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Region-Specific Rapid Regulation of Aromatase Activity in Zebra Finch Brain

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Region-Specific Rapid Regulation of Aromatase Activity in Zebra Finch Brain

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Physiological Science

by

Devon Comito

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ABSTRACT OF THE THESIS

Region-Specific Rapid Regulation of Aromatase Activity in Zebra Finch Brain

by

Devon Comito

Master of Science in Physiological Science

University of California, Los Angeles, 2015

Professor Barney A. Schlinger, Chair

Recent studies have shown that rapid modulation of the estrogen synthesizing enzyme, aromatase, regulates estrogen production in neurons to affect behavior and cognition. In songbirds, aromatase is expressed at high levels in the hippocampus (HP), hypothalamus (HYP), and caudomedial nidopallium (NCM), where estrogens have been shown to affect learning and memory (HP), reproductive behavior (HYP), and auditory processing (NCM). Previous studies, largely in the HYP of quail, show that aromatase activity is down-regulated by Ca^{2+}-dependent phosphorylation. Here, using zebra finches (*Taeniopygia guttata*), we ask if similar mechanisms are at work in the songbird HYP and if there are sex differences and differences in aromatase modulation between the HYP, HP and NCM. We quantified the conversion of [^{3}H]androstenedione to estrone with a well-established *in vitro* assay to measure the effects of Ca^{2+}, Mg^{2+}, ATP, and an inhibitor of kinase activity in homogenates and partially purified brain fractions. We report a rapid down-regulation of aromatase activity in the presence of

phosphorylating conditions across all three brain regions and both sexes. However, regional differences were seen in response to individual cofactor concentrations, some of which were improved by partial purification of the homogenates. Furthermore, while low concentrations of ATP inhibited aromatase activity, unexpectedly, inhibition was no longer seen with high ATP concentrations. These results provide evidence for a regional and temporal specificity in aromatase activity that has not been observed in songbirds.

The thesis of Devon Comito is approved.

Arthur P. Arnold

Thomas J. O'Dell

Barney A. Schlinger, Committee Chair

University of California, Los Angeles

2015

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Introduction

It is well known that estrogens are created by the enzyme aromatase in peripheral tissues, such as the ovary. These estrogens circulate widely to create potent and long lasting effects via slow acting transcriptional mechanisms. More recently, however, it has been found that estrogens can be produced in neurons to affect behavior on a rapid time scale (Roepke *et al.*, 2011), with important roles in reproductive behavior (Balthazart *et al.*, 2009), spatial learning and memory (Oberlander *et al*., 2004), and other cognitive processes (Balthazart & Ball, 2013). Birds have been especially useful animal models for investigating estrogen effects on brain and behavior (Schlinger & Balthazart, 2013), with songbirds in particular demonstrating elevated aromatase mRNA and protein expression in the hippocampus (HP), hypothalamus (HYP), and caudomedial nidopallium (NCM) (Saldanha *et al.,* 1998; Saldanha *et al.,* 2000). Whereas there is evidence that estrogens can fluctuate over a rapid time-scale within the songbird brain (Remage-Healey *et al.*, 2008) similar to a neurotransmitter (Balthazart & Ball, 2006), less is known about the mechanisms by which estrogen synthesis is rapidly modulated (Remage-Healey *et al.* 2012; Cornil *et al*., 2012).

Transcriptional regulation of aromatase activity can take hours to days, and is too slow for these transient estrogen dependent mechanisms. Instead, posttranslational modifications may be controlling enzyme activity. Previous studies have shown that aromatase activity can be regulated by Ca^{2+} dependent phosphorylation within minutes (Balthazart *et al.*, 2003). On depolarization, there is an increase in intracellular Ca^{2+}. This can then lead to phosphorylation of the aromatase enzyme via several different protein kinases, ultimately inhibiting its activity. This reaction requires Ca^{2+} and Mg^{2+} serving as cofactors as well as ATP, which serves as a phosphate donor. This mechanism has been identified in the HYP of Japanese quail (Balthazart

et al., 2001), in cell lines transfected with human aromatase (Charlier *et al.*, 2011), as well as in whole telencephalon of zebra finches (Cornil *et al.*, 2012). Not only is aromatase reversibly inhibited in the presence of these cofactors, but addition of kinase inhibitors prevents this inhibition (Balthazart *et al.*, 2003).

Local estrogen synthesis is implicated in sensory processing and/or behavioral activation by actions within discrete regions of the songbird brain. In particular, estrogens in the HP affect spatial learning and memory (Rensel *et al.*, 2013), in the HYP they influence reproductive behavior (Cornil *et al.*, 2012), and in the NCM they affect auditory processing (Remage-Healey *et al.*, 2012). Whereas previous studies have made regional comparisons in aromatase expression and ultrastructural compartmentalization of aromatase in these brain regions, no one has assessed the rapid mechanisms modulating aromatase activity in these brain regions separately. While we expect aromatase activity to be regulated by Ca^{2+} -dependent phosphorylations throughout the brain, we also hypothesize that aromatase activity may be differentially regulated in each brain region.

In order to test these hypotheses, we quantified the conversion of [^{3}H]androstenedione to estrone using the well established *in vitro* tritiated water assay. Since previous studies have indicated that Ca^{2+}, Mg^{2+}, and ATP are necessary for phosphorylation to occur, we first tested the effects of the concentrations of these factors individually in homogenates of each brain region. Next we tested the combined effects of phosphorylating factors and the effect of a protein kinase C (PKC) inhibitor on aromatase activity. In that local estrogen synthesis might activate behaviors differently in males and females (Peterson *et al.*, 2005; Konkle & Balthazart, 2011), we also tested for sex differences in the rapid regulation of aromatase across all three brain

regions. Finally, to refine our biochemical measures, we used both whole tissue homogenates as well as partially purified fractions enriched with microsomal and synaptosomal aromatase.

Materials and Methods

Subjects

Experiments were performed on 53 non-breeding, adult (>100 days of age) zebra finches taken from our UCLA colony. This includes 35 males and 18 females. Birds were kept on a 14:10 light:dark cycle. They received food (seed, supplements) and water ad libitum. To prevent the effects of anesthesia on enzyme activity, animals were sacrificed using rapid decapitation. All procedures were approved by the UCLA Chancellor's Animal Research Committee.

Dissections

After removal from the skull, brains were placed upside down on a cooled petri dish on ice. The HYP was first dissected from the medio-ventral part of the brain. Two parasaggital cuts were made just medial to each optic tectum approximately 2mm deep. A third coronal cut was made caudal to the optic tectum, at the level of the oculomotor neurons. This allowed the hindbrain to be completely removed. A coronal cut approximately 2mm deep rostral to the preoptic area, at the level of the tractus septomesencephalicus, was then made. The block of tissue was then removed from the brain with a transverse cut along the anterior commissure and was frozen on dry ice immediately. The brain was righted and the HP was then dissected from the skull as described previously (Saldanha *et al.,* 1998). The anterior portion of the brain was removed with a razor blade. Next, two parasaggital cuts were made 1mm from each side of the midline. The section medial to the two cuts (the HP) was then peeled off the top surface starting from the

posterior. The two HP were then immediately frozen on dry ice. Once the HP was removed, the caudal region of the telencephalon containing the NCM was removed and frozen on dry ice. The optic tecti were removed to allow the brain to lay flat. Starting at the dorsal surface, a U shape was cut starting just lateral to the HP and ending medial of the tractus dorso arcopallius.

Homogenates

On the day of the assay, each brain region was homogenized on ice in an all glass potter tube. In assays testing whole homogenates, brains were homogenized in 4^{o}C KTH buffer (150mM KCl, 10mM Tris-Hepes, pH 7.2). In experiment 4, whole homogenates were prepared in 4^{o}C KTH-Sucrose buffer (150mM KCl, 10mM Tris-Hepes, 320mM Sucrose, pH 7.2). To equilibrate protein concentration across tissues, each HP was homogenized in 700ul KTH buffer, each HYP was homogenized in 1000ul KTH buffer, and each NCM was homogenized in 1400ul KTH buffer. In assays testing partially purified fractions, brains were homogenized in 4^{o}C KTH-Sucrose buffer. Pilot experiments were tested on pooled homogenates from 4 male brains. In order to ensure an adequate protein concentration, all four HP were homogenized in 1400ul KTH-Sucrose buffer, HYP were homogenized in 2000ul KTH-Sucrose buffer, and NCM were homogenized in 2800ul KTH-Sucrose buffer. In Experiment 5, samples from 6 males and 6 females were homogenized independently. To ensure an adequate volume and protein concentration, each HP was homogenized in 500ul KTH-Sucrose buffer, each HYP was homogenized in 650ul KTH-Sucrose buffer, and each NCM was homogenized in 900ul KTH-Sucrose buffer. After homogenization, samples were centrifuged at 1,000G for 15 minutes at 4^{o}C. Supernatant was removed to new microcentrifuge tubes. This supernatant was then used as the "homogenate" sample.

Aromatase Activity Assay

After homogenization, aromatase activity was measured by quantifying the amount of tritiated water produced by aromatization of [1β-^{3}H]androstenedione (Roselli and Resko, 1991). At all times, samples were kept on ice. Activity was assayed in microcentrifuge tubes. Each tube first received 100ul of homogenate, followed by100ul of KTH buffer containing experimental factors. Assays also included two extra "background" samples that received 100ul homogenate from 4 random individuals, 100ul KTH buffer, and 10ul of the aromatase inhibitor fadrozole (0.41mg/ml dissolved in water)(Wade *et al.*, 1994). All samples were then briefly vortexed and preincubated for 10 minutes in a 37^{o}C shaking water bath. After 10 minutes, samples were transferred back to ice and received 50ul [1β-^{3}H]androstenedione (specific activity = 24.0 Ci/mmol; PerkinElmer, Waltham, MA) to reach a final concentration of 25nM, a value which has been shown previously to be a saturating concentration in quail (Baillen & Balthazart,1997). All samples then received 50ul NADPH for a final concentration of 1.2mM. Substrate and cofactor concentrations are based on the final volume of 300ul per tube. Samples were immediately transferred back into the 37^{o}C shaking water bath to start the assay. After a 10 minute incubation, samples were removed to ice to stop the reaction and were centrifuged at 4^{o}C for 10 minutes at 10,000G (9600rpm^{-1}). The supernatant was removed and transferred into fresh microcentrifuge tubes while the remaining protein pellet was saved in the -80^{o}C freezer to be analyzed for protein concentration at a later time.

To quantify the formation of tritiated H_2O, 600ul of 10% trichloroacetic acid with 2% charcoal was then added to all supernatant samples. All samples were then centrifuged at 5000G, 4^{o}C for

15 minutes. Supernatant was then run through Dowex cation exchange columns made of Pasteur pipettes plugged with glass beads and filled with 1.4ml dowex cation exchange resin AG 50W-X4, 100-200 mesh (Biorad, Hercules, CA) and rinsed 3 times with 600ul MilliQ water. Effluent was collected in 20ml scintillation vials and 15ml liquid scintillation cocktail was added to each sample. Samples remained in the dark for a minimum of 4 hours and were counted for 4 minutes each. Protein pellets were assayed with the ThermoFisher Scientific Pierce BCA protein assay kit (Waltham, MA). Aromatase activity was expressed as disintegrations per minute (DPM) per mg protein after correction of the counts for quenching, recovery, blank values, and percentage of tritium in β position in the substrate.

Pilot Experiments 1-3: Effects of Ca^{2+}, ATP and Mg^{2+} on aromatase activity in HP, HYP, and NCM homogenates

Three preliminary experiments were carried out to determine potential effects of Ca^{2+}, ATP, and Mg^{2+}, factors previously shown to influence the phosphorylation of aromatase activity of hypothalamic aromatase activity in other species. Experiments were carried out in each brain region on whole homogenates (n = 3 males) and/or partially purified fractions (n = 4 males, pooled and assayed in duplicate).

First, we tested the effects of Ca^{2+} concentration on aromatase activity in whole homogenates and partially purified fractions. We prepared 6 microcentrifuge tubes, one for each concentration, with 100ul of sample. Then 50ul of buffer containing Ca^{2+} was added. We examined 5 different Ca^{2+} concentrations: 0mM, 0.1mM, 0.5mM, 1mM, and 2mM. To establish a null treatment condition, we also included a treatment with 0mM Ca^{2+} plus 0.5mM EGTA, a known Ca^{2+} chelator. Next, 50ul of buffer containing ATP and Mg^{2+} (final concentration of

0.5mM) was added to all samples. All concentrations are based on the final volume of the preincubation reaction, 200ul.

Second, we tested the effects of ATP concentration of aromatase activity in whole and partially purified homogenates. Six different ATP concentrations were tested: 0mM, 0.1mM, 0.5mM, 1mM, 2mM, and 5mM. Samples received 100ul homogenate, followed by 50ul of the corresponding ATP solution, followed by 50ul of buffer containing Mg^{2+} (final concentration of 0.5mM).

Third, we tested the effects of 6 different Mg^{2+} concentrations on aromatase activity in whole homogenates. Final concentrations tested were 0mM, 1mM, 2mM, 6mM, 10mM, and 14mM. Samples received 100ul homogenate, followed by 50ul Mg^{2+}, followed by 50ul of ATP (final concentration of 0.5mM).

Experiment 4: Effects of combined phosphorylating factors on aromatase activity on HP, HYP, and NCM whole homogenates

HP, HYP, and NCM were dissected from 6 males and 6 females and homogenized as previously described. Three different conditions were tested: control, Ca^{2+}/Mg^{2+}/ATP (phosphorylating conditions), and Ca^{2+}/Mg^{2+}/EGTA (phosphorylating conditions with a Ca^{2+} chelator). Control samples received 100ul homogenate and 100ul KTH buffer. Ca^{2+}/Mg^{2+}/ATP groups received 100ul sample, 50ul of 0.5mM Ca^{2+}/Mg^{2+}/ATP, and 50ul KTH buffer. Ca^{2+}/Mg^{2+}/EGTA groups received 100ul sample, 50ul of 0.5mM Ca^{2+}/Mg^{2+} and 50ul of 0.5mM EGTA. Concentrations of Ca^{2+}, Mg^{2+}, ATP, and EGTA are final concentrations based on previous studies in quail preoptic area (Konkle and Balthazart, 2011).

Experiment 5: Effects of a PKC inhibitor on aromatase activity in HP, HYP, and NCM homogenates

This experiment tested the effects of Bisindolylmaleimide (Bis) (Calbiochem, Merck, Nottingham UK), a known PKC inhibitor, on whole and partially purified homogenates at two ATP concentrations in 6 males and 6 females. Samples were tested in the presence or absence of 10uM Bis (Balthazart, 2003) at both 1mM ATP and 5mM ATP concentrations, based on results from Experiment 2. In addition, based on the results from experiments 1 and 3, all samples received KTH buffer containing 6mM Mg^{2+} and 1mM Ca^{2+}. As a result, there were four different treatment conditions: 1mM ATP with Bis, 1mM ATP without Bis, 5mM ATP with Bis, and 5mM ATP without Bis. Reported concentrations represent the final concentration based on the preincubation volume of 200ul.

Aliquots of 100ul were separated into the four groups mentioned above. Samples first received 50ul of Bis or KTH buffer. Since a small amount of DMSO was needed to dissolve Bis into solution, all control samples received KTH buffer with an equal amount of DMSO. Then 50ul of KTH buffer containing Ca^{2+}/$Mg^{2+/}$ATP was added. The preincubation and incubation were carried out as previously described.

Statistical Analyses

In all experiments, aromatase activity was assessed using linear mixed models. Bird identity was always entered as a random factor. In pilot experiments, brain region and ion concentration were set as fixed factors. For experiment 4, treatment and sex were used as fixed factors. Experiment 5 used treatment, ATP concentration, and sex as fixed factors. Brain region was not a factor in experiments 4 and 5 since each region was tested in a separate assay. Data was first tested for

normality and was logarithmically transformed if it was not normally distributed. If a significant main effect was found ($p < .05$), a least significant difference post-hoc test was performed. All statistical analyses were performed using SPSS 22 (IBM Statistics, 2014). All figures show raw data mean values ± 1 SE.

Results

Experiment:1 Effects of Ca^{2+} on aromatase activity in HP, HYP, and NCM homogenates

Although aromatase activity appears to decrease with the addition of Ca^{2+}, there was no significant effect of Ca^{2+} concentration on aromatase activity in whole homogenates in any of the three brain regions (HP: $F_{5,10} = .584$, $P = .713$; HYP: $F_{5,10} = 2.164$, $P = .140$; NCM: $F_{5,10} = 1.392$, $P = .306$; Figure 1). However, when the experiment was repeated on a pooled sample of partially purified homogenates, a drastic inhibition was seen in NCM (Figure 2). Samples receiving 0.1mM Ca^{2+} showed a 70% decrease in aromatase activity in comparison with samples containing the Ca^{2+} chelator, EGTA, and 0mM Ca^{2+}. After 0.1mM Ca^{2+}, aromatase activity remained at a steady low level and no further enzyme inhibition was seen in the NCM. For all subsequent experiments we used 1mM Ca^{2+} as this value remains physiologically relevant and appears to reduce enzyme activity across all brain regions.

Experiment 2: Effects of ATP on aromatase activity in HP, HYP, and NCM homogenates

In whole homogenates, ATP concentration had a significant effect on aromatase activity in HP ($F_{5,10} = 4.279$, $P = .018$)(Figure 3). In HP, low ATP levels decreased aromatase activity. Specifically, post hoc analysis revealed that activity was significantly inhibited until 0.5mM ATP. Concentrations higher than 0.5mM ATP slowly started to increase aromatase activity.

Addition of 5mM ATP significantly increased aromatase activity to levels above those seen with 0.5mM, 1mM, and 2mM ATP. In the NCM, there was no effect of ATP ($F_{5,10} = 2.804$, $P = .078$), although there appears to be a strong inhibition at 1mM ATP and a loss of inhibition at 2-5mM ATP. There was also no effect of ATP concentration on aromatase activity in the HYP ($F_{5,10} = .398$, $P = .840$).

When this experiment was repeated in a pooled sample of partially purified homogenates, results were amplified in the HP and NCM (Figure 4). Aromatase activity decreased from 0 to 1mM ATP concentrations. From 1-5mM ATP, aromatase activity significantly increased to values above control samples. Those with 5mM ATP appeared significantly higher than all concentrations. In NCM, control aromatase activity more than doubled at 5mM ATP, and in HP, activity increased to levels 25% greater than seen in controls. Although ATP showed no significant inhibition on aromatase activity in partially purified HYP samples, aromatase activity did increase slightly at high ATP concentrations.

Because of the divergent effects of high and low concentrations of ATP on aromatase activity, in subsequent experiments, we tested both 5mM and 1mM ATP concentrations to assess phosphorylating conditions.

Experiment 3: Effects of Mg^{2+} on aromatase activity in HP, HYP, and NCM homogenates

Mg^{2+} showed a significant effect on aromatase activity in whole homogenates of all three brain regions (HP- $F_{5,10} = 5.468$, $P = .011$; HYP- $F_{5,10} = 3.73$, $P = .036$; NCM-$F_{5,10} = 7.12$, $P = .004$) (Figure 5). A steady decrease in activity can be seen from 0mM Mg^{2+} to 14mM Mg^{2+} concentrations. As 6mM Mg^{2+} was the minimal value required to significantly inhibit aromatase

activity while remaining within physiologically ranges, this concentration was chosen for future experiments.

Experiment 4: Effects of combined phosphorylating factors on aromatase activity on HP, HYP, and NCM whole homogenates

When samples received a combination of Ca^{2+}, Mg^{2+} and ATP, a significant treatment effect was seen in all three brain regions (HP: $F_{2,20} = 34.001$, $P < .0001$, Figure 6; HYP: $F_{1, 20} = 8.143$, $P = .003$, Figure 7; NCM: $F_{2,20} = 6.879$, $P = .005$; Figure 8). In addition, the HYP showed a significant sex difference ($F_{1,10} = 7.739$, $P = .019$). Males showed higher overall aromatase activity than females in the HYP. This sex difference was not seen in HP ($F_{1,10} = .031$, $P = .864$) or NCM ($F_{1,10} = 3.748$, $P = .082$).

Post hoc analysis revealed that in HP, samples receiving Ca^{2+}/Mg^{2+}/ATP in both males and females were significantly lower than the control and Ca^{2+}/Mg^{2+}/EGTA samples. In the HYP, female samples receiving Ca^{2+}/Mg^{2+}/ATP showed a significant decrease in activity in comparison to the control and Ca^{2+}/Mg^{2+}/EGTA. Male samples receiving Ca^{2+}/Mg^{2+}/ATP were significantly lower than Ca^{2+}/Mg^{2+}/EGTA but not significantly lower from the control. In NCM, only female Ca^{2+}/Mg^{2+}/ATP were significantly lower than Ca^{2+}/Mg^{2+}/EGTA. None of the brain regions showed a significant difference between control and Ca^{2+}/Mg^{2+}/EGTA samples, although often times Ca^{2+}/Mg^{2+}/EGTA samples appeared higher in activity than control. These results support that phosphorylating conditions together lower aromatase activity throughout the brain.

Experiment 5: Effects of a PKC inhibitor on aromatase activity in HP, HYP, and NCM homogenates

While no sex effects were seen, the effects of Bis and ATP varied across brain regions in whole homogenates. HP whole homogenates showed significant Bis effects ($F_{1,31}$ = 11.640, P = .002) (Figure 9). Bis increased aromatase activity. While there was no significant effect of ATP ($F_{1,31}$ = .411, P = .526) or sex effect ($F_{1,10}$ = .442, P = .521), there was a significant ATP*Sex interaction ($F_{1,31}$= 5.725, P = .023) in the HP as well. The addition of 5mM ATP increased overall aromatase activity in males but not in females. When looking at the HYP, only a significant ATP effect can be seen ($F_{1, 30.262}$ = 11.668, P = .002) (Figure 10). In both males and females, samples run with 5mM ATP had higher activity than those run with 1mM ATP. There was no significant effect of Bis ($F_{1, 30.262}$ = .178, P = .676) and there were no sex effects ($F_{1, 10.127}$ = .037, P = .852). The NCM was similar to the HP in that it showed a significant Bis effect ($F_{1,31}$ = 18.773, P < .0001)(Figure 11). It was also similar to the HYP in that it showed a significant ATP effect ($F_{1,31}$ = 34.81, P < .0001). However, unlike the HP and HYP, the NCM also showed a significant ATP*Bis interaction ($F_{1,31}$ = 4.985, P = .033). The effects of Bis in samples treated with 5mM ATP were nullified. Control samples and Bis treated samples at 5mM ATP had similar activity levels.

Due to the differences seen between whole homogenates and partially purified homogenates in the validation experiments, this set of experiments was then repeated on partially purified homogenates. Partially purified homogenates of each region showed very similar effects. There was a significant Bis effect in all regions (HP: $F_{1,31}$ = 117.278, P < .0001, Figure 12; HYP: $F_{1, 30.664}$ = 35.826, P < .0001, Figure 13; NCM: $F_{1,31}$ = , P < .0001, Figure 14). Bis again increased aromatase activity in comparison with control samples. In addition, all regions showed a significant ATP effect (HP: $F_{1, 31}$ = 39.864, P < .0001; HYP: $F_{1,30.664}$ = 23.787, P <

.0001; NCM: $F_{1, 31} <$, P = .0001). Once again, partial purification appears to have clarified and amplified the results.

Discussion

Overall, these results further support the idea of rapid aromatase modulation by Ca^{2+}-dependent phosphorylation. Within minutes, aromatase in all three brain regions was inhibited in the presence of phosphorylating factors such as Ca^{2+}, Mg^{2+}, and ATP. When a protein PKC inhibitor was added to the mix, this inhibition was no longer seen. Whereas previous studies described rapid modulation of brain aromatase in quail HYP and zebra finch telencephalon (Balthazart *et al.*, 2003; Cornil *et al.*, 2012), this is the first time a region specific comparison has been made in a songbird brain. While the nature of aromatase inhibition appears to be consistent, there are regional and species differences in the effects of various cofactors. Moreover, we have evidence that high concentrations of ATP reverse the expected inhibition of aromatase.

Enzymes such as tyrosine hydroxylase can be modulated by divalent cations (Ames *et. al.*, 1978; Albert *et al.*, 1984). Originally it was proposed that aromatase may be modified by posttranslational processes similar to those seen in tyrosine hydroxylase. Early evidence indicated that divalent cations may control aromatase activity (Hochberg *et al.*, 1986; Steimer & Hutchison, 1981). As a result, many of the first experiments in the avian brain explored the effects of phosphorylating factors on aromatase activity. Most early evidence for rapid aromatase modulation in the brain has come from *in vitro* studies of whole homogenates of Japanese quail (*Coturnix japonica*) HYP. Therefore, we first examined whole homogenates of zebra finch brain. The present study allowed us to make three main comparisons: 1) a comparison between quail

HYP and zebra finch HYP, 2) a comparison between quail HYP and zebra finch HP and NCM, and 3) a species specific comparison between zebra finch HP, HYP, and NCM.

Experiments in quail HYP reported the rapid inhibition of aromatase activity in the presence of Ca^{2+}, Mg^{2+}, and ATP (Balthazart *et al.*, 2001). However, when the individual effects of Ca^{2+}, Mg^{2+}, and ATP were tested in the present study, species and regional differences can be seen. Aromatase activity in zebra finch HYP was only affected by Mg^{2+}. No effect of Ca^{2+} concentration or ATP concentration can be seen in zebra finch HYP. Similar to zebra finch HYP, whole homogenates of the zebra finch NCM only responded to Mg^{2+} concentration as well. On the other hand, aromatase activity in whole homogenates of zebra finch HP was affected by both Mg^{2+} and ATP concentrations**.** Although the addition of Mg^{2+} clearly inhibits aromatase activity across species and brain regions, the role and availability of Ca^{2+} and ATP are still unclear.

The lack of a noticeable Ca^{2+} effect was surprising given that previous research has shown aromatase in the quail HYP as well as in the zebra finch NCM to be affected by an increase in intracellular Ca^{2+} (Balthazart *et al.*, 2001; Remage-Healey *et al.*, 2011). Since large populations of aromatase are found in the synapse and cell body (Schlinger, 1989), we wondered if a partial purification of whole homogenates would help clarify these unexpected results by enriching the samples with synaptosomes and microsomes. When the effect of Ca^{2+} was retested in partially purified fractions of the NCM, a drastic inhibition was seen with the addition of just 0.1mM Ca^{2+}. However, partial purification did not change the effects of Ca^{2+} on the HP or HYP. This may indicate a true species and regional difference in aromatase modulation in the zebra finch.

These results question the dynamics of kinase and cofactor availability throughout the different regions. Previous studies have found regional differences in Ca^{2+} concentration and regional differences in the regulation of Ca^{2+} uptake in rat brain (Hartmann *et al*, 1996; Jensen *et al.*, 1990). It is possible that intracellular Ca^{2+} availability naturally differs between each brain region in the present study. Perhaps each region has a different Ca^{2+} buffering capacity. Furthermore, it has been suggested that control of phosphorylation may be indirectly dependent on the interaction of aromatase with additional enzymes and modulatory factors (Charlier *et al.*, 2015). Perhaps these factors vary between brain regions as well. Future experiments are needed to characterize regional cofactor distribution in the songbird brain.

When the combination of Ca^{2+}/Mg^{2+}/ATP was applied to whole homogenates, an equally robust inhibition on enzyme activity was seen in all brain regions. While this combined effect is in accord with previous findings in quail HYP (Balthazart *et al.*, 2001; Konkle & Balthazart, 2011; Cornil *et al.*, 2012), these results further question the dynamics of kinase and cofactor availability throughout the songbird brain. Although the combination of Ca^{2+}/Mg^{2+}/ATP resulted in an inhibition across all zebra finch brain regions, no significant difference was seen between control samples and samples receiving Ca^{2+}/Mg^{2+}/EGTA in any of the brain regions. In quail HYP, a similar study found an overall increase in aromatase activity with the addition of EGTA as well as a sex difference in the effects of EGTA (Konkle & Balthazart, 2011). Although the conditions in the current experiment were not identical to those tested in quail, these results further suggest differences in Ca^{2+} availability within specific regions and between quail and zebra finch brain.

While there appear to be differences in Ca^{2+} between zebra finch and quail, the current study also suggests possible differences in ATP use and regulation between species and brain

regions. Once again, partial purification changed the results seen in the NCM. While no ATP effect was seen in NCM whole homogenates, a strong effect was seen in partially purified fractions. Results in the HP and HYP remained consistent with findings in whole homogenates. This indicates the buffering capacity and response to change in the NCM may vary the most in comparison to the HYP and HP.

Furthermore, while effects seen in zebra finch HP partially purified fractions were consistent with those seen in whole homogenates, purification amplified the effects of ATP. In partially purified HP, an inhibition was seen at low ATP concentrations. After 1mM ATP, activity increased to values well above control levels. While previous studies have not specifically examined the effect of ATP concentration on aromatase activity in brain, previous work has shown that the addition of high but physiological levels of ATP help decrease aromatase activity to less than half the activity seen in controls (Balthazart *et al.*, 2001). In cell lines expressing aromatase, 8mM ATP decreases enzyme activity to almost zero (Charlier *et al.*, 2011). Also, zebra finch telencephalic synaptosomes receiving phosphorylating conditions with 2mM ATP showed a stronger inhibition than those receiving the same conditions with only 1mM ATP (Cornil *et al.*, 2012). It is possible that ATP is activating a separate pathway that is then causing this increase in enzyme activity, but reasons for this difference and increase in activity are still unclear. Future studies may study effects of ATP concentration on regional synaptosomal preparations. The HP, which shows the greatest effect of ATP on aromatase activity in both whole homogenates and partially purified samples, is also a region different from the HYP and NCM in that it has low somal aromatase and high synaptosomal aromatase (Peterson *et al.*, 2005). Therefore, this effect may be more specific to reactions occurring at the synapse.

Further support for species and regional differences in cofactor availability is seen when testing the effects of Bis on aromatase activity. Previous studies have shown that kinase inhibitors prevent inhibition of aromatase activity while in the presence of phosphorylating factors (Balthazart *et al.*, 2003). When the effect of a PKC inhibitor was tested on whole homogenates in the zebra finch HYP, only a significant ATP effect was seen. On the contrary, the HP showed a significant Bis and ATP*Sex interaction while the NCM showed a significant Bis*ATP interaction. Interestingly, while sex differences have been shown in quail HYP (Konkle & Balthazart, 2011), this is the first time a sex difference has been seen in regulation of aromatase activity in zebra finches. Although this difference was not consistent throughout the present study, it is possible that there may be differential modulation of aromatase activity in females and males. As a whole, these results indicate that not only are there potential regional differences in cofactor regulation, but there may also be sex differences in cofactor regulation in zebra finch brain.

When the experiment was repeated in partially purified fractions however, an overall ATP effect and an overall Bis effect can be seen in all zebra finch brain regions. This result further confirms aromatase is fundamentally controlled by Ca^{2+}-dependent phosphorylation in both zebra finch and quail brain. Previous studies indicated that Ser/Thr kinases are most likely phosphorylating the aromatase enzyme itself (Balthazart, 2001; Balthazart, 2003), and the present results support the role of PKC in aromatase phosphorylation and inhibition. However, these findings also highlight potential differences in the effect of ATP in zebra finch and quail brain.

In conclusion, these results provide evidence for a species, regional, and temporal specificity in aromatase activity that has not been observed in birds. Although it is clear that

Ca^{2+}-dependent phosphorylation inhibits enzyme activity, the mechanism is more complex and sensitive than originally perceived. There may be regional differences in cofactor use and availability. This is also the first time a bimodal effect of ATP has been shown on brain aromatase activity. Future studies plan to test regional differences in living explants. Although the enzyme has the ability to be modulated by Ca^{2+}-dependent phosphorylations in each of these regions, the mechanisms used in intact, living neurons may be different. These studies help reveal underlying mechanisms whereby estrogen levels can fluctuate rapidly in brain to exert actions as a neuromodulator.

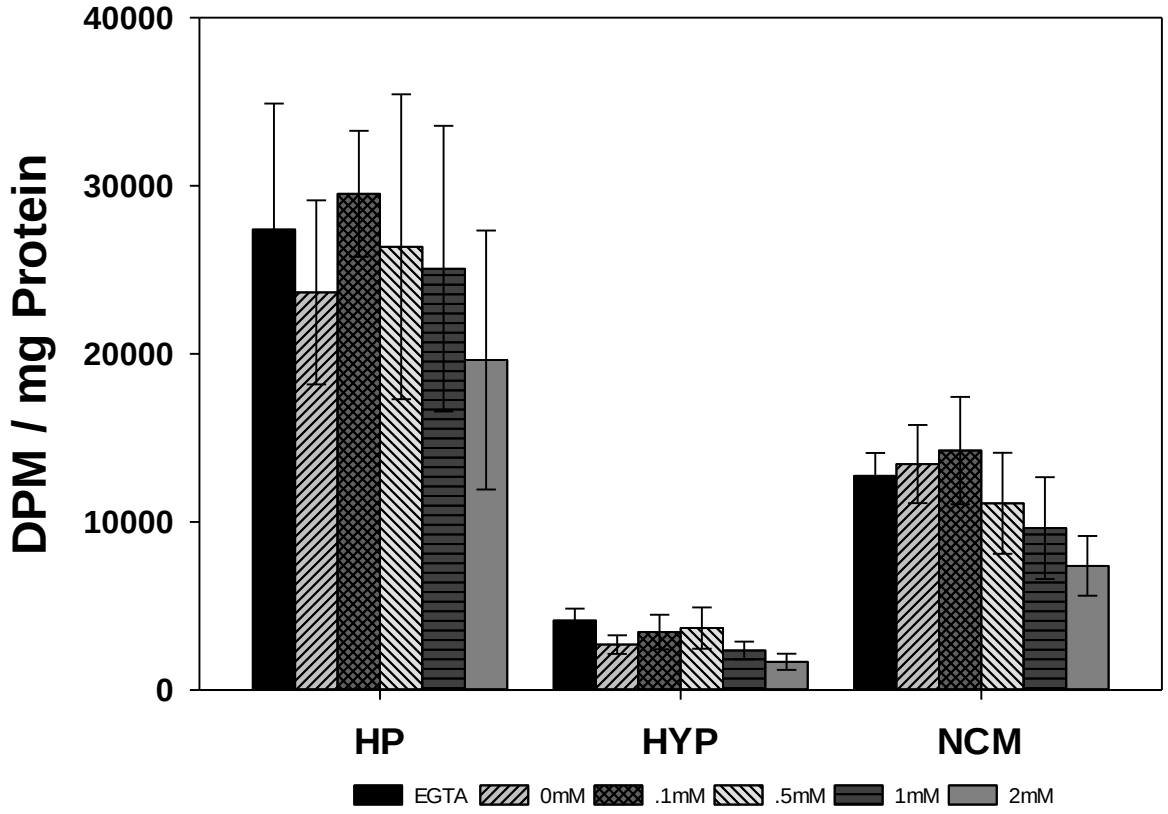

Figure 1: Effect of Ca^{2+} on aromatase activity in whole homogenates of HP, HYP, and NCM

Ca^{2+} was tested at 6 concentrations in each brain region ranging from EGTA and no Ca^{2+} through 2mM Ca^{2+}. Each bar represents mean activity levels at a specific [Ca^{2+}], measured in disintegrations per minute (DPM) and adjusted per mg protein in each sample after a ten minute preincubation and a 10 minute incubation with substrate. Error bars are +/- 1 SEM. Letters over bars indicate significance ($p < .05$). n = 3 males.

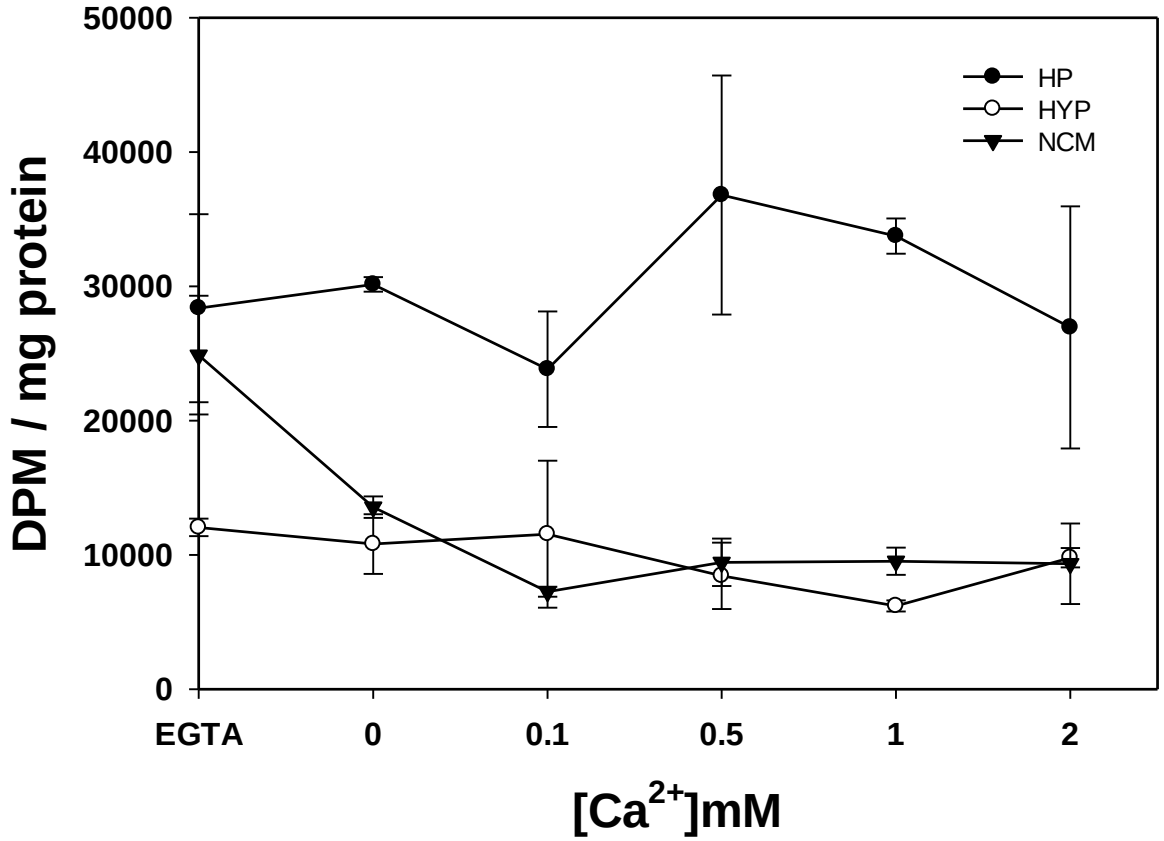

Figure 2: Effect of Ca^{2+} on aromatase activity in partially purified homogenates of HP, HYP, and NCM

Respective regions from 4 male zebra finches were pooled and homogenized. Samples were purified and S1 was preincubated 10min with 6 [Ca^{2+}] and incubated for an additional 10 minutes with substrate. Each point represents mean activity levels at a specific [Ca^{2+}], measured in DPM and adjusted per mg protein in each sample. Error bars are +/- 1 SEM. Samples were assayed in duplicate.

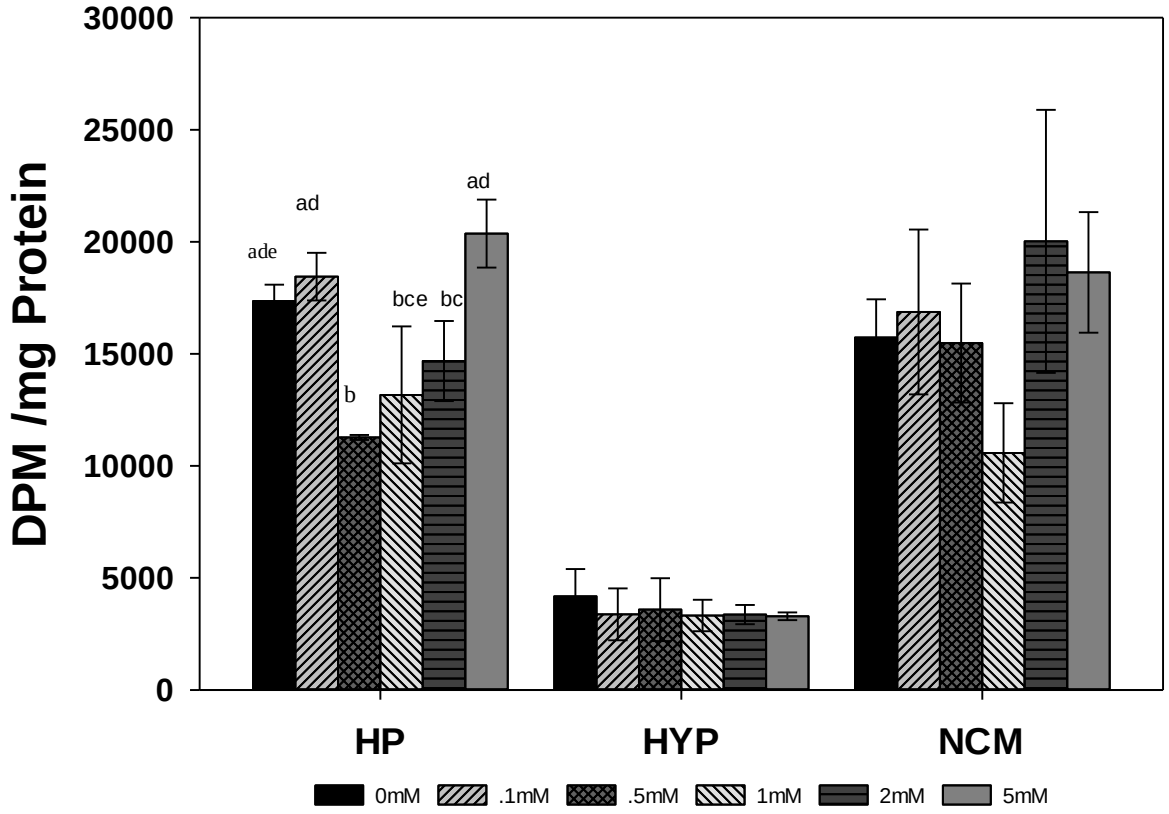

Figure 3: Effect of ATP concentration on aromatase activity in wholes homogenates of HP, HYP, and NCM

Aromatase activity measured in disintegrations per minute (DPM) and adjusted per mg protein in each sample. Whole homogenates from each region were treated with 6 ATP concentrations, ranging from 0mM to 5mM ATP. Each bar represents mean activity levels at a specific [ATP]. Samples received a 10 minute preincubation followed by a 10 minute incubation. Error bars are +/- 1 SEM. Letters over bars indicate significance ($p < .05$). n = 3 males.

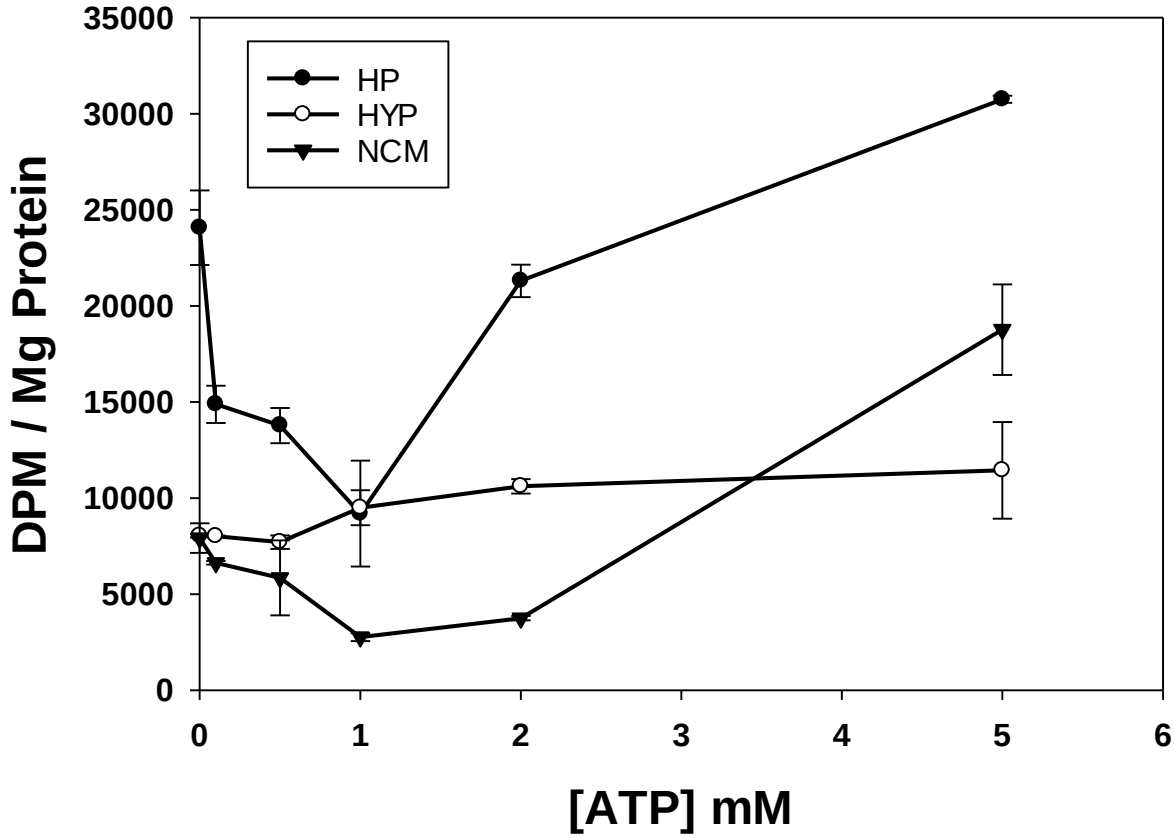

Figure 4: Effects of ATP concentration on aromatase activity of partially purified homogenates of HP, HYP, and NCM

Respective regions from 4 male zebra finches were pooled and homogenized. Samples were partially purified and S1 was preincubated 10min with 6 different [ATP] ranging from 0mM to 5mM. Each point represents mean activity levels at a specific [ATP]. Enzyme activity was quantified by amount of tritiated water produced and measured in DPM and adjusted per mg protein in each sample. Error bars are +/- 1 SEM. Samples were assayed in duplicate.

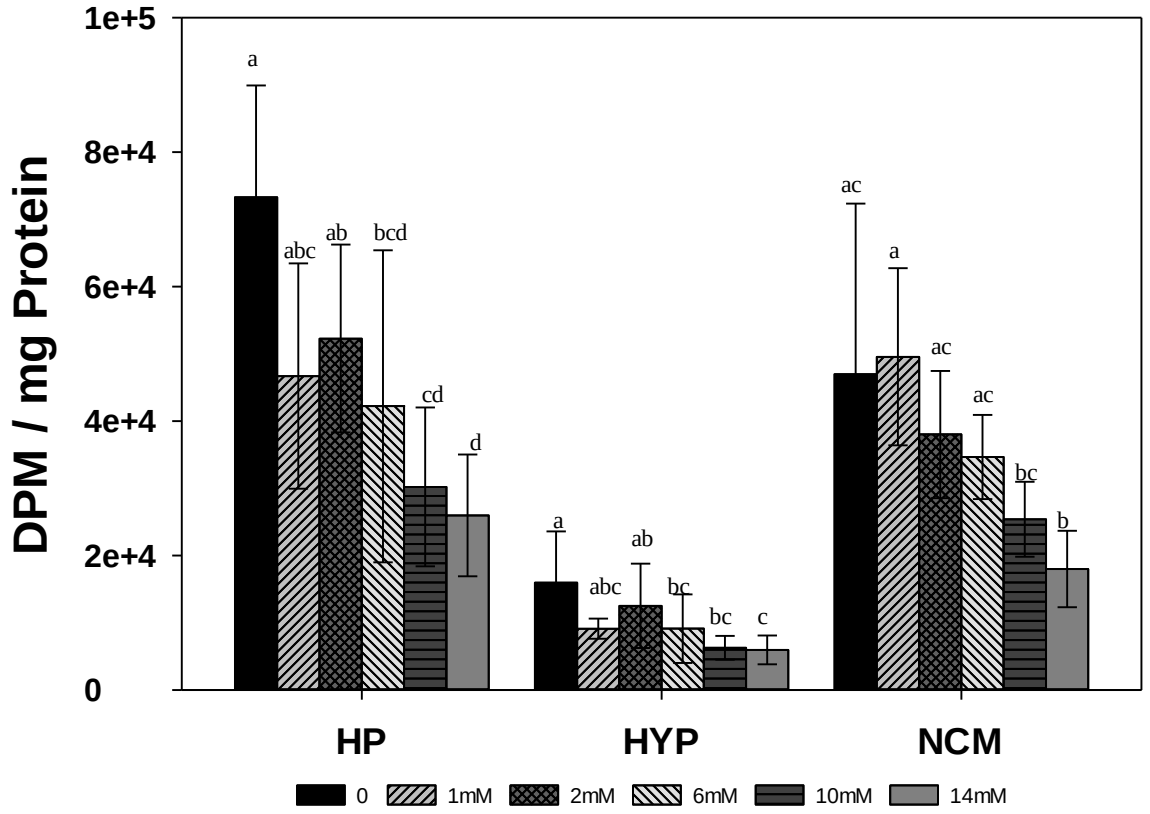

Figure 5: Effects of Mg^{2+} concentration on aromatase activity in whole homogenates of HP, HYP, and NCM

Aromatase activity quantified by production of tritiated water after a 10 minute preincubation and 10 minute incubation with substrate. Aromatase activity is measured in disintegrations per minute (DPM) and adjusted per mg protein in each sample. Whole homogenates from each region were treated with 6 Mg^{2+} concentrations, ranging from 0mM to 14mM Mg^{2+}. Each bar represents mean activity levels at a specific [Mg^{2+}]. Error bars are +/- 1 SEM. Letters over bars indicate significance ($p < .05$). n = 3 males.

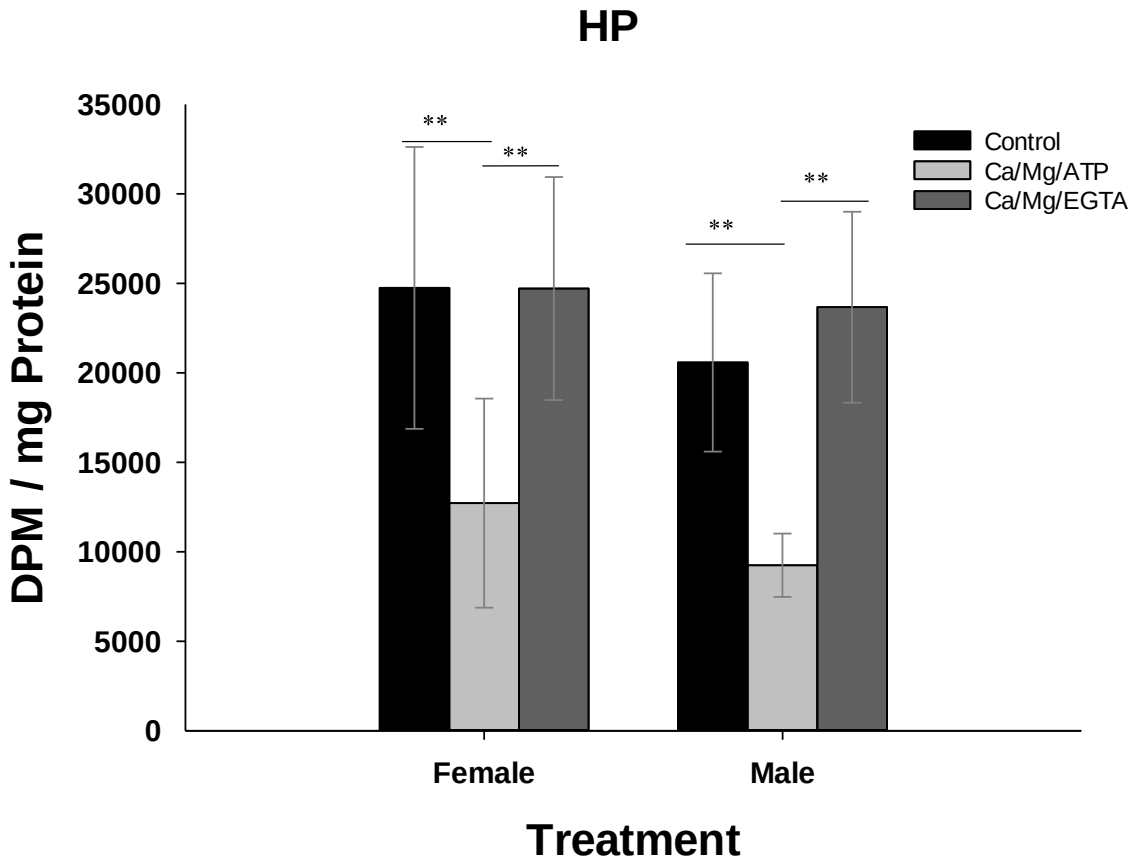

Figure 6: Effects of Ca^{2+}/Mg^{2+}/ATP on aromatase activity in HP whole homogenates

Aromatase activity was measured in whole homogenates of male (n = 6) and female (n = 6) zebra finches. Samples received a10 minute preincubation followed by a 10 minute incubation. Samples were treated with control conditions, phosphorylating conditions, or phosphorylating conditions in the presence of EGTA. Activity is measured in DPM per mg protein in each sample. Bars represent the mean activity +/- 1SEM. Significance: *P < .05, ** P < .01, *** P < .001. There was an overall treatment effect, p < .0001.

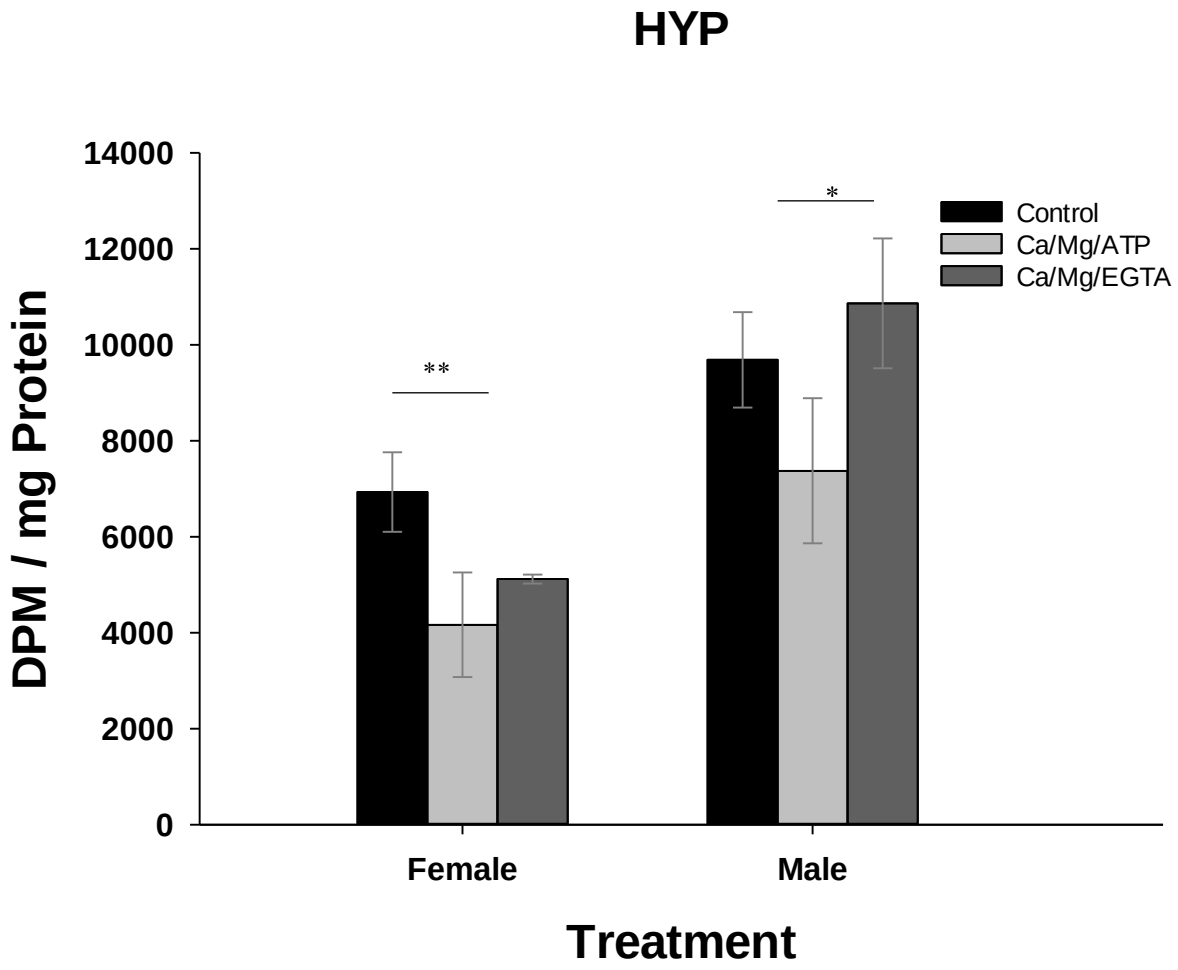

Figure 7: Effects of Ca^{2+}/Mg^{2+}/ATP on aromatase activity in HYP whole homogenates

Aromatase activity was measured in whole homogenates of male (n = 6) and female (n = 6) zebra finches. Samples received a10 minute preincubation followed by a 10 minute incubation. Samples were treated with control conditions, phosphorylating conditions, or phosphorylating conditions in the presence of EGTA. Activity is measured in DPM per mg protein in each sample. Bars represent the mean activity +/- 1SEM. Significance: *P < .05, ** P < .01, *** P < .001 There was an overall treatment effect, P = .003 as well as a sex effect, P = .019.

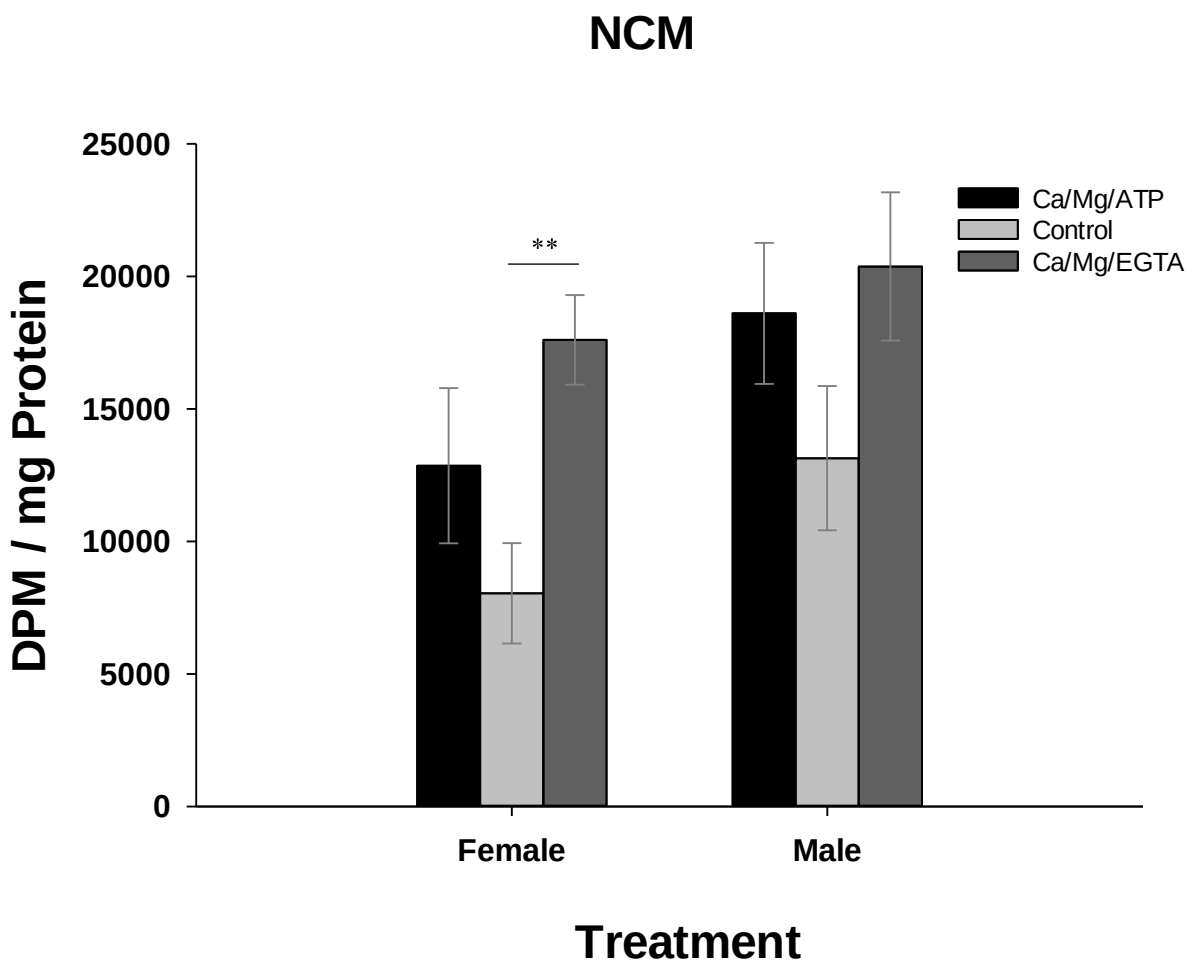

Figure 8: Effects of Ca^{2+}/Mg^{2+}/ATP on aromatase activity in NCM whole homogenates

Aromatase activity was measured in whole homogenates of male (n = 6) and female (n = 6) zebra finches. Samples received a10 minute preincubation followed by a 10 minute incubation. Samples were treated with control conditions, phosphorylating conditions, or phosphorylating conditions in the presence of EGTA, indicated by bar color. Activity is measured in DPM per mg protein in each sample. Bars represent the mean activity +/- 1SEM. Significance: *P < .05, ** P < .01, *** P < .001 There was an overall treatment effect, P = .005.

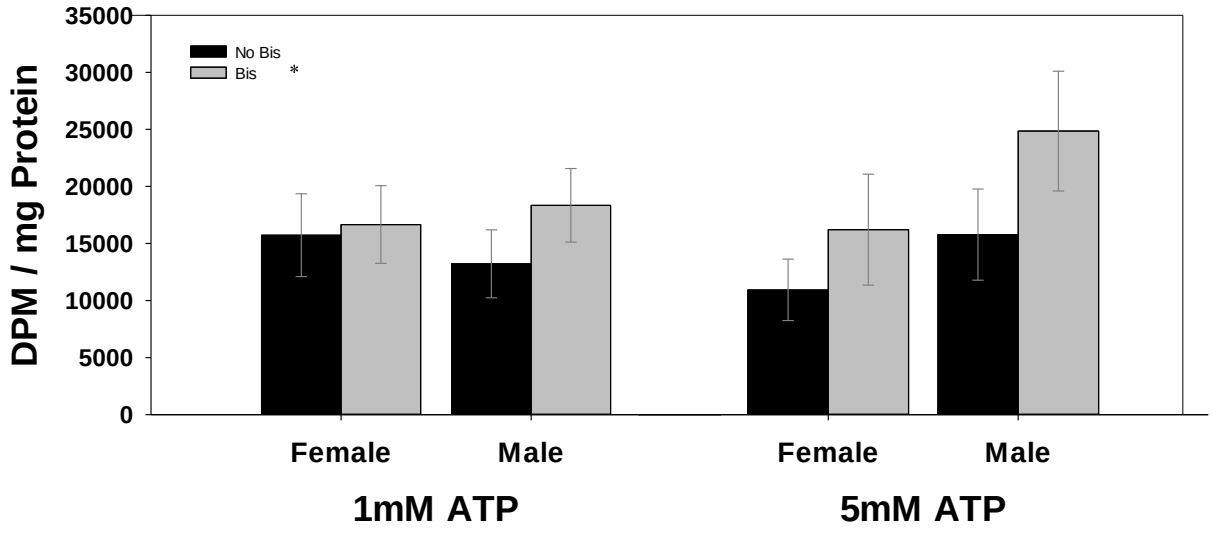

Figure 9: Effects of Bis and ATP on aromatase activity in HP whole homogenates

HP samples were preincubated for 10 minutes in the presence or absence of Bis and at 1mM or 5mM ATP. Activity was measured in whole homogenates in males (n =6) and females (n =6). Enzyme activity is measured in DPM per mg protein in each sample. Bars represent mean activity level +/- 1 SEM after a 10 minute incubation with substrate. An asterisk indicates an overall Bis effect, P = .002. There is also an ATP*Sex interaction as addition of 5mM ATP increased overall aromatase activity in males but not in females mM ATP, P = .023.

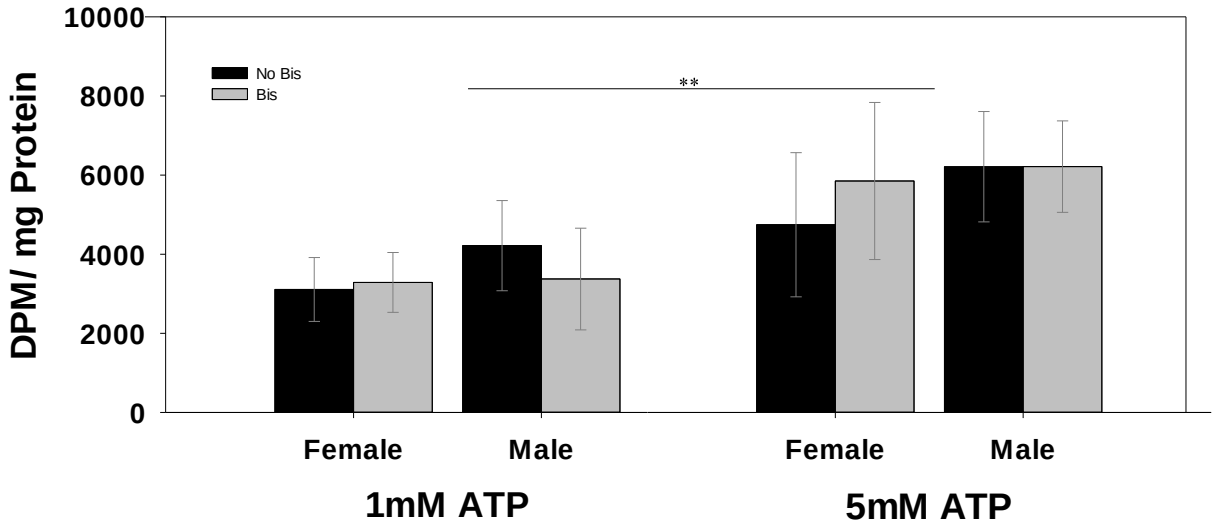

Figure 10: Effects of Bis and ATP on aromatase activity in HYP whole homogenates

HYP samples were preincubated for 10 minutes in the presence or absence of Bis and at 1mM or 5mM ATP. Activity was measured in whole homogenates in males (n =6) and females (n =6). Enzyme activity is measured in DPM per mg protein in each sample. Bars represent mean activity level +/- 1 SEM after a 10 minute incubation with substrate. Asterisks indicate a significant ATP effect, P = .002.

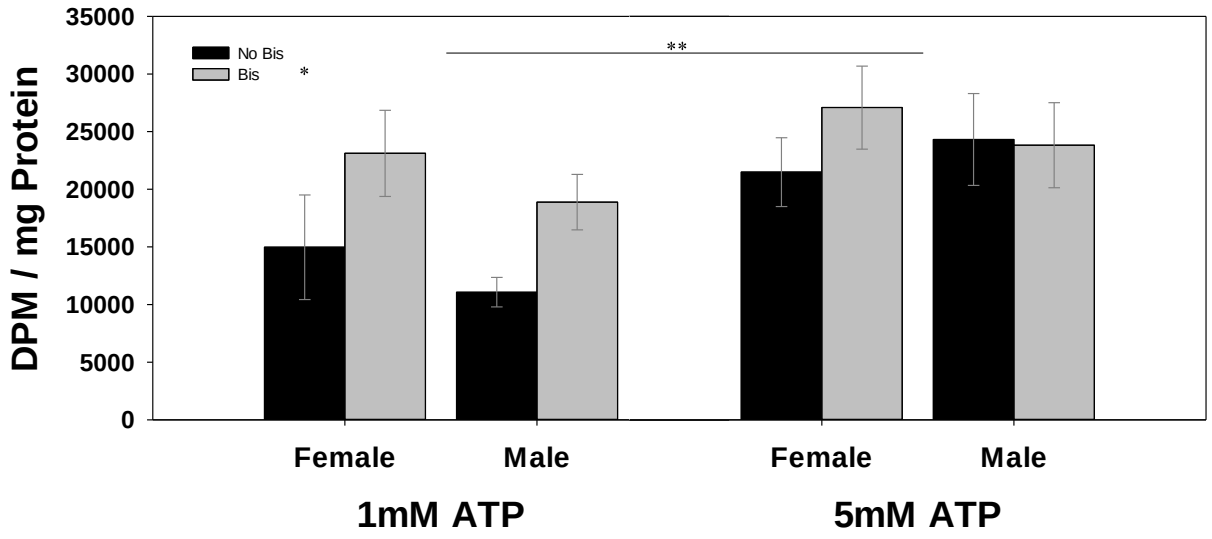

Figure 11: Effects of Bis and ATP on aromatase activity in NCM whole homogenates

Zebra finch NCM samples were preincubated for 10 minutes in the presence or absence of Bis and at 1mM or 5mM ATP. Activity was measured in whole homogenates in males (n =6) and females (n =6). Enzyme activity is measured in DPM per mg protein in each sample. Bars represent mean activity level +/- 1 SEM after a 10 minute incubation with substrate. An asterisk next to the legend indicates an overall Bis effect, $P < .0001$. Asterisks over bars indicate a significant ATP effect, $P = .002$. There is also a significant ATP*Bis interaction, $P = .033$.

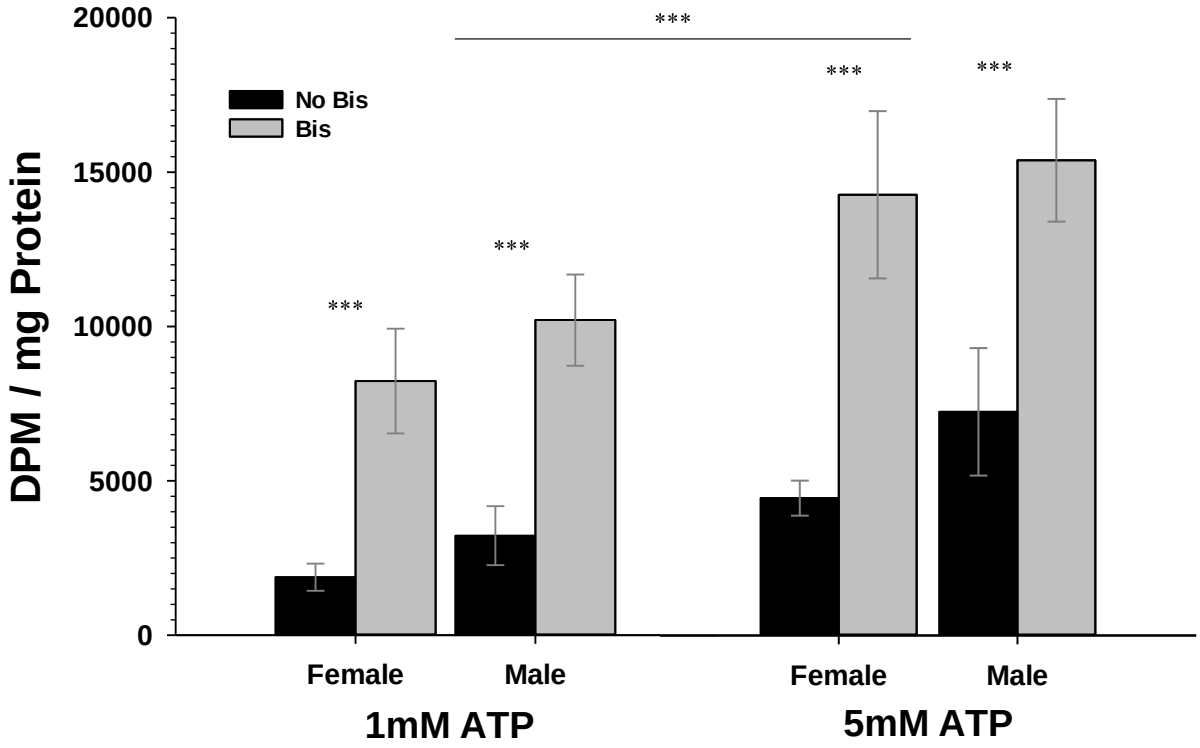

Figure 12: Effects of Bis and ATP on aromatase activity in HP partially purified fractions

Zebra finch HP samples were purified and preincubated for 10 minutes in the presence or absence of Bis and at 1mM or 5mM ATP. Activity was measured in males (n =6) and females (n = 6). Enzyme activity is measured in DPM per mg protein in each sample. Bars represent mean activity level +/- 1 SEM after a 10 minute incubation with substrate. Significance: *P < .05, ** P < .01, *** P < .001. There is an overall ATP effect and an overall Bis effect in both males and females.

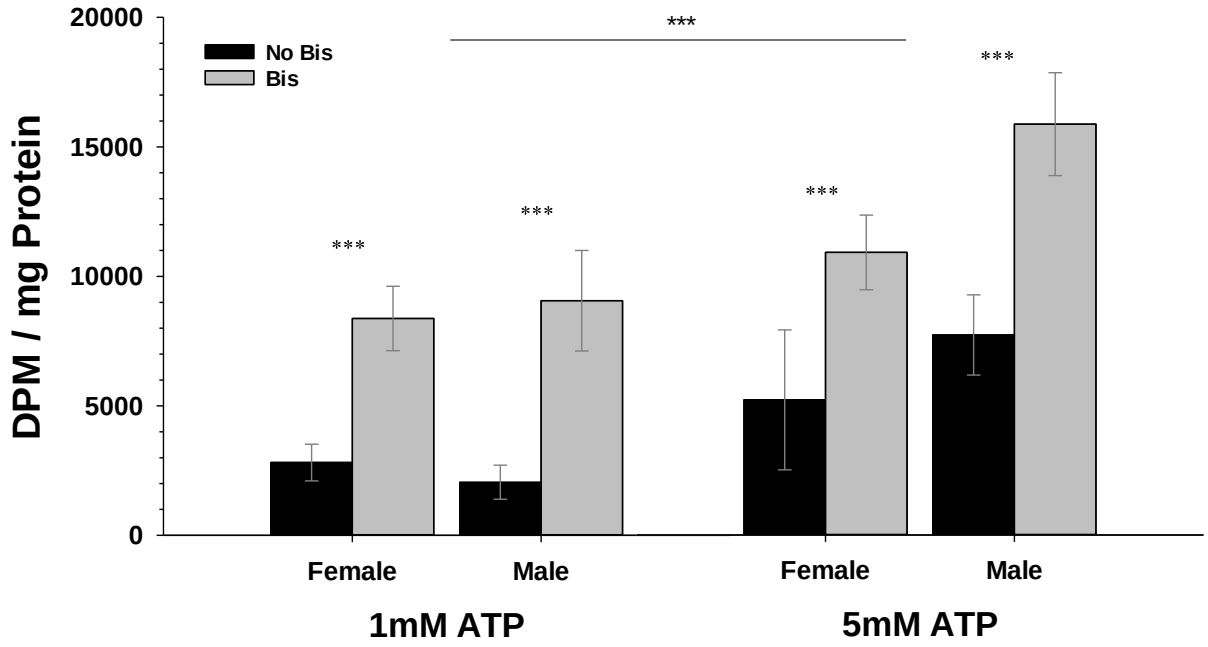

Figure 13: Effects of Bis and ATP on aromatase activity in HYP partially purified fractions

Zebra finch HYP samples were purified and preincubated for 10 minutes in the presence or absence of Bis and at 1mM or 5mM ATP. Activity was measured in males (n =6) and females (n =6). Enzyme activity is measured in DPM per mg protein in each sample. Bars represent mean activity level +/- 1 SEM after a 10 minute incubation with substrate. Significance: *P < .05, ** P < .01, *** P < .001. There is an overall ATP effect and an overall Bis effect in both males and females.

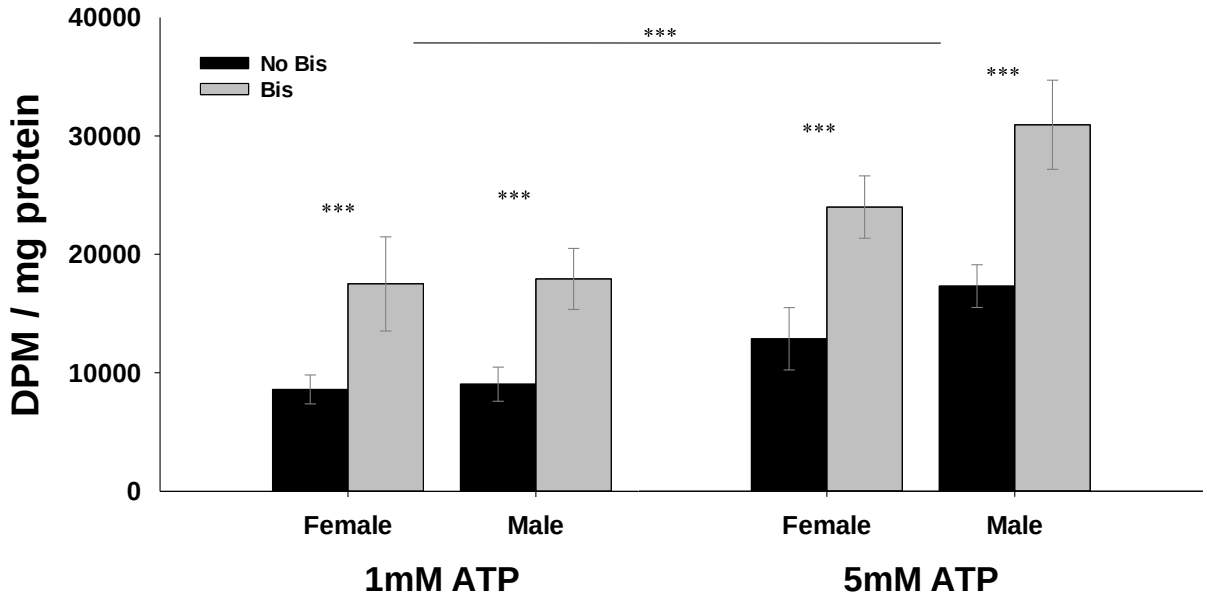

Figure 14: Effects of Bis and ATP on aromatase activity in NCM partially purified fractions

Zebra finch NCM samples were purified and preincubated for 10 minutes in the presence or absence of Bis and at 1mM or 5mM ATP. Activity was measured in males (n =6) and females (n =6). Enzyme activity is measured in DPM per mg protein in each sample. Bars represent mean activity level +/- 1 SEM after a 10 minute incubation with substrate. Significance: *P < .05, ** P < .01, *** P < .001. There is an overall ATP effect and an overall Bis effect in both males and females, P < .0001.

References

Albert KA, Helmer-Matyjek E, Nairn AA, Muller TH, Haycock JW, Greene LA, Goldstein M, Greengard P. Calcium/phospholipid-dependent protein kinase (protein kinase C) phosphorylates and activates tyrosine hydroxylase. *Proc Natl Acad Sci USA* 1984; 81:7713-7717.

Ames MM, Lerner P, Lovenberg W. Tyrosine hydroxylase: activation by protein phosphorylation and end product inhibition. *J Biol Chem* 1978; 253: 27-31.

Baillien M, Balthazart J. A direct dopaminergic control of aromatase activity in the quail preoptic area. *J. Steroid Biochem. Molec. Biol.* 1997; 63(1-3):99-113.

Balthazart J, Baillien M, Ball GF. Rapid and reversible inhibition of brain aromatase activity. *Journal of Neuroendocrinology*. 2001; 13:63-73.

Balthazart J, Baillen M, Ball GF. Phosphorylation processes mediate rapid changes of brain aromatase activity. *Journal of Steroid Biochemistry and Molecular Biology*. 2001 79; 261-277.

Balthazart J, Baillien M, Charlier TD, Ball GF. Calcium-dependent phosphorylation processes control brain aromatase in quail. *European Journal of Neuroscience*. 2003; 17:1591-1606.

Balthazart J, Ball GF. Is brain estradiol a hormone or a neurotransmitter? *TRENDS in Neurosciences*. 2006 May; 29(5): 241-249.

Balthazart J, Taziaux M, Holloway K, Ball GF, Cornil CA. Behavioral effects of brain-derived estrogens in birds. *Ann N Y Acad Sci.* 2009 Apr; 1163: 31-48

Balthazart J and Ball GF. Balthazart B, Ball GF, eds. *Brain Aromatase, Estrogens, and Behavior*, Oxford Univ. Press, Oxford (2013).

Charlier TD, Harada N, Balthazat J, Cornil CA. Human and quail aromatase activity is rapidly and reversibly inhibited by phosphorylating conditions. *Endocrinology*. 2011 Nov; 152(11):4199-210.

Charlier TD, Cornil CA, Patte-Mensah C, Meyer L, Mensah-Nyagan AG, Balthazart J. Local modulation of steroid action: rapid control of enzymatic activity. *Front Neurosci.* 2015 Mar; 9:83.

Cornil CA, Leung CH, Pletcher ER, Naranjo KC, Blauman SJ, Saldanha CJ. Acute and specific modulation of presynaptic aromatization in the vertebrate brain. *Endocrinology* 2012 Jun; 153(6): 2562-2567.

Cornil CA, Ball GF, Balthazart J. Rapid control of male typical behaviors by brain-derived estrogens. *Front Neuroendocrinol.* 2012 Oct; 33(4): 425-446.

Hartmann H, Velbinger K, Eckert A, Muller WE. Region-specific downregulation of free intracellular calcium in the aged rat brain. *Neurobiolody of Aging* 1996; 12(4): 557-563.

Hochberg Z, Bick T, Pelman R, Brandes JM, Barzilai D. The dual effect of calcium on aromatatization by cultured human trophoblast. *J Steroid Biochem* 1986; 24:1217-1219.

Jensen J, Lynch G, Baudry M. Regional differences in the activation of synaptosomal mitochondrial Ca^{2+} uptake by spermine in rat brain. *Brain Res.* 1990 Jul 23;523(2):313-5

Konkle ATM, Balthazart J. Sex differences in the rapid control of aromatase activity in the quail preoptic area. *J Neuroendocrinol*. May 2011; 23(5): 424-434.

Oberlander JG, Schlinger BA, Clayton NS, Saldanha CJ. Neural aromatatization accelerates the acquisition of spatial memory via and influence on the songbird hippocampus. *Horm Behav*. 2004 Apr; 45(4):250-8.

Peterson RS, Yarram L, Schlinger BA, Saldanha CJ. Aromatase is pre-synaptic and sexually dimorphic in the adult zebra finch brain. *Proc Biol Sci.* Oct 2005; 272(1576):2089-96.

Remage-Healey L, Maidment NT, Schlinger BA. Forebrain levels fluctuate rapidly during social interactions. *Nat Neurosci*. 2008 Nov; 11(11):1327-34.

Remage-Healey L, Oyama RK, Schlinger BA. Elevated aromatase activity in forebrain synaptic terminals during song. *J. Neuroendocrinol.* 2009 Mar; 21(3):191-9.

Remage-Healey L, Dong S, Maidment NT, Schlinger BA. Presynaptic control of rapid estrogen fluctuations in the songbird auditory forebrain. *The Journal of Neuroscience*. 2011 Jul; 31(27):10034-10038.

Remage-Healey L., Saldanha CJ, Schlinger, BA. Estradiol synthesis and action at the synapse: evidence for "synaptocrine" signaling. *Frontiers in Endocrinology*. Sep 2011; 2(28): 1-13.

Remage-Healey L, Dong, SM, Chao A, Schlinger BA. Sex-specific, rapid neuroestrogen fluctuations and neurophysiological actions in the songbird auditory forebrain. *Journal of Neurophysiology*. March 2012; 107(6): 1621-1631.

Rensel MA, Salwiczek L, Roth J, Schlinger BA. Context-specific effects of estradiol on spatial learning and memory in the zebra finch. *Neurobiol Learn Mem*. Feb 2013; 100C: 41-47.

Roepke TA, Ronnekleiv OK, Kelly MJ. Physiological consequences of membrane-initiated estrogen signaling in the brain. *Front Biosci* 2011;16:1560-1573.

Roselli CE, Resko JA. *In vitro* assay of aromatase activity in the central nervous system. In: Greenstein B, eds. *Neuroendocrine Research Methods*, vol2. Switzerland: Harwood Academic Publishers, 1991: 937-951.

Saldanha CJ, Popper P, Micevych PE, Schlinger BA. The passerine hippocampus is a site of high aromatase: inter- and intraspecies comparisons. *Horm Behav* 1998; 34:85-97.

Saldanha CJ, Tuerk MJ, Kim YH, Fernandes AO, Arnold AP, Schlinger BA. Distribution and regulation of telencephalic aromatase expression in the zebra finch revealed with a specific antibody. *J. Comp. Neurol*. 2000; 423: 619-630.

Schlinger BA, Callard GV. Localization of aromatase in synaptosomal and microsomal subfractions of quail (Coturniz coturnix japonica) brain. *Neuroendocrinology* 1989 Apr;49(4):434-41.

Schlinger BA, Balthazart J. Aromatase and behavior: concepts gained from studies of aromatase in the avian brain. In: Balthazart B, Ball GF, eds. *Brain Aromatase, estrogens, and Behavior*, Oxford Univ. Press, Oxford (2013) p. 169-198

Steimer T, Hutchison JB. Micromethods for the *in vitro* study of steroid metabolism in the brain using radiolabelled tracers. In: Greenstein B, eds. *Neuroendocrine Research Methods*, vol 2. Switzerland: Harwood Academic Publishers, 1991: 875-919.

Wade J, Schlinger BA, Hodges L, Arnold AP. Fadrozole: a potent and specific inhibitor of aromatase in the zebra finch brain. *Gen Comp Endocrinol.* 1994 Apr; 94(1):53-61.