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Phosphoregulation of Aromatase in the Zebra Finch Brain

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Phosphoregulation of Aromatase in the Zebra Finch Brain

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by

Lara Hovsepian-Ruby

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ABSTRACT OF THE THESIS

Phosphoregulation of Aromatase

in the Zebra Finch Brain

by

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Master of Science in Physiological Science

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Professor Barnett Schlinger, Chair

Recent research demonstrates rapid neurotransmitter-like estradiol signaling in the brain. This form of neuronal communication is supported by findings that the estrogen synthetic enzyme aromatase is present within presynaptic terminals of the vertebrate CNS and is subject to rapid regulation. Previous work on zebra finch neural aromatase provides evidence for phosphorylation-dependent inhibition of activity as well as evidence for phosphorylation-dependent stimulation of activity. To clarify this discrepancy, in the present study we use *in vitro* assays to measure aromatase activity in homogenates or partially purified fractions of male zebra finch HP and NCM and examined the effects of acid phosphatase (AP) on aromatase activity under various phosphorylating conditions. We report that under low or modest phosphorylating conditions, AP causes an increase in basal aromatase activity, whereas in the presence of high phosphorylating conditions, aromatase activity is inhibited by AP. Furthermore, our data suggests that AP acts by direct dephosphorylation of the Y361 residue of aromatase.

The thesis of Lara Hovsepian-Ruby is approved.

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Introduction

Despite the typical characterization of steroids as systemic, slow-acting regulators of peripheral and central tissue, a growing body of evidence demonstrates rapid neuromodulatory actions of estrogens (Woolley 2007). These membrane-initiated estrogen effects have been demonstrated to occur on post-synaptic membranes of discrete neuronal populations within the hippocampi (HP) (Saldanha *et al.* 1998) and caudal nidopallia (NCM) (Saldanha *et al.* 2013) of many species of oscine songbirds, where they are linked to both auditory processing as well as spatial learning and memory (Remage-Healey *et al*. 2012; Rensel *et al.* 2013).

Acute control over estrogen synthesis near post-synaptic estrogen receptors has been shown to be enzyme-dependent (Remage-Healey *et al*. 2011). Aromatase Cytochrome P_{450} is the only enzyme known to catalyze the synthesis of estradiol and estrone from testosterone and androstenedione (AE), respectively. While circulating estrogen levels reflect peripheral aromatase concentration, (Balthazart *et al*. 1993; Roselli *et al*. 1989, 1997), transcriptional regulation of aromatase occurs too slowly to reflect rapid estrogen fluctuation observed in the vertebrate brain. Rather, several studies suggest that neuroestrogen concentration is modulated by rapid activation or deactivation of aromatase following phosphorylation.

In vitro studies on Japanese quail have shown that preoptic-hypothalamic aromatase activity is rapidly down-regulated under conditions that promote Ca^{2+}-dependent kinase phosphorylation (Balthazart *et al.* 2003). Treatment with specific kinase inhibitors, including inhibitors of protein kinase C and protein kinase A, shows the inhibitory effects of phosphorylating conditions on aromatase to be reversible (Balthazart *et al.* 2003; Comito *et al*. 2016). Similarly, phosphorylating conditions reversibly inhibit aromatase activity in human cells lines transfected with aromatase (Charlier *et al.* 2011). *In vivo*, these conditions are analogous to

the process where neuronal depolarization increases intracellular Ca^{2+} levels, which then activate kinase protein(s) that phosphorylate and thus inhibit the enzyme aromatase. Such inhibitory phosphoregulation leads to decreased conversion of androgens to estrogens, and thus, reduced extracellular estrogen signaling.

By contrast, studies on non-neuronal mammalian tissue show evidence of activation of the enzyme aromatase upon phosphorylation. It has been suggested that of the multiple phosphorylation sites on aromatase, certain residues must be constitutively phosphorylated and others dephosphorylated in order for the enzyme to be optimally active (Balthazart *et al*. 2005). Recent work shows that phosphorylation of one aromatase residue in particular - Y361, which is necessary for substrate binding and enzymatic activity (Lo *et al.* 2014) - accelerates the catalytic activity of the protein and thus provides a possible target for rapid regulation of local estrogen synthesis (Ghosh *et al.* unpublished data).

We recently observed these contrasting effects of phosphorylation on enzymatic aromatase in zebra finch HP and NCM (Comito *et al*. 2016). To explain these results, we hypothesize that phosphorylation regulates aromatase bidirectionally. We tested this idea using specific pharmacological treatments, including treatment with acid phosphatase (AP), a general protein phosphatase, and protein tyrosine phosphatase 1B (PTP1B), a phospho-tyrosine protein phosphatase. Furthermore, we used site-specific anti-phospho-Y361 (PY361Ab) and anti-non-phospho-Y361 (nPY361 Ab) antibodies in order to pair phosphorylation status of the Y361 residue of aromatase with enzymatic activity.

Materials and Methods

Subjects

This study was done using adult male zebra finch (>100 days of age) housed communally in our colony at the University of California, Los Angeles. All birds were kept on a 14:10 light:dark cycle and provided water, seeds, supplements, grit, and cuttlebone *ad libitum.* All protocols were approved by the UCLA Chancellor's Animal Research Committee.

Dissections

Brain dissections were adapted from *Comito et al.* 2016. After sacrificing the bird using rapid decapitation, the brain was removed from the skull and placed dorsal side down on a chilled petri dish. In order to facilitate dissection of the HYP, the optic lobes were first removed by cutting the optic tecta. Two 2 mm deep coronal cuts were then made, one rostral to the hindbrain and the other caudal to the anterior commissure, and a transverse cut was used to remove the HYP-containing block of tissue between these coronal cuts. The brain was then repositioned so that the dorsal side was facing up, and the rostral telencephalon was removed using a razor blade. To collect the HP, two parasagittal cuts approximately 3 mm from either side of the midline (about 2 mm deep) were made. The anterior region between the two cuts was isolated as the HP. Finally, the NCM-containing region was isolated by removing 3 mm of the most caudal portion of tissue in the telencephalon. All tissue samples were frozen on dry ice immediately after dissection and stored at -80°C until the day of the assay.

Sample Preparation

Just prior to the aromatase assay, each brain tissue was thawed and homogenized on ice in separate glass potter tubes (15-20 strokes). KTH-sucrose buffer (150 mM KCl, 10 mM Tris-HEPES, 320 mM Sucrose, pH 7.2) volumes that would yield equivalent protein concentrations

across different sized brain regions were previously identified in our lab (*Comito et al.* 2016); for whole homogenate preparations, HP tissue was homogenized in 700uL/sample and NCM in 1400uL/sample. To prepare supernatant fractions (S1) enriched in synaptosomes and microsomes (Schlinger and Callard 1989; Rohmann *et al.* 2007), HP tissue was homogenized in 350uL/sample and NCM in 700uL/sample. The homogenate was then centrifuged at 1000g for 15 minutes at 4°C, the supernatant transferred to a new tube for use during the assay, and the pellet discarded.

Aromatase Assay

Aromatase activity was measured *in vitro* by quantifying tritiated water production upon aromatization of [1ß-^{3}H] AE. All samples underwent three 10-minute incubations in a shaking (89-90 rpm) water bath set to 37°C. On ice, KTH buffer containing experimental factors was added to 50uL of whole homogenate or S1. AP was obtained from Sigma-Aldrich, St Louis, MO, USA; Cat. number P-1146. PTP1B was obtained from Cayman Chemical Company, Ann Arbor, MI, USA; specific activity = 15 units/mg. In all cases, ATP was supplemented with 1mM MgCl2 and 0.5 mM CaCl2 (ATP/Mg/Ca). 75mM Fadrozole was added to negative control tubes before all samples were placed in the first incubation. After 10 minutes, the samples were immediately returned to ice and again, KTH containing experimental factors was added to each tube as specified below. After the second incubation, [1ß-^{3}H] AE (25nM final concentration; specific activity = 24.0 Ci/mmol; PerkinElmer, Waltham, MA, USA) was quickly added to all tubes, followed by NADPH (1.2mM final concentration), and samples were placed back in the water bath for their final incubation. Samples were then centrifuged at 10,000g for 10 minutes at 4°C to separate protein pellets from tritiated water-containing supernatant. Protein pellets were stored in -20°C for later analysis of protein concentration using the ThermoFisher Scientific Pierce

bicinchoninic acid protein assay kit (Waltham, MA, USA). The supernatant was then transferred to new tubes and combined with 600uL of 10% trichloroacetic acid supplemented with 2% charcoal, and centrifuged at 5000g for 15 minutes at 4°C. The supernatant was purified as described in Baillien and Balthazart 1997, effluent collected in 20mL scintillation vials and mixed with 15mL scintillation fluid, and left in the dark for a minimum of three hours. In the last step, samples were counted using a Packard TriCarb 2100TR scintillation counter for 4 minutes each. Output was recorded in disintegration per minute, which was analyzed alongside protein concentrations of each sample to present aromatase activity as disintegration per minute per mg protein after correcting for background activity measured in the presence of Fadrozole.

Western Blot

Y361 phosphorylation status of aromatase was determined by western blot analysis using polyclonal PY361 and nPY361 antibodies, both gifted by the Debashis Ghosh Laboratory (Syracuse, NY, USA) and designed to specifically recognize phosphorylation or non-phosphorylation (respectively) of aromatase at this residue. Aromatase S1 samples were resolved by SDS-PAGE prior to being electrophoretically transferred to a nitrocellulose membrane. After blocking in TBST supplemented with 3% nonfat milk (w/v), the membrane was incubated with nPY361Ab or PY361Ab diluted 1:1000 in TBST supplemented with 1% nonfat milk (w/v) for 1 hour at room temperature. Subsequently, the membrane was extensively washed with TBST prior to incubation with horseradish peroxidase-linked goat-anti-rabbit IgG (Bio-rad) diluted 1:5000 in TBST supplemented with 1% nonfat milk (w/v) for 1 hour at room temperature. The membrane was again washed extensively in TBST, and visualized by enhanced chemiluminescence detection using the Clarity Western ECL Substrate Kit (Bio-rad).

Statistical Analysis

Aromatase activity was assessed using repeated measures two-way ANOVA. We used a within-subject design. Bird identity was always entered as a random factor, while concentrations of ATP, AP, and PTP1B were all set as fixed factors. Brain region was not a factor because brain regions were tested in separate assays. Data in Figures 1C and 2B were not analyzed statistically since subjects were pooled for assay. All statistical analyses were performed using GraphPad Prism (La Jolla, CA, USA). If a significant main effect was found ($p < 0.05$), pairwise multiple comparisons were performed. All figures show normalized data mean values ± SEM.

Results

Bidirectional effects of phosphorylation on aromatase

To determine whether the enzyme aromatase is activated or deactivated upon phosphorylation, we measured aromatase activity in NCM whole homogenates treated with various concentrations of ATP (0, 1, or 5mM) in the presence or absence of AP (0.4 units/tube) (Fig. 1B). Surprisingly, we found that varying phosphorylating conditions in control tubes not containing AP does not significantly affect aromatase activity. Upon addition of 0.4 units of AP per tube, a differential response is observed; AP induces an increase in enzymatic activity under low or moderate phosphorylating conditions (0 or 1mM ATP, respectively), but a dramatic decrease under high phosphorylating conditions (5mM ATP). A similar trend is observed in partially purified hippocampal supernatant treated with .04 units of AP per tube (Fig. 1C). An increase in AP to 4 units per tube eliminates this trend and causes a nearly 100% decrease in aromatase activity regardless of ATP concentration.

Aromatase modulation by direct phosphorylation

We next asked whether AP acts directly on the aromatase enzyme to modulate its activity, or if it works indirectly by modulating the phosphorylation status of endogenous kinases and/or phosphatases. We reasoned that if AP acts indirectly, no changes in aromatase activity should be observed if AP is added to samples after they are incubated in phosphorylating conditions and then phosphorylations are blocked by chelating calcium with 1mM EGTA. We observe instead that in supernatant fractions of NCM and HP, 0.004 units of AP per tube induces an increase in aromatase activity under moderate phosphorylating conditions, and a decrease in activity under high phosphorylating conditions (Fig. 2B). We interpret this as evidence for direct dephosphorylation of aromatase by AP.

Elevation of aromatase activity by phosphorylation of residue Y361

In order to determine the effect of Y361 phosphorylation status on aromatase activity, we ran a split experiment on supernatant fractions of NCM and HP. Half of each sample was run through an aromatase assay to measure enzymatic activity, and the other half in a western blot probing for phosphorylation at residue Y361. We find that in both NCM and HP, the treatment with 4 units of AP largely decreases aromatase activity compared to samples treated with 0 or 0.004 units AP (Fig. 3A). Figure 3B shows that PY361 Ab recognizes both the aromatase monomer at 65kDa and the dimer at 130kDa only in samples treated with 0 or 0.004 units AP, indicating that the decrease in aromatase activity induced by 4 units AP is likely mediated by dephosphorylation at this residue.

In a separate experiment, NCM and HP tissue treated with 5mM ATP and 0.4 units AP (which has been shown to dramatically inhibit aromatase activity (Fig. 1B)) were run on a western blot and incubated with PY361 Ab or nPY361 Ab (data not shown). While control samples containing no AP were labeled by PY361 Ab and not nPY361 Ab, treated tubes were labeled by nPY361 Ab and not Y361 Ab, demonstrating the compatibility of the antibodies with zebra finch aromatase.

To further explore if aromatase activity can be decreased by targeting all tyrosine residues, we treated NCM supernatants with PTP1B. Unexpectedly, no significant effects of PTP1B on aromatase activity were observed.

Discussion

Discovery of rapid estrogen fluctuation in the brains of many vertebrate species has prompted work on the mechanisms of acute aromatase regulation. A substantial amount of research indicates that modulation of this estrogen-synthetic enzyme is largely governed by phosphoregulation, a process in which terminal phosphate group are transferred from ATP to specific aromatase residues. However, evidence for both enzyme activation and inhibition by phosphorylation confound our understanding of aromatase regulation (Cornil *et al.* 2012; Comito *et al.* 2016; Catalano *et al.* 2009; Barone *et al.* 2012).

Here, we show a rather novel effect of phosphorylation on aromatase activity. Increasing Ca^{2+}-dependent enzymatic phosphorylation by supplementing NCM whole homogenates with increasing concentrations of ATP does not significantly decrease aromatase activity, as predicted by previous research on zebra finches (Fig 1B). Despite consistently high enzymatic activity regardless of phosphorylating conditions, it is likely that the phosphorylation status of aromatase is indeed altered by varying conditions. It is possible that the ATP is inducing equal and oppositional phosphorylation effects on aromatase by simultaneously regulating endogenous kinases and phosphatases that act on aromatase either directly or indirectly to modulate its phosphorylation status. As ATP concentration increases, so too would the rate of aromatase phosphorylation/dephosphorylation, but equal rates of modulation by kinases/phosphatases would yield a net effect on aromatase phosphorylation, thus having no significant effect on aromatase activity.

This explanation is supported by idea that not one specific phosphorylation site is responsible for aromatase activity (Charlier *et al.* 2011). Instead, it's likely that acute regulation of aromatase by phosphorylation occurs at a combination of enzyme hotspots responsible for

direct effects on its reaction mechanism (Ghosh *et al.* unpublished data). It is likely that certain hotspots must be constitutively phosphorylated and other dephosphorylated to maintain protein stability and optimal activity (Balthazart *et al.* 2005).

To gain a better understanding of how phosphorylating and dephosphorylating conditions work in tandem to regulate aromatase activity, we decided to treat neuronal tissue with a general protein phosphatase in low, moderate, and high phosphorylating conditions. Elevated serum levels of one phosphomonoesterase in particular, AP, has been indicated in estrogen-dependent cancers such as mammary carcinoma and prostate tumors (Filmus *et al.* 1984; Lee *et al.* 1987), where the lysosomal acid hydrolase likely plays a role in degradative changes in the malignant cells (Brandes *et al.* 1967). These data provide an interesting link between estrogen production and phosphatase activity.

Interestingly, we find that while AP increases aromatase activity (and thus, estrogen production) in low to moderate phosphorylating conditions, the opposite effect is observed under high phosphorylating conditions (Fig. 1B). Given that protein activity is oftentimes regulated by phosphorylation, it is likely that the AP is itself becoming phosphorylated and thus deactivated under high phosphorylating conditions. In addition to bidirectional regulation of aromatase by AP based on phosphorylating conditions, we find that AP has differential effects based on concentration used. While low levels of AP (0 - 0.004 units per tube) regulate aromatase bidirectionally, high levels of AP (4 units per tube) dramatically reduce activity regardless of phosphorylating conditions (Fig. 1C, Fig. 2B, Fig. 3A).

Our data implies that aromatase in certain tumor cells may respond differently to AP from blood serum depending on ATP concentration. Although extracellular concentrations of adenosine remain relatively constant in most tissues, a rapid 100-fold increase can be observed in

response to inflammation from solid tumor microenvironments (Ohta *et al.* 2006). This increase in ATP, which occurs endogenously from osteocyte activity, has been shown to exert inhibitory effects on breast cancer cells (Jiang *et al.* 2015). Our data implies the possibility that the ATP increase that occurs as part of a cellular stress response works to combat the AP-induced increases in aromatase activity that feed estrogen-dependent tumors.

Previous work shows that the residue Y361, which is located in the proximal cavity where aromatase's electron transfer partner is presumed to bind, is a very likely target of phosphorylation by certain kinases (Ghosh *et al.* unpublished data). Given that phosphorylation of this site has been noted in human breast cancer cell lines (Catalano *et al.* 2009), we asked whether AP-induced changed in aromatase activity are dependent on regulation of the phosphorylation status of this site. Our western blot analysis shows that 4.0 units per tube of AP strip the critical Y361 residue of its phosphate group (Fig. 3A). On the other hand, aromatase in tubes containing 0 or 0.004 units/tube of AP showed significantly higher activity than those treated with high levels of AP, and the Y361 residue in these samples remained phosphorylated (Fig. 3A). We postulate that this aromatase hotspot requires a stable phosphate group in order to properly participate in the enzymatic reaction mechanism. It is also possible that stripping Y361 of its phosphate group causes denaturation of aromatase. Increased aromatase activity in samples treated with 0.004 units AP can potentially be explained by the role of AP in stripping phosphate groups from other critical residues that remain constitutively dephosphorylated during aromatase activity.

The involvement of additional tyrosine residues in aromatase regulation is demonstrated by our finding that PTP1B does not significantly alter aromatase activity. If Y361 was the only critical tyrosine hotspot involved in the reaction mechanism, aromatase activity would have been

inhibited by PTP1B regardless of phosphorylating conditions, but this is not what we found. Our results indicate that one or more additional tyrosine residues likely remain phosphorylated during enzymatic activity; thus, PTP1B simultaneously imposes oppositional regulatory effects on aromatase. Alternatively, it is also possible that not enough PTP1B was used to induce a significant effect.

In conclusion, these data provide evidence for direct, bidirectional control of aromatase activity via phosphoregulation. In addition to the Y361 residue, additional enzyme hotspots participating in the reaction mechanism of aromatase likely exist and provide an exciting avenue of future research. Insight into the regulatory mechanisms surrounding estrogen production will one day be a key tool in understanding and treating certain estrogen-dependent cancers.

Figure 1: Bidirectional effects of aromatase phosphorylation

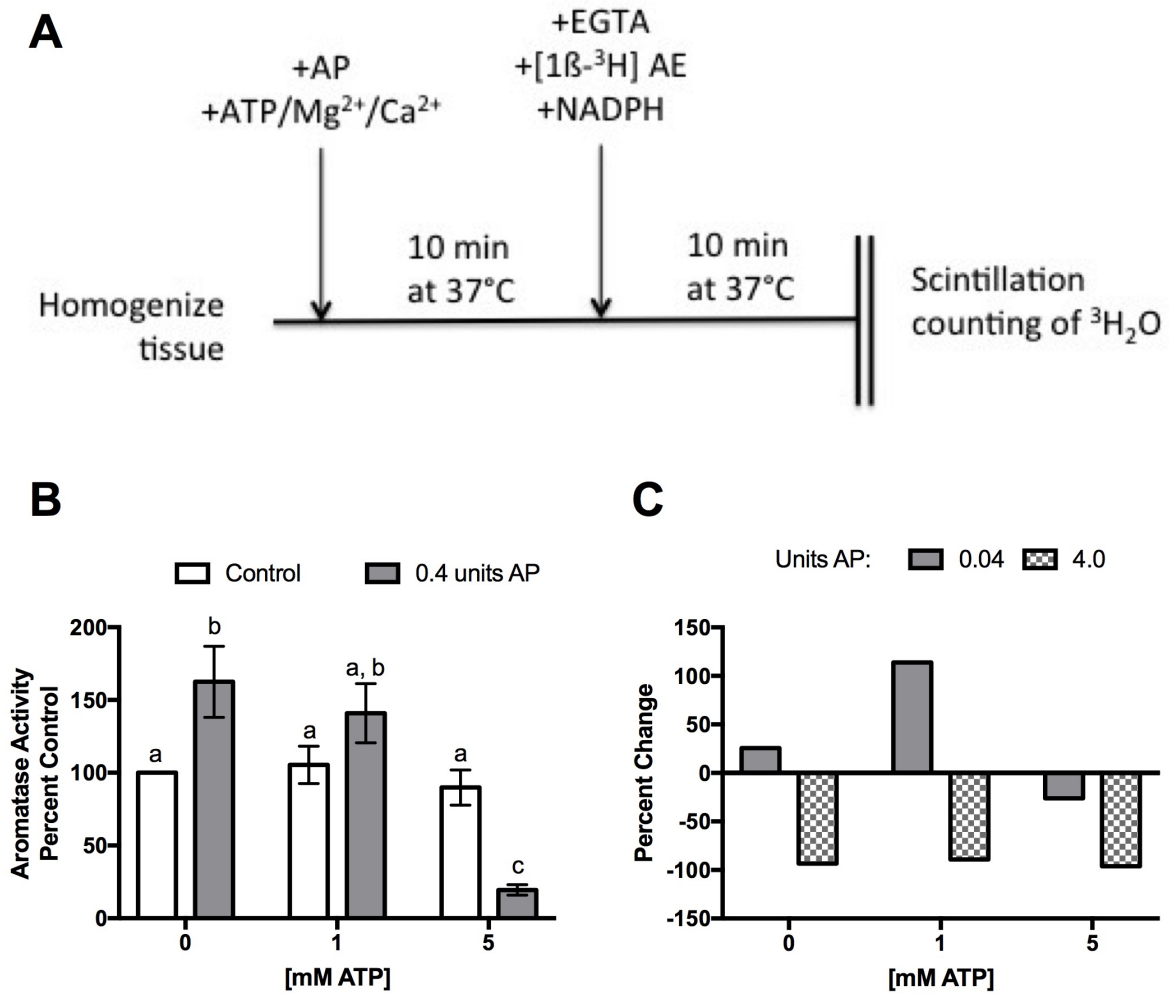

Fig. 1: (A) Schematic presentation of the experimental protocol. **(B)** Effects of acid phosphatase on whole homogenates of NCM in the presence or absence of ATP supplemented with Mg^{2+}/Ca^{2+}; $n = 3$ males; Data represent mean activity ± SEM; letters over bars indicate significance. **(C)** Percent change in aromatase activity of HP supernatant relative to non-AP-containing controls. Samples were pooled ($n = 7$ males) and assayed singly.

Figure 2: Direct aromatase phosphorylation by acid phosphatase

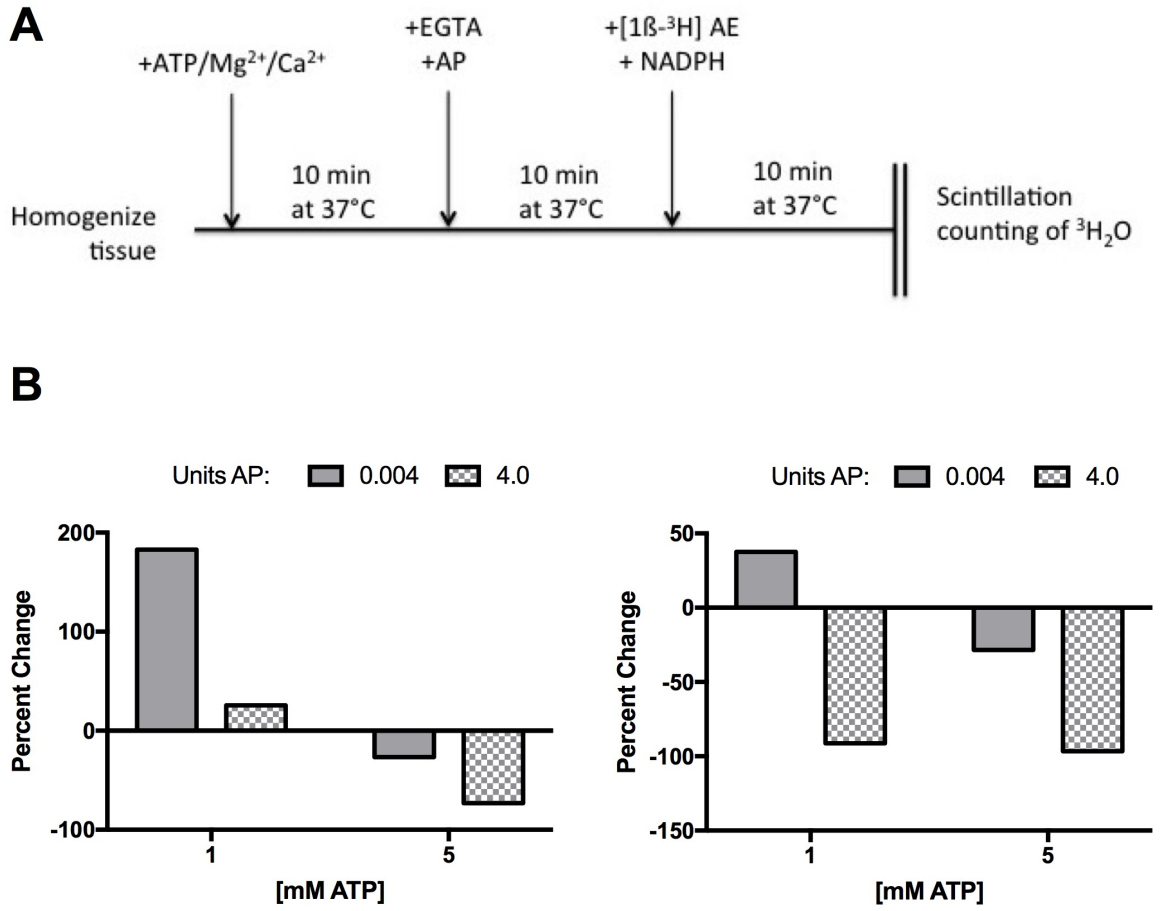

Fig. 2: (A) Schematic presentation of the experimental protocol. **(B)** Percent change in aromatase activity of NCM (left) and HP (right) supernatant relative to non-AP-containing controls. Samples were pooled ($n = 5$ males for NCM, $n = 7$ males for HP) and assayed singly.

Figure 3: Acid phosphatase-induced Y361 residue dephosphorylation

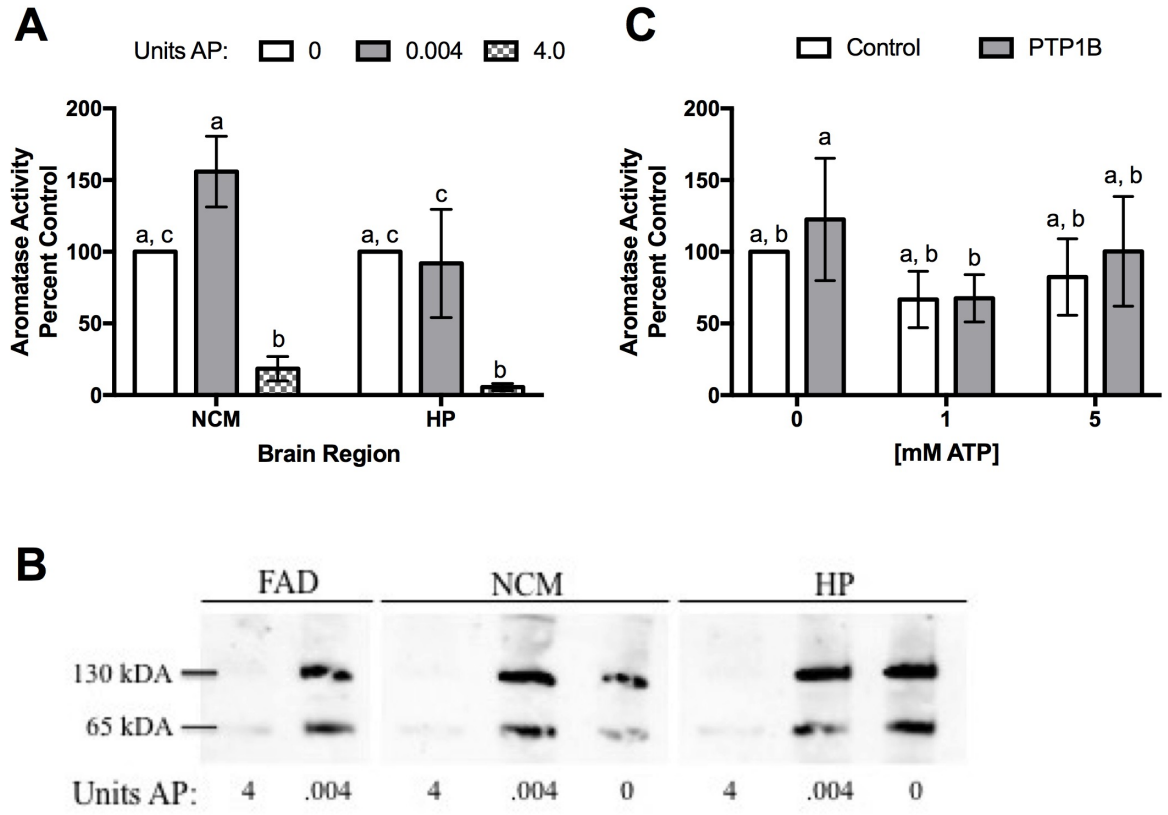

Figure 3: Effects of acid phosphatase on supernatants of NCM and HP; $n = 3$ males; Data represent mean activity ± SEM; letters over bars indicate significance. **(B)** Western blot analysis of samples from (A). Membrane was incubated with anti-phosphoryl-Y361 antibody. **(C)** Ability of PTP1B (.01ug/tube) to rescue aromatase activity in supernatants of NCM incubated in the presence or absence of various concentrations of ATP supplemented with Mg^{2+}/Ca^{2+}; $n = 5$ males; Data represent mean activity ± SEM.

References

Baillien, M., Balthazart, J. (1997). A direct dopaminergic control of aromatase activity in the quail preoptic area. *Journal of Steroid Biochemistry and Molecular Biology*, 63(1-3): 99-113.

Balthazart, J., Baillien, M., & Ball, G. F. (2005). Interactions between kinases and phosphatases in the rapid control of brain aromatase. *Journal of Neuroendocrinology*, 17(9), 553–559.

Balthazart, J., Baillien, M., Charlier, T. D., & Ball, G. F. (2003). Calcium-dependent phosphorylation processes control brain aromatase in quail. *European Journal of Neuroscience*, 17(8), 1591–1606.

Balthazart, J., Foidart A. (1993). Brain aromatase and the control of male sexual behavior. J Steroid Biochem Mol Biol 44:521–540

Barone I,, Giordano, C., Malivindi, R., Lanzino, M., Rizza, P., Casaburi, I., Bonofiglio, D., Catalano, S., Ando, S. (2012). Estrogens and PTP1B function in a novel pathway to regulate aromatase enzymatic activity in breast cancer cells. *Endocrinology*, 153(11):5157-5166.

Brandes D, Sloan KW, Anton E, Bloedorn F. (1967). The effect of x-irradiation on the lysosomes of mouse mammary gland carcinomas. *Cancer Research*, 27: 731-746.

Catalano, S., Barone, I., Giordano, C., Rizza, P., Qi, H., Gu, G., Malivindi, R., Bonofiglio, D., Ando, S. (2009). Rapid estradiol/ERalpha signaling enhances aromatase enzymatic activity in breast cancer cells. *Molecular Endocrinology*, 23(10):1634-1645.

Charlier T. D., Harada N., Balthazart J. and Cornil C. A. (2011). Human and quail aromatase activity is rapidly and reversibly inhibited by phosphorylating conditions. *Endocrinology,* 152, 4199–4210.

Comito, D., Pradhan, D. S., Karleen, B. J., & Schlinger, B. A. (2016). Region-specific rapid regulation of aromatase activity in zebra finch brain. *Journal of Neurochemistry*, 136(6), 1177–1185.

Cornil C. A., Leung C. H., Pletcher E. R., Naranjo K. C., Blauman S. J. and Saldanha C. J. (2012). Acute and specific modulation of presynaptic aromatization in the vertebrate brain. *Endocrinology,* 153, 2562–2567.

Filmus, J. E., Podhajcer, O. L., Mareso, E., Guman, N., Mordoh, J. (1984). Acid phosphatase in human breast cancer tissue. *Cancer,* 53:301-305.

Jiang., J. X., Riquelme, M. A., Zhou, J. Z. (2015). ATP, a double-edged sword in cancer. *Oncoscience*, 2(8): 673-674.

Lee, C., Wang., M., Murphy, G. P., Chu, M. (1978). A solid-phase fluorescent immunoassay for human prostatic acid phosphatase. *Cancer Research*, 38: 2871-2878.

Lo, J., Di Nardo, G., Griswold, J., Egbuta, C., Jiang, W., Gilardi, G., Ghosh, D. (2013). Structural basis for the functional roles of critical residues in human cytochrome p450 aromatase. *Biochemistry*, 52(34):5821-5829.

Ohta, A., Gorelik, E., Prasad, S. J., Ronchese, F., Lukashev, D., Wong, M. K., Huang, X., Caldwell, S., Liu, K., Smith, P., Chen, J. F., Jackson, E. K., Apasov, S., Abrams, S., Sitkovsky, M. (2006). A2A adenosine receptor protects tumors from antitumor T cells. *PNAS*, 103:13132–13137.

Remage-Healey, L., Dong, S. M., Chao, A., & Schlinger, B. A. (2012). Sex-specific, rapid neuroestrogen fluctuations and neurophysiological actions in the songbird auditory forebrain. *Journal of Neurophysiology*, 107(6), 1621–1631.

Remage-Healey, L., Saldanha, C. J., & Schlinger, B. A. (2011). Estradiol synthesis and action at the synapse: evidence for "synaptocrine" signaling. *Frontiers in Endocrinology*, 2, 1-13.

Rensel, M. A., Salwiczek, L., Roth, J., & Schlinger, B. A. (2013). Context-specific effects of estradiol on spatial learning and memory in the zebra finch. *Neurobiology of Learning and Memory*, 100(C), 41–47.

Rohmann, K. N., Schlinger, B. A., & Saldanha, C. J. (2007). Subcellular compartmentalization of aromatase is sexually dimorphic in the adult zebra finch brain. *Journal of Neurobiology*, 67(1), 1–9.

Roselli, C.E., Resko, J.A. (1997). Sex differences in androgen-regulated expression of cytochrome P450 aromatase in the rat brain. *The Journal of Steroid Biochemistry and Molecular Biology,* 61:365–374.

Roselli, C.E., Resko, J.A. (1989). Testosterone regulates aromatase activity in discrete brain areas of male rhesus macaques. *Biology of Reproduction*, 40:929–934.

Saldanha C. J., Popper P., Micevych P. E. and Schlinger B. A. (1998). The passerine hippocampus is a site of high aromatase: Inter- and intraspecies comparisons. *Hormones and Behavior*, 34, 85–97.

Saldanha C. J., Remage-Healey L. and Schlinger B. A. (2013). Neuroanatomical distribution of aromatase in birds: Cellular and subcellular analyses, in Brain aromatase, estrogens and behavior. *Oxford University Press,* 100–114.

Schlinger B. A. and Callard G. V. (1989). Localization of aromatase in synaptosomal and microsomal subfractions of quail (Coturnix coturnix japonica) brain. *Neuroendocrinology*, 49, 434–441.

Woolley, C.S. (2007). Acute effects of estrogen on neuronal physiology. *The Annual Review of Pharmacology and Toxicology*, 47:657–80.