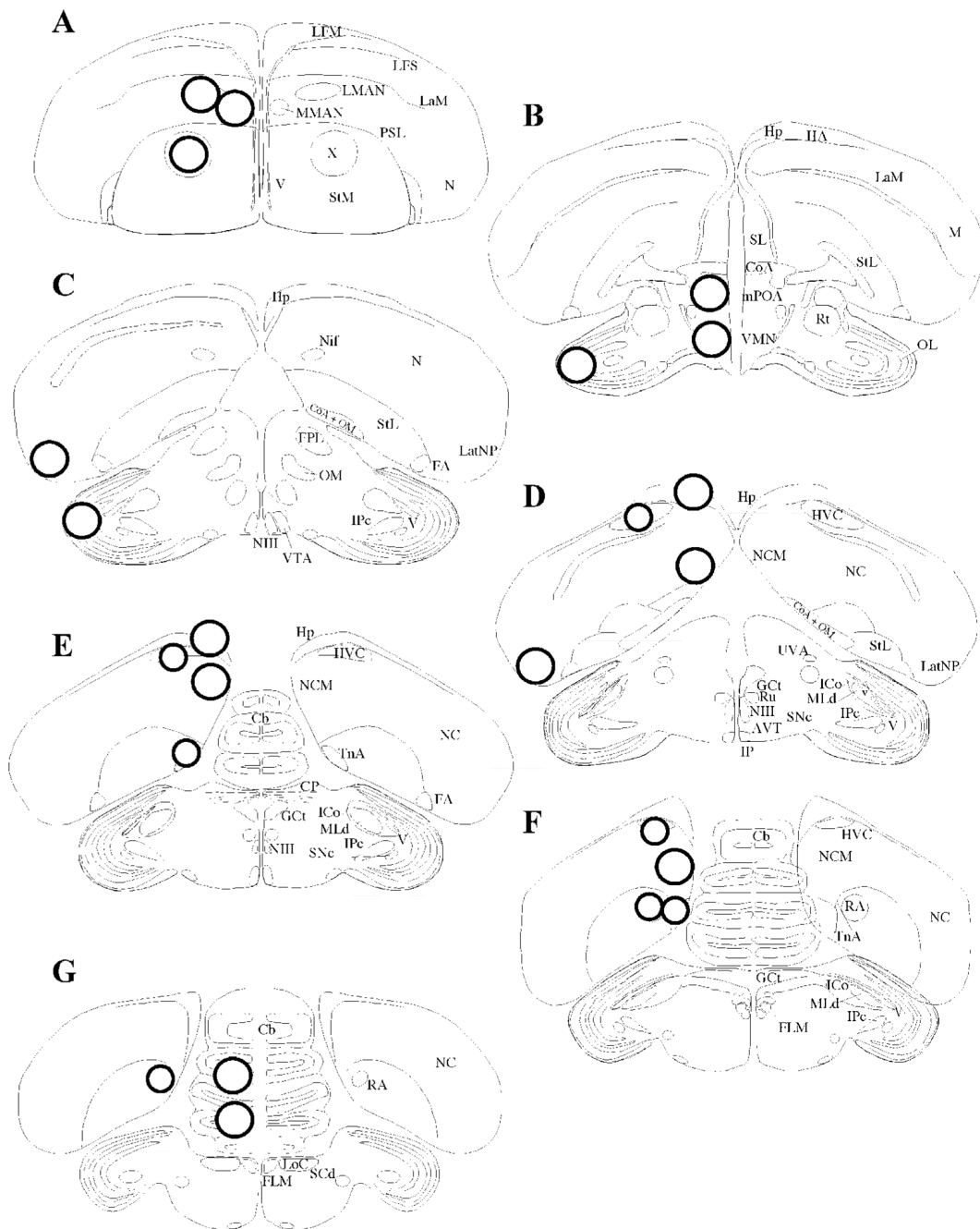
Figure 1.
Schematic representation of the location of the punches, as identified by circles in the transversal sections. (A–H) Sections arranged in a rostral to caudal order. Abbreviations: Cb, cerebellum; CoA, anterior commissure; CP, posterior commissure; FA, fronto-arcopallial tract; FLM, fasciculus longitudinalis medialis (medial longitudinal bundle); GCt, mesencephalic central gray (periaqueductal gray); HA, accessory part of the hyperpallium; Hp, hippocampus; ICo, intercollicular nucleus; IPc, parvocellular part of the isthmi nucleus; LaM, mesopallial lamina; LatNP, lateral nidopallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; LoC, locus ceruleus; LPS, pallial-subpallial lamina; LSt, lateral striatum; M, mesopallium; MFL, supreme frontal lamina; MLd, lateral mesencephalic nucleus; MMAN,

medial magnocellular nucleus of the anterior nidopallium; mPOA, median preoptic area; MSt, medial striatum; N, nidopallium; NC, caudal nidopallium; NCM, caudal medial nidopallium; NIf, nucleus interface of the nidopallium; NIII, oculomotor nerve; OL, optic lobe; OM, occipito-mesencephalic tract; RA, robust nucleus of arcopallium; Rt, nucleus rotundus; Ru, red nucleus; SCd, dorsal subceruleus nucleus; SFL, superior frontal lamina; SL, lateral septal nucleus; SNc, substantia nigra, pars compacta; TnA, nucleus taeniae of the amygdala; UVA, nucleus uveaformis; V, ventricle; VMN, ventromedial nucleus of the hypothalamus; VTA, ventral tegmental area; X, Area X
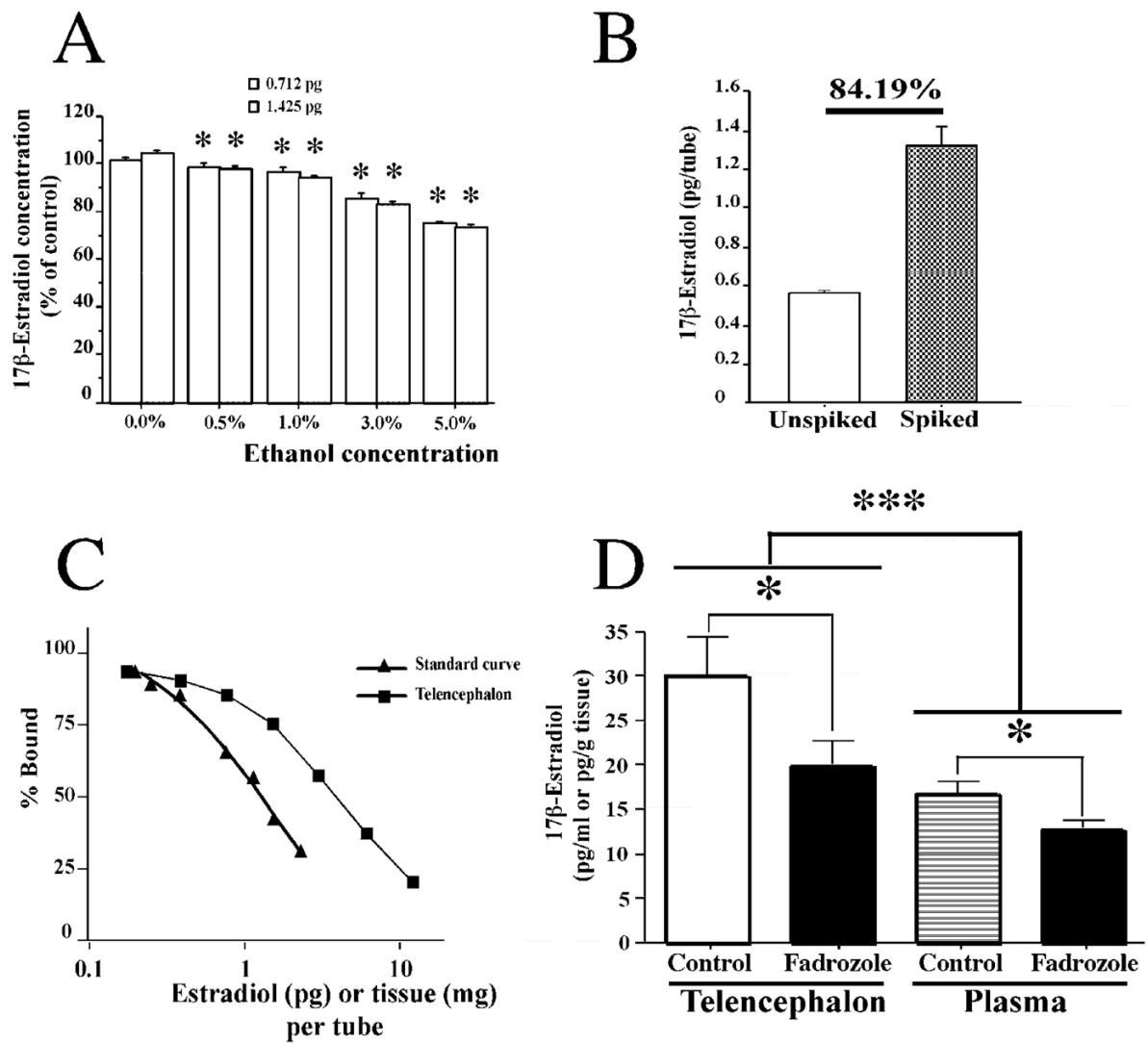
Figure 2.
A. Bar graph representing the effect of increasing concentration of ethanol (0, 0.5, 1, 3, and 5%) on the E_2 RIA. Two concentrations of E_2 were used (0.712 and 1.425 pg per tube). *p<0.05 vs 0% ethanol (control). **B.** Bar graph representing the recovery of a known amount of E_2 (0.9 pg per tube) that was added to telencephalon samples prior to solid phase extraction and then measured via RIA. The recovery was 84.19%. **C.** Comparison of serial diluted brain tissue and standard curve to confirm that solid phase extraction effectively removed interfering substances. **D.** Bar graph representing the effect of fadrozole treatment on E_2 concentrations in the plasma and in the telencephalon and the significant difference between E_2 level in telencephalon and plasma. *p<0.05 vs control; *** p<0.001 vs telencephalon
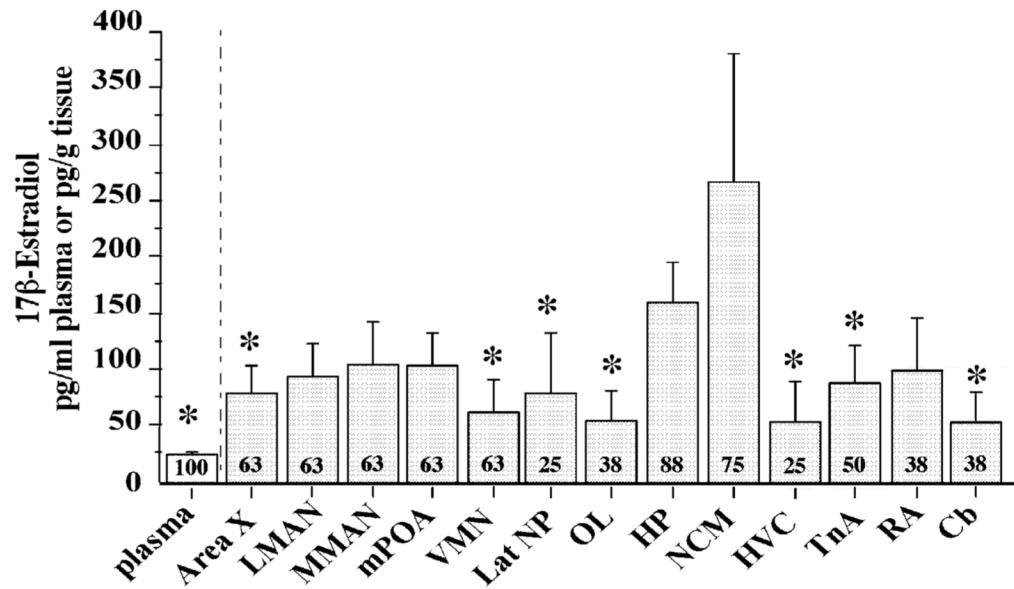
Figure 3.
Bar graph representing the E_2 concentrations in the plasma and in 13 different brain regions in adult male zebra finches. Numbers indicate the percentage of detectable samples. *$p<0.05$ vs NCM
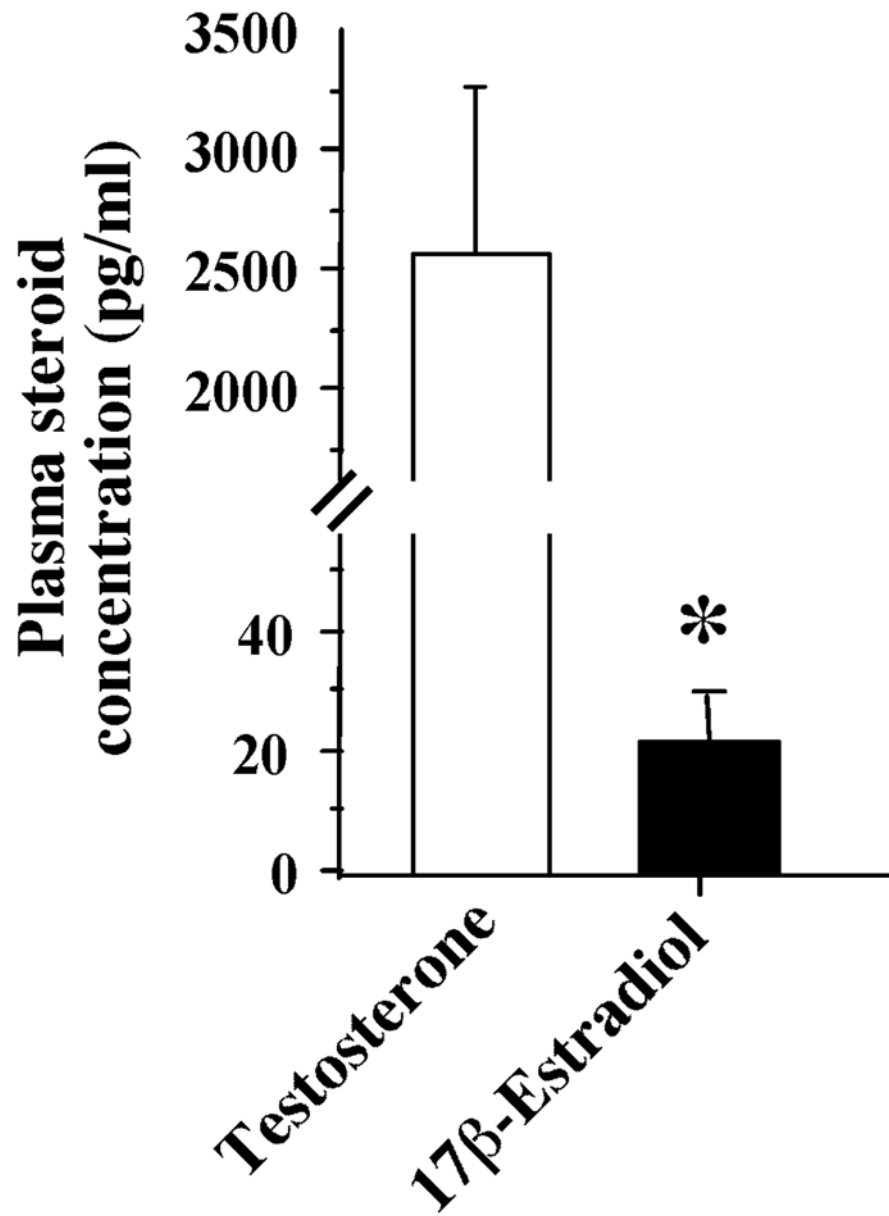
Figure 4.
Bar graph representing the plasma concentration of testosterone and estradiol. *p<0.05 vs testosterone

Table 1

Number (range) and weight (mean ± standard error of the mean) of punches from the 13 different brain regions.

	Number of punches (range)	Weight (mg) mean ± SEM	Aromatase expression
X	2 – 6	5.42 ± 0.13	0
LMAN	2 – 6	5.53 ± 0.21	0
MMAN	2 – 6	4.78 ± 0.11	+
mPOA	4 – 6	4.74 ± 0.07	++
VMN	4 – 6	4.61 ± 0.11	++
HP	8	4.41 ± 0.08	+++
LatNP	6	5.05 ± 0.11	0
OL	8 – 10	5.86 ± 0.10	0
NCM	8	5.21 ± 0.17	+++
HVC	4 – 8	4.18 ± 0.07	F
TnA	6	3.75 ± 0.11	++
RA	6 – 8	3.70 ± 0.08	F
Cb	8 – 10	5.06 ± 0.08	+

Note: We indicated the level of aromatase expression in the different brain regions (Saldanha et al., 2000), from no aromatase-immunoreactive cells (0) to high level of immunopositive cells (+++). While aromatase cell bodies are not observed in some regions, these regions present aromatase-immunoreactive fibers (F; Peteron et al., 2005)