

# Estrogen Provision by Reactive Glia Decreases Apoptosis in the Zebra Finch (*Taeniopygia guttata*)

Colin J. Saldanha,<sup>1,2</sup> Kevin N. Rohmann,<sup>1</sup> Luckshman Coomaringam,<sup>1</sup> Ryan D. Wynne<sup>1</sup>

<sup>1</sup> Department of Biological Sciences, Lehigh University, 111 Research Drive, Bethlehem, Pennsylvania 18015

<sup>2</sup> Program in Cognitive Science, Lehigh University, Bethlehem, Pennsylvania 18015

Received 10 November 2004; accepted 23 December 2004

**ABSTRACT:** Upregulation of aromatase (estrogen synthase) in glia around the site of neural injury may limit neural degeneration. Systemic administration of estrogen limits neural damage, but the specific role of local estrogen provision in this effect is unclear. In male zebra finches, we tested the effect of local aromatase inhibition and estrogen replacement on type of cellular degeneration and the distance of this degeneration from the source of insult. Subjects received injections of the aromatase inhibitor fadrozole into one telencephalic lobe and fadrozole and estradiol into the contralateral lobe. Seventy-two hours later, we used Fluoro-Jade B and TUNEL to label dying and apoptotic cells, respectively. Since each subject was its own control, we were able to assess the influence of local estrogen replacement in relative distinction from circulating steroids and constitutive aromatization. Cellular degeneration around the lesion was measured with

Fluoro-Jade B, TUNEL, and indirectly with aromatase expression. Additionally, the glial nature of aromatase-positive cells around the injury was queried by colocalization with vimentin. The estrogen replaced injury had fewer apoptotic cells clustered more closely around the injury compared to the hemisphere injected with fadrozole alone. Since Fluoro-Jade B and TUNEL labeled similar numbers of cells, and the distance of these cells from the injection was identical, we suggest that estrogen replacement functions primarily to restrict apoptosis in the current paradigm. Lastly, aromatase-positive cells around injuries co-localize vimentin, establishing their glial nature. Thus, glial estrogen provision at sites of neural insult may be critical in limiting the cellular degeneration caused by injury via an inhibition of apoptosis. © 2005 Wiley Periodicals, Inc. *J Neurobiol* 00: 000–000, 2005

**Keywords:** stroke; songbird; lesion; pyknosis; repair

## INTRODUCTION

The vertebrate brain is responsive to steroids including 17 $\beta$ -estradiol (E<sub>2</sub>) throughout life (Arnold and Gorski, 1984; McEwen and Alves, 1999; McEwen, 2001; Wise, 2003). Earlier work established a pivotal role for E<sub>2</sub> in the organization and

activation of circuits necessary for sexual and aggressive behaviors in several vertebrates (MacLusky and Naftolin, 1981; Adkins-Regan and Watson, 1990; Balthazart et al., 1992). More recent work has expanded the role of this steroid into such arenas as neuromuscular control, pain, learning, memory, and mood (McEwen, 2001; Morrison, 2003; Wise, 2003; Maggi et al., 2004; Morgan et al., 2004). Estrogenic modulation of the central nervous system appears to be multifaceted and well conserved across vertebrates. Included in the suite of physiological parameters modulated by E<sub>2</sub> is an emerging role for this steroid in the protection of

Correspondence to: C.J. Saldanha (colin@lehigh.edu).

Contract grant sponsor: NIH; contract grant number: RO1 042767 (C.J.S.).

© 2005 Wiley Periodicals, Inc.  
Published online in Wiley InterScience  
(www.interscience.wiley.com).  
DOI 10.1002/neu.20147

the brain from degeneration (Cho et al., 2003; Merchenthaler et al., 2003).

In some vertebrates, neural insult by ischemia, mechanical injury, or excitotoxicity results in the expression of aromatase (estrogen synthase) in glial cells around the site of damage (Garcia-Segura et al., 1999; Azcoitia et al., 2001; Peterson et al., 2001). This up-regulation appears functional. Neural injury is exaggerated in aromatase knockout mice relative to heterozygous conspecifics and in rats subjected to systemic aromatase inhibition (Garcia-Segura et al., 2001). Indeed, peripheral administration of estrogen or aromatizable androgens decreases neural injury in rats, and replacement of peripheral estrogens ameliorates the effect of aromatase inhibition supporting a role for aromatase in limiting neural damage (Garcia-Segura et al., 2003).

Cellular degeneration following neural injury typically involves two distinct phases. First, necrotic cell death characterized by a compromise of the integrity of the cell membrane and swelling of the cell, occurs as an immediate consequence of insult (Nicotera and Lipton, 1999). A subsequent round of death follows in susceptible cells neighboring the lesion site. This degeneration is characterized by apoptotic cell death and typically occurs over an extended period of time (Tang and Porter, 1996). We have shown that inhibition of aromatase immediately around the injury site increases apoptosis in zebra finches (Wynne and Saldanha, 2004). These data suggest that local E<sub>2</sub> provision may limit the amount of delayed cell death, thereby reducing injury. However, the involvement of general degeneration and apoptosis in this paradigm remains to be directly tested. Further, though strongly suggested, the idea that locally up-regulated aromatase in glial cells is neuroprotective by providing high levels of E<sub>2</sub> to local circuitry remains unevaluated.

Songbirds (order: Passeriformes) are excellent models for these studies. Injury-induced aromatase is rapid and robust in the zebra finch (*Taeniopygia guttata*), where transcription and translation of this gene are detectable as early as 24-h post-insult (Peterson et al., 2001) and persist at least 7-days post-insult (Peterson et al., 2004). Secondly, although glial aromatase is detectable in primary dissociated cell cultures of developing telencephalon (Schlinger et al., 1994), it is only detectable *in vivo* following disruption of the neuropil (Peterson et al., 2001, 2004; Wynne and Saldanha, 2004). Further, in male zebra finches, the brain may be the only source of E<sub>2</sub> available to modulate central and peripheral processes (Schlinger and Arnold, 1991, 1992). Lastly, the high expression of neuronal aromatase at several well-

defined telencephalic loci in this species makes it possible to experimentally discriminate between injury-induced glial and constitutive neuronal aromatase expression (Shen et al., 1995; Saldanha et al., 2000; Peterson et al., 2001). In the present report, we tested the influence of aromatase inhibition and simultaneous E<sub>2</sub>-replacement on generalized cellular degeneration and apoptosis in adult male zebra finches.

## METHODS

Subjects were six adult (>90 days) male zebra finches purchased from a local breeder (Canary Bird Farms, Old Bridge, NJ) and housed at the Biological Sciences Animal Facility, Lehigh University. Birds were held in same-sex groups (two–four per group) in cages (18" × 18" × 16") under a 14:10 LD cycle (lights on at 0600 h). The room temperature was maintained at 72° ± 2F, and food, grit, water, and a cuttlebone were available *ad libitum*. All housing and experimental protocols used in this study were approved by Lehigh University Institutional Animal Care and Use Committee (IACUC).

### General Experimental Design

We have previously shown that administration of fadrozole increases neural damage and apoptosis relative to saline (Wynne and Saldanha, 2004). In the present study, we asked if this effect was due to a decrease in local E<sub>2</sub>. Each subject served as its own control. The potential role of localized E<sub>2</sub>-provision on injury was tested by injecting the aromatase inhibitor fadrozole (Wade et al., 1994) into one hemisphere and fadrozole + E<sub>2</sub> into the contralateral hemisphere. Based upon previous research (Peterson et al., 2001; Wynne and Saldanha, 2004), all animals were sacrificed 72-h post-injury, when aromatase transcription and translation is robust, and when fadrozole administration alone increases injury twofold relative to saline (Wynne and Saldanha, 2004). In each animal, we tested the influence of E<sub>2</sub>-replacement on (a) cellular degeneration around the lesion site, (b) distance of degenerating cells from the lesion and injection, and (c) aromatase expression in glia and neurons. A detailed description of each experimental procedure follows.

### Neural Injury and Drug Delivery

Subjects ( $n = 6$ ) were anesthetized with ketamine/xylazine (0.08 ml per bird with 0.3 and 16 mg/mL ketamine and xylazine) and positioned in a stereotaxic apparatus with the head angled at 45°. The cranium was exposed by midline incision and an 18G needle was used to create a bilateral craniotomy. Injections were targeted at the entopallial nucleus 2 mm anterior to the pineal gland and 3 mm lateral to the midline (Stokes et al., 1974). The entopallial nucleus was selected as a target since it is devoid of constitutive

aromatase (Shen et al., 1995; Saldanha et al., 2000). Thus, the only aromatase detected immediately surrounding the injury site ought to be a direct consequence of neural disruption (Peterson et al., 2001, 2004; Wynne and Saldanha, 2004). A 50- $\mu$ L 22s Hamilton syringe (Hamilton Company, Reno, NV) was positioned at the surface of the brain at an angle of 45°, and lowered 2.5 mm ventrally where it resided for 120 s. At this point, 5  $\mu$ L of either a 10 mg/mL solution of fadrozole (50  $\mu$ g total) in steroid-suspension vehicle (SSV; 9 mg NaCl, 5 mg sodium carboxymethylcellulose, 4  $\mu$ l polysorbate 80, 9  $\mu$ l benzyl alcohol in 1 ml distilled water) or 5  $\mu$ l of a 200  $\mu$ g/ml E<sub>2</sub> solution in 10 mg/ml fadrozole (1  $\mu$ g E<sub>2</sub> and 50  $\mu$ g fadrozole total) in SSV was injected into one of the two hemispheres. The concentration of fadrozole used was based upon previous research showing a reliable inhibition of neural aromatase activity following oral administration (Saldanha et al., 2004). The amount of E<sub>2</sub> delivered was half that used in previous studies that showed neuroanatomical changes in response to local E<sub>2</sub> implants in birds (Grisham et al., 1994). In two birds, fadrozole was first delivered into the right hemisphere followed by fadrozole + E<sub>2</sub> into the left. The next two birds first received fadrozole into the left hemisphere followed by fadrozole + E<sub>2</sub> into the right. The last two birds first received fadrozole + E<sub>2</sub> delivered to the right hemisphere followed by fadrozole to the left. The needle remained in the brain for 60 s and was then retracted. The scalp was repositioned over the cranium and sealed with Collodion Flexible (EM Science, Gibbstown, NJ). Following surgery, the birds recovered from anesthesia under a heat lamp, and were subsequently used for neuroanatomical analyses.

## Tissue Preparation

Following the 72-h recovery period, subjects were deeply anesthetized with 1.5 ml of ketamine/xylazine and transcardially perfused with 5 ml 0.1 M phosphate buffer (PB) followed by 40 ml of 4% paraformaldehyde (pH 7.35) containing 15% picric acid. The abdominal cavity was opened to reveal the testes which appeared well developed in all birds, suggesting physiological levels of circulating androgens (Farner and Wingfield, 1980). The brains were removed and immersed in 4% paraformaldehyde (48 h, 4°C), embedded in 8% gelatin, re-immersed in 4% paraformaldehyde (72 h, 4°C), cryoprotected in 30% sucrose in PB (72 h, 4°C) and sectioned on a cryostat. Five sets (A–E) of 50- $\mu$ m coronal sections were collected into a high-sucrose, polyethylene glycol solution (Watson et al., 1986) and the sections were stored at –20°C until ready for use. Sets of coronal sections were used to determine (a) general degeneration using Fluoro-Jade B, (b) apoptotic degeneration using TUNEL, (c) extent of diffusion of fadrozole and E<sub>2</sub> using aromatase expression, and (d) glial (vs. neuronal) nature of aromatase expressing cells around injury using double-label immunocytochemistry. Other sets of sections were used to optimize protocols for data collection or as replicates.

## General Degeneration

To determine the extent of neural injury achieved by the lesions and associated injections, we used Fluoro-Jade B, a poly-anionic fluorescein derivative believed to bind to positively charged molecules in dying neurons and glia (Schmued and Hopkins, 2000; Butler et al., 2002; Anderson et al., 2003). Sections were mounted onto subbed slides and allowed to air dry for 24 h, immersed in 100% ethanol for 3 min, 70% ethanol for 1 min, and distilled water for 1 min. Sections were then washed in 0.06% potassium permanganate (15 min), distilled water (1 min), and then stained with 0.001% Fluoro-Jade B (Chemicon, Temecula, CA; 30 min) under low light conditions. Following staining, sections were rinsed in distilled water (3  $\times$  1 min), air dried, cleared with xylene and coverslipped.

## Apoptotic Degeneration

To determine the extent of programmed cell death induced by the mechanical injuries and injections, sections were exposed to terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) which labels the 3' ends of degenerating DNA, thereby permitting visualization of apoptotic nuclei. The TUNEL Apoptosis Detection Kit was purchased from Upstate Biotechnology (Waltham, MA). Briefly, sections were washed in 0.1 M phosphate buffer saline (PBS) 1 $\times$  for 30 min at room temperature, exposed to 0.036% H<sub>2</sub>O<sub>2</sub> (10 min) and washed in PBS (3  $\times$  15 min). Sections were then incubated with terminal deoxynucleotidyl transferase (TdT) buffer (30 mM Tris-HCl buffer, pH 7.4, containing 140 mM sodium cacodylate, 1 mM cobalt chloride) for 20 min followed by incubation in TdT end labeling cocktail containing TdT buffer, biotin-dUTP, and TdT at a 90:5:5 ratio overnight at 37°C. Once incubation in the cocktail was complete, the reaction was terminated by immersing the sections in 0.1 M PBS for 10 min, and then incubated in a 1:200 avidin-biotin complex (Vectastain, Vector Laboratories, Burlingame, CA) in 0.3% PBT for 60 min at room temperature. Sections were then washed in 0.1 M PBS 1 $\times$  for 45 min, and incorporated biotin was visualized using the Vector SG substrate kit (Vector Laboratories) for peroxidase. Following the color reaction, sections were washed 1 $\times$  for 5 min in 0.1% PBT, dehydrated through graded alcohols (70, 95, 95, 100, 100%), cleared with xylene, and coverslipped.

## Aromatase Expression

In order to determine if the administered fadrozole and E<sub>2</sub> remained local to the injection site, we used immunocytochemistry with a specific antibody against aromatase using previously published protocols (Saldanha et al., 2000). Briefly, sections were washed several times in 0.1 M PB, rinsed in 0.036% H<sub>2</sub>O<sub>2</sub>, washed, immersed in 10% normal goat serum (60 min) and incubated in 1:5000 AZAC for 48 h at 4°C. Specific binding of the antibody was amplified by incubation in 1:200 biotinylated goat anti-rabbit IgG, 1:100 avidin-bio-

tin-HRP, and the immunoprodut developed with diaminobenzidine activated by peroxide (Saldanha et al., 2000). Sections were washed several times, mounted onto subbed slides, air dried, dehydrated, cleared, and coverslipped.

### Co-Expression of Glial Markers and Aromatase

To determine the nature of cells expressing aromatase around the injury tract, we used double-label immunocytochemistry with an antibody against the glial marker vimentin (Developmental studies Hybridoma Bank, Iowa City, IA) and aromatase. Sections were washed in 0.1 M PB (6 × 5 min), incubated in 0.036% H<sub>2</sub>O<sub>2</sub> (10 min) to exhaust endogenous peroxidase, washed in 0.1 M phosphate buffer containing 0.1% Triton X100 (0.1% PBT; 3 × 15 min), and incubated in 10% normal goat serum in 0.3% PBT (1 h). Sections were then incubated in a primary antibody cocktail containing 1:2500 AZAC (Saldanha et al., 2000) and 1:750 anti-vimentin in 0.3% PBT (48 h, 4°C). Each run included a subset of sections that were exposed to AZAC only (no vimentin control). Sections were washed 3 × 15 min in 0.1% PBT to remove excess primary and then incubated in a secondary antibody cocktail containing 1:50 mouse-adsorbed, goat anti-rabbit cyanine-5 (CY-5) and 1:50 rabbit-adsorbed, goat anti-mouse cyanine-2 (CY-2; Jackson Immunochemicals, West Grove, PA) in 0.3% PBT for 2 h at room temperature under foil. Sections were then washed in 0.1% PBT for a total of 2 h, mounted, dehydrated, and coverslipped.

### Confocal Microscopy

Sections stained with the fluorescein-derivative Fluoro-Jade B and those double-stained with antibodies against vimentin and aromatase were inspected under a scanning confocal microscope. Sections were observed using a 25× or 40×/1.4 NA plan apo objective on an inverted microscope (Zeiss Axiovert 200M) equipped with a Zeiss LSM510 META scan head. Argon ion, 543 HeNe, and 633 HeNe lasers were used to generate the 488 (not used), 543 (Fluoro-Jade B and CY-2), and 633 (CY-5) lines used for excitation, and pinholes were typically set to 1–1.5 airy units. For Fluoro-Jade B, we collected 3–9 images of nonoverlapping fields of cells around a visible needle tract per injury per bird at low power (total of 56 photomicrographs for analysis). Additionally, we collected 3 images of nonoverlapping fields of labeled cells per injury per bird at high power (total of 36 photomicrographs for analysis). For vimentin/aromatase, high-power images were collected from areas adjacent to the injury and within the ventromedial nucleus (VMN), approximately 3 mm distal to the lesion. Images were exported and stored as TIFF files.

### Light Microscopy

Sections stained for TUNEL to label apoptotic nuclei and those stained for aromatase expression were examined

under a light microscope. Sections were viewed under 100× or 200× and digitized images were captured using a DXM1200 camera mounted atop a Nikon EM1000 light microscope. For TUNEL, we captured 2–12 frames of nonoverlapping field of labeled nuclei per injury per bird at high power (total of 81 photomicrographs of analysis). For aromatase expression, we captured 4–14 images of nonoverlapping fields of cells around the needle tract per injury per bird at low power (total of 82 photomicrographs for analysis). At high power, 5 images of nonoverlapping fields of cells were captured around the injury and in the VMN (total of 120 photomicrographs for analysis). Digitized images were exported and stored as TIFF files.

### Data Collection and Analysis

To measure the effect of E<sub>2</sub> replacement on the type of degeneration caused by the injections, high-power photomicrographs of Fluoro-Jade B and TUNEL were rendered using NIH Image J. An experimenter blind to the source of the photomicrographs counted the number of labeled Fluoro-Jade B and TUNEL cells in each picture. Data from pictures within individual injuries and subjects were averaged, and group means representing the number of Fluoro-Jade B and TUNEL labeled cells were computed across treatment. Group means were analyzed using two-way ANOVA with treatment (fadrozole vs. fadrozole + E<sub>2</sub>) and type of cell death (Fluoro-Jade B vs. TUNEL) both as within subject variables.

To measure the effect of E<sub>2</sub> replacement on the extent of degeneration, low-power photomicrographs of Fluoro-Jade B and TUNEL were rendered using NIH Image J. In each photomicrograph, an experimenter measured the distance between the needle tract and the 10 most distal labeled cells that were visible. These distances were averaged within picture and hemisphere to yield group means across fadrozole and E<sub>2</sub>-replaced treatments. Data were analyzed using two-way ANOVA with treatment and type of cell death both as within subject variables (see above).

As an independent confirmation of extent of degeneration, we used low-power photomicrographs of aromatase expression to quantify the area of degeneration around injection sites. An experimenter blind to the condition of the animals rendered the photographs using Image J and measured the halo of degeneration in each picture. The area of degeneration, visible as a halo of negative staining immediately surrounding the lesion (Peterson et al., 2004; see Fig. 3) was summed across individual injuries (× 250) and compared across treatments using one-way ANOVA and Fisher LSD.

To ascertain if the drug delivery remained local to the injection site, we used the intensity of aromatase immunoprodut in individual glia in the area of damage and individual neurons within the VMN, a locus approximately 3 mm away from the lesion. Aromatase expression is responsive to fadrozole and E<sub>2</sub>, in that a compromise in aromatase activity results in an increase in immunoprodut (presumably due to negative feedback)

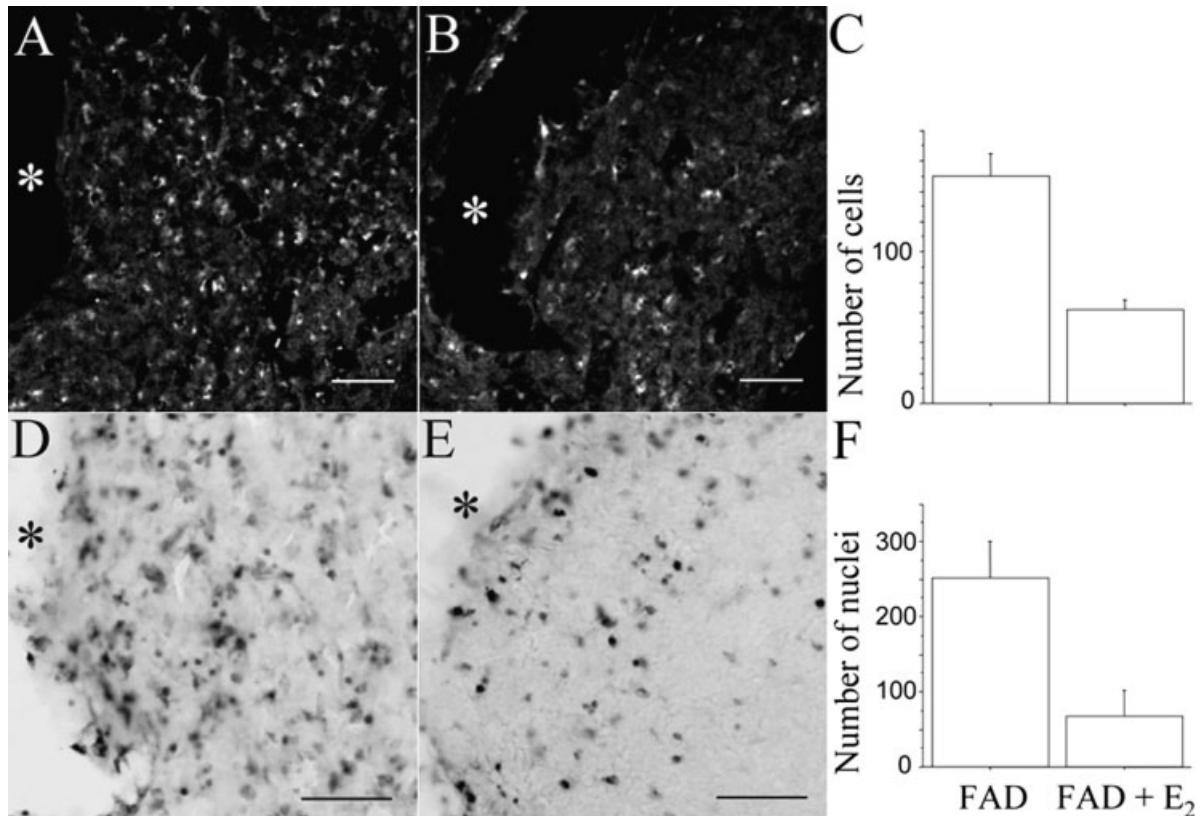
and treatment with E<sub>2</sub> decreases aromatase immunoproduct (Harada et al., 1999). The optical density of immunostain (hereafter: intensity) was measured using Image J in approximately 100 glia per injury per bird and 100 neurons per hemisphere within the VMN per bird. The intensity of unstained areas in between labeled cells was measured as an index of background and these numbers were either subtracted from or divided into the intensity of immunostain to yield a measure of relative optical density (ROD). ROD was averaged across samples within individual injuries. Data were compared using two-way ANOVA with treatment and cell type (glia or neurons) both as within subject variables.

**RESULTS**

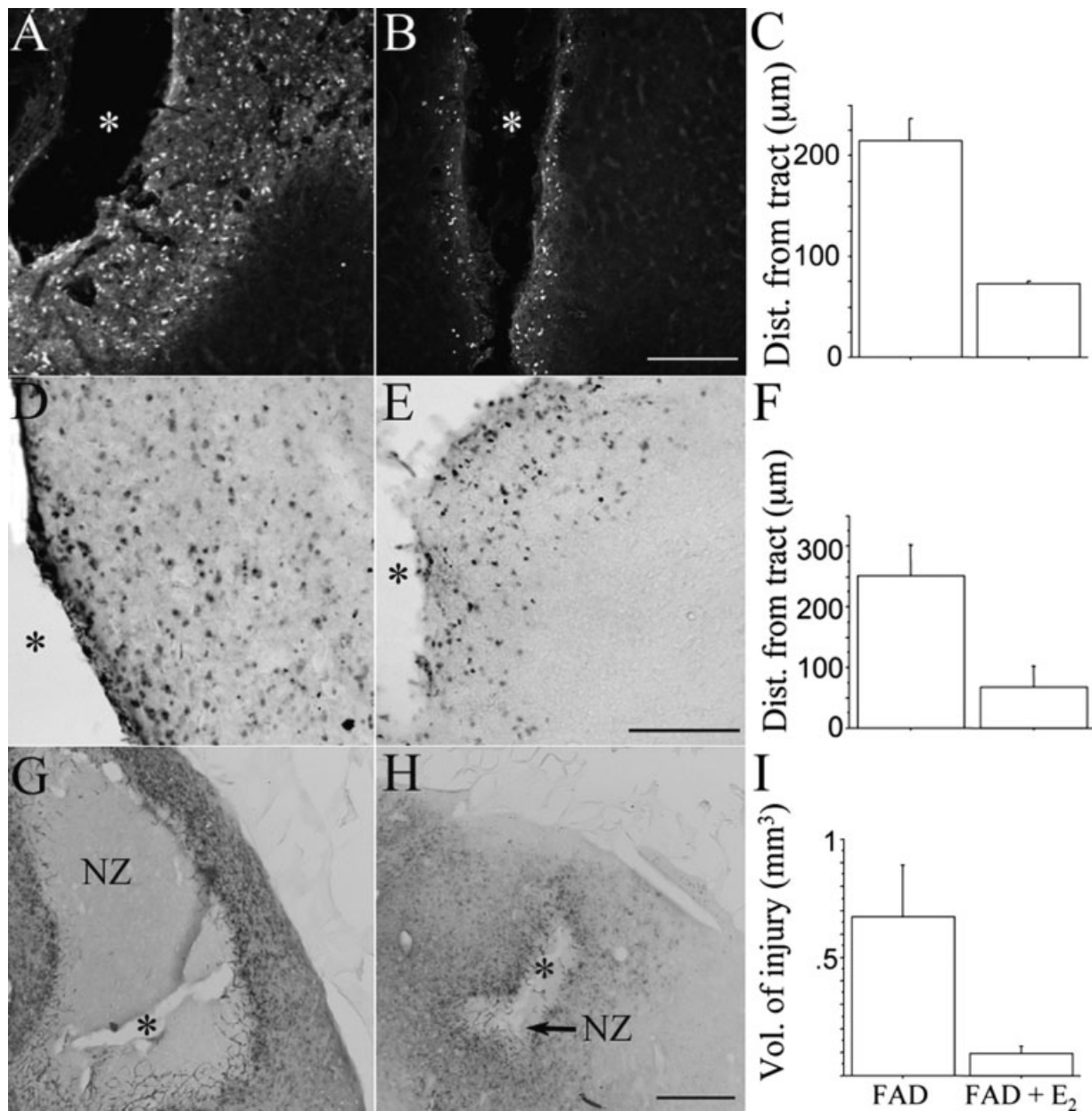
All data presented herein reflect means ± S.E.M. In general, the injury caused by injection of fadrozole alone appeared more extensive and severe than the lesion associated with injection of fadrozole and E<sub>2</sub>. Specifically, hemispheres injected with fadrozole had more degenerating cells labeled with Fluoro-Jade B

and TUNEL relative to the lobe administered E<sub>2</sub> (F<sub>(1,5)</sub> = 43.978; p = 0.0012; Fig. 1). There was no effect of type of death (Fluoro-Jade B vs. TUNEL, F<sub>(1,5)</sub> = 1.405; p = 0.289), but a modest interaction between treatment and type of cell death; (F<sub>(1,5)</sub> = 8.012; p = 0.04). The source of the significant interaction term was a difference between E<sub>2</sub>-replaced Fluoro-Jade B and fadrozole-associated TUNEL (p < 0.05; least square means).

These data are supported by the extent of injury as assessed by the distance of Fluoro-Jade B and TUNEL-labeled cells from the injury tract. In lobes injected with fadrozole, labeled cells were found further away from the needle tract relative to hemispheres that received E<sub>2</sub>-replacement (F<sub>(1,5)</sub> = 55.745; p = 0.0007; Fig. 2). There was no effect of type of cell death (F<sub>(1,5)</sub> = 0.054; p = 0.83) or an interaction between treatment and type of cell death (F<sub>(1,5)</sub> = 0.223; p = 0.66). Thus, the data suggest that E<sub>2</sub>-replacement decreases the number of necrotic and apoptotic cells, thereby limiting the extent of cellular degeneration around neural damage.



**Figure 1** The number of cells undergoing generalized cellular degeneration (A, B, C) including but not limited to apoptosis (D, E, F) is larger around the fadrozole (FAD)-associated injury (A, D) compared to E<sub>2</sub>-associated injury (B, E). Histograms (C, F) of the difference in the number of cells undergoing generalized cellular degeneration as assessed by Fluoro-Jade B and apoptosis as determined by TUNEL. \*Represents needle tract. Scale bar = 50 μm for all panels.

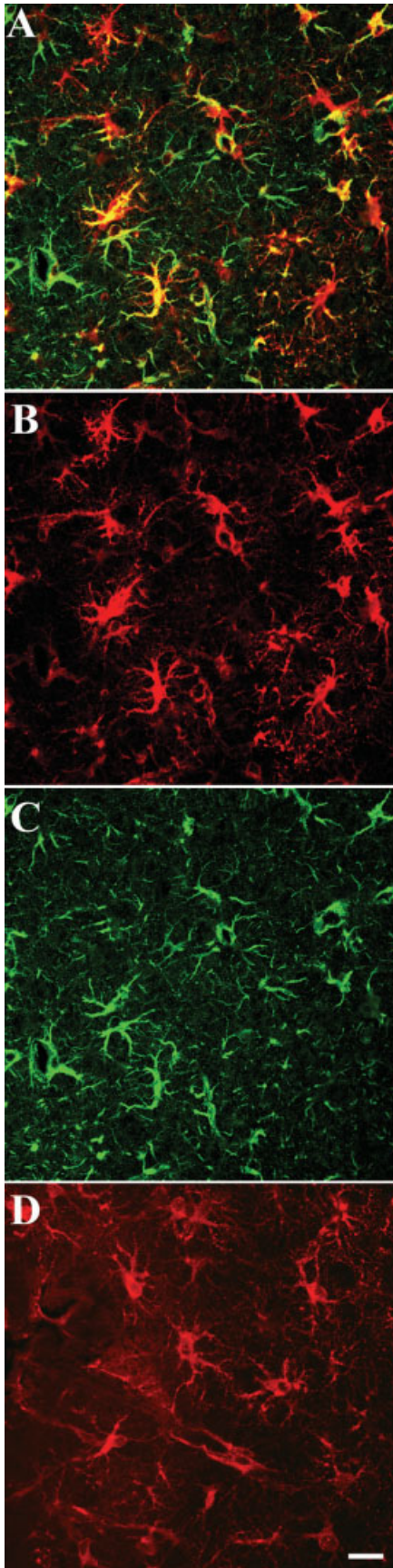


**Figure 2** Replacement with E<sub>2</sub> (B, E, H) decreases the extent of generalized cellular degeneration (A, B) and apoptosis (D, E) from the lesion tract (\*) relative to fadrozole (FAD) alone (A, D, G). The extent of presumptive necrotic tissue (G, H) as evidenced by negative staining around the lesion was also lower in the E<sub>2</sub>-treated hemisphere. Histograms (C, F, I) depicting the difference in distance of cells undergoing generalized cellular degeneration as measured by Fluoro-Jade B, apoptosis as assessed by TUNEL, and the extent of presumptive necrotic tissue as determined by negative staining in aromatase stained sections. NZ = presumed necrotic zone. Scale bar = 200 µm in panels (A) and (B); 50 µm in (D) and (E); and 250 µm in (G) and (H).

The extent of damage resulting from injections was also assessed indirectly using visibly damaged tissue on sections processed for aromatase expression. These data showed a similar pattern of variation between treatments relative to measures of Fluoro-Jade B and TUNEL. Specifically, the total volume of

injury was greater around the fadrozole-injected lobe relative to the hemisphere that received fadrozole and E<sub>2</sub> ( $F_{(1,10)} = 6.937$ ;  $p = 0.02$ ; Fig. 2).

In order to judge whether the administered drugs remained local to the injection site, we used an indirect measure of aromatase content in glia around the



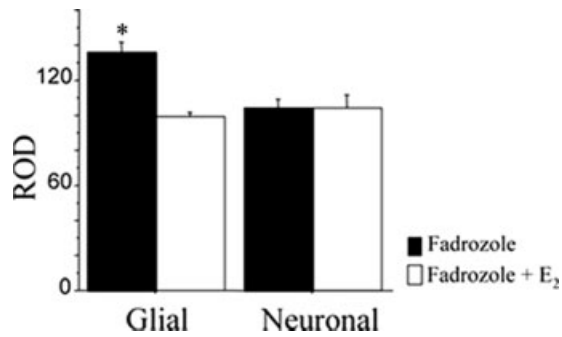
injury and neurons at a locus about 3 mm away. Both methods of standardization (subtraction of vs. division by background) yielded similar patterns of results. Here we report the relative optical density of aromatase immunostain after subtraction of nonspecific staining. Two-way repeated measures ANOVA revealed a significant effect of treatment ( $F_{(1,5)} = 45.498$ ;  $p = 0.0011$ ), no effect of cell type ( $F_{(1,5)} = 3.621$ ;  $p = 0.115$ ) but a significant interaction between these variables ( $F_{(1,5)} = 43.202$ ;  $p = 0.0012$ ; Fig. 4). Post hoc tests reveal that both main effect and the interaction are due to higher relative optical density of immunostain in glia surrounding the fadrozole-associated injury relative to all other groups. Thus, glia around the fadrozole injury were most darkly stained and were darker than contralateral pools of neuronal aromatase and the glia surrounding the  $E_2$ -replaced injury.

Tissue double-labeled with antibodies against aromatase and vimentin allowed for classification of aromatase expressing cells around the site of injury by means other than general morphology. All aromatase-positive cells around the injury site showed colocalized vimentin staining. This staining was specific to the monoclonal vimentin antibody as evidenced by lack of CY-2 signal in sections that served as a no-vimentin-primary antibody control [Fig. 3(D)]. Lack of vimentin immunoprotein in neuronal pools expressing aromatase such as those examined in the VMN showed that vimentin staining was specific to reactive glia (data not shown).

## DISCUSSION

The administration of aromatase inhibitor alone or aromatase inhibitor with  $E_2$ -replacement into disparate hemispheres of the same brain, permitted the evaluation of local aromatization and estrogen provision. We have learned that cells that up-regulate aromatase in response to mechanical injury also express the glial marker vimentin. These cells function to limit the extent of neural degeneration by apparently increasing local levels of  $E_2$ . Locally elevated estro-

**Figure 3** (A) Aromatase cells around injury co-express glial proteins (yellow). Panels (B) and (C) reveal the identical field of cells viewed through the red [aromatase (B)] and green [vimentin (C)] channels alone. (D) Aromatase positive glia with no anti-vimentin control in a section with both emission channels visible (note the absence of green in the image). Scale bar = 20  $\mu\text{m}$  and is identical in all panels.



**Figure 4** Histogram of relative optical density (ROD) of aromatase expression in glial (injury-induced) and neuronal (constitutive) cells in fadrozole vs. fadrozole + E<sub>2</sub> injected hemispheres. Column with \* is significantly different from all others which do not differ from each other ( $p < 0.05$ ).

gens may decrease neurodegeneration by inhibiting programmed cell death in response to mechanical injury.

In avian species, the identification of aromatase-expressing cells around injury sites as glial, was limited to morphological criteria (Peterson et al., 2001, 2004; Wynne and Saldanha, 2004). Using double-label immunocytochemistry with antibodies specifically raised against songbird aromatase (Saldanha et al., 2000) and vimentin (Alvarez-Buylla et al., 1987), the present data reveal that the vast majority of aromatase-expressing cells around the site of injury also co-localize the glial marker vimentin [Fig. 3(A)]. We are confident in the authenticity of immunoprotein achieved in these protocols. The aromatase antibody has been previously characterized using absorption, no-primary, and Western Blot analyses (Saldanha et al., 2000). In the present study, omission of the monoclonal primary antibody against vimentin from the reaction completely eliminated CY-2 signal around the site of injury (no primary control; Fig. 3). Additionally, in double-labeled tissue, aromatase-expressing neurons show no evidence of vimentin immunoprotein pointing to the specificity of this antibody in labeling glia and not neurons. Thus, aromatase expression around the site of neural injury is restricted to reactive glia.

The present data strongly suggest that reactive glia protect the brain from degeneration caused by mechanical insult. Earlier studies have supported the idea that aromatization, most likely within these very glia, is neuroprotective (Garcia-Segura et al., 2001; Peterson et al., 2001; Azcoitia et al., 2002; Wynne and Saldanha, 2004). This hypothesis was directly tested in the current experiment, and reveal that locally elevated E<sub>2</sub> rescues the degeneration caused by mechanical injury (Figs. 1, 2). These findings are

in excellent agreement with previous reports where systemic estrogen administration decreased the amount of neural damage in response to injury in rodents (Simpkins et al., 1997; Toung et al., 1998; Rusa et al., 1999; Garcia-Segura et al., 2001; Rau et al., 2003a). However, systemic manipulations leave open the possibility that aromatization at peripheral sites and/or constitutive neuronal aromatization acting on peripheral substrates could contribute to the observed neuroprotective effects. To the best of our knowledge, this is the first study to reveal that local aromatization in glial cells immediately surrounding injury may protect the brain via a paracrine increase in estrogens.

The idea that treatments in the current paradigm remained local to the delivery site is supported by measures of aromatase optical density. Specifically, glial aromatase around the site of fadrozole delivery was found to be more intense than around the site of E<sub>2</sub> replacement (Fig. 4). This effect was not apparent in pools of neuronal aromatase in the VMN, a locus about 3 mm away. These findings suggest the possibility that while lesion sites were exposed to different levels of E<sub>2</sub> (resulting in differences in ROD), nearby sites of constitutive neuronal aromatization did not differ in the level of E<sub>2</sub> exposure. We therefore conclude that our treatments did indeed isolate the role of up-regulated aromatase in glia around the site of injury in distinction from constitutive neuronal aromatase.

This E<sub>2</sub>-dependent neuroprotection could involve apoptotic and necrotic pathways. The data suggest that the number and distance of apoptotic cells around the needle tract is statistically indistinguishable from the number and distance of pyknotic cells. Specifically, we were unable to detect differences in the extent of degeneration as measured using TUNEL (apoptosis) or Fluoro-Jade B (generalized cell death). Thus, apoptosis is sufficient to account for all degeneration observed in the current paradigm. We recognize that this interpretation requires further rigorous testing perhaps by varying the type of neural insult, dose of fadrozole and E<sub>2</sub> used, and importantly the survival time after insult. Further, we do not know whether TUNEL-labeled cells co-localize Fluoro-Jade B. These experiments are currently underway. Nevertheless, the current results are in good agreement with other studies since work in rodents suggests that peripheral estrogens can severely inhibit apoptosis as early as 8 h following injury (Rau et al., 2003a). The biochemical cascades that ensue may involve caspase activity as has been suggested by several studies in rodents (Jover et al., 2002; Monroe et al., 2002;

Rau et al., 2003b; Yune et al., 2004). We do not know, however, if E<sub>2</sub> protects the brain by a similar pathway in birds. Experiments that explore the time course, regulation, and mechanism of estrogenic neuroprotection in songbirds are currently underway in our laboratory.

Locally elevated estrogen may protect the brain by actions on traditional intranuclear receptors or via membrane pathways, an effect that may involve ER $\alpha$  rather than ER $\beta$  (Wise, 2002). Indeed, ER $\alpha$  and androgen receptor (AR) are prominent in astrocytes and microglia, respectively, following injury in the rat (Garcia-Ovejero et al., 2002). The expression of steroid receptors around sites of neural injury in the songbird awaits evaluation.

In summary, the present data strongly implicate estrogen provision by reactive glia around neural damage as a mechanism that limits the extent of degeneration. Further research will examine the biochemical and cellular pathways recruited by locally elevated estrogens towards limiting programmed cell death in this *in vivo* model for neuronal degeneration.

## REFERENCES

- Adkins-Regan E, Watson JT. 1990. Sexual dimorphism in the avian brain is not limited to the song system of songbirds: a morphometric analysis of the brain of the quail (*Coturnix japonica*). *Brain Res* 514:320–326.
- Alvarez-Buylla A, Buskirk DR, Nottebohm F. 1987. Monoclonal antibody reveals radial glia in adult avian brain. *J Comp Neurol* 264:159–170.
- Anderson KJ, Fugaccia I, Scheff SW. 2003. Fluoro-jade B stains quiescent and reactive astrocytes in the rodent spinal cord. *J Neurotrauma* 20:1223–1231.
- Arnold AP, Gorski RA. 1984. Gonadal steroid induction of structural sex differences in the central nervous system. *Annu Rev Neurosci* 7:413–442.
- Azcoitia I, DonCarlos LL, Garcia-Segura LM. 2002. Estrogen and brain vulnerability. *Neurotox Res* 4:235–245.
- Azcoitia I, Garcia-Ovejero D, Chowen JA, Garcia-Segura LM. 2001. Astroglia play a key role in the neuroprotective actions of estrogen. *Prog Brain Res* 132:469–478.
- Balthazart J, De Clerck A, Foidart A. 1992. Behavioral demasculinization of female quail is induced by estrogens: studies with the new aromatase inhibitor, R76713. *Horm Behav* 26:179–203.
- Butler TL, Kassed CA, Sanberg PR, Willing AE, Pennyacker KR. 2002. Neurodegeneration in the rat hippocampus and striatum after middle cerebral artery occlusion. *Brain Res* 929:252–260.
- Cho JJ, Iannucci FA, Fraile M, Franco J, Alesius TN, Stefano GB. 2003. The role of the estrogen in neuroprotection: implications for neurodegenerative diseases. *Neuro Endocrinol Lett* 24:141–147.
- Farner DS, Wingfield JC. 1980. Reproductive endocrinology of birds. *Annu Rev Physiol* 42:457–472.
- Garcia-Ovejero D, Veiga S, Garcia-Segura LM, DonCarlos LL. 2002. Glial expression of estrogen and androgen receptors after rat brain injury. *J Comp Neurol* 450:256–271.
- Garcia-Segura LM, Azcoitia I, DonCarlos LL. 2001. Neuroprotection by estradiol. *Prog Neurobiol* 63:29–60.
- Garcia-Segura LM, Veiga S, Sierra A, Melcangi RC, Azcoitia I. 2003. Aromatase: a neuroprotective enzyme. *Prog Neurobiol* 71:31–41.
- Garcia-Segura LM, Wozniak A, Azcoitia I, Rodriguez JR, Hutchison RE, Hutchison JB. 1999. Aromatase expression by astrocytes after brain injury: implications for local estrogen formation in brain repair. *Neuroscience* 89:567–578.
- Grisham W, Mathews GA, Arnold AP. 1994. Local intracerebral implants of estrogen masculinize some aspects of the zebra finch song system. *J Neurobiol* 25:185–196.
- Harada N, Honda SI, Hatano O. 1999. Aromatase inhibitors and enzyme stability. *Endocr Relat Cancer* 6:211–218.
- Jover T, Tanaka H, Calderone A, Oguro K, Bennett MV, Etgen AM, Zukin RS. 2002. Estrogen protects against global ischemia-induced neuronal death and prevents activation of apoptotic signaling cascades in the hippocampal CA1. *J Neurosci* 22:2115–2124.
- MacLusky NJ, Naftolin F. 1981. Sexual differentiation of the central nervous system. *Science* 211:1294–1302.
- Maggi A, Ciana P, Belcredito S, Vegeto E. 2004. Estrogens in the nervous system: mechanisms and nonreproductive functions. *Annu Rev Physiol* 66:291–313.
- McEwen BS. 2001. Estrogens effects on the brain: multiple sites and molecular mechanisms. *J Appl Physiol* 91:2785–2801.
- McEwen BS, Alves SE. 1999. Estrogen actions in the central nervous system. *Endocr Rev* 20:279–307.
- Merchenthaler I, Dellovade TL, Shughrue PJ. 2003. Neuroprotection by estrogen in animal models of global and focal ischemia. *Ann NY Acad Sci* 1007:89–100.
- Monroe DG, Berger RR, Sanders MM. 2002. Tissue-protective effects of estrogen involve regulation of caspase gene expression. *Mol Endocrinol* 16:1322–1331.
- Morgan MA, Schulkin J, Pfaff DW. 2004. Estrogens and non-reproductive behaviors related to activity and fear. *Neurosci Biobehav Rev* 28:55–63.
- Morrison JH. 2003. Aging and mammalian cerebral cortex: monkeys to human. *Alzheimer Dis Assoc Disord* 17 (Suppl 2):S51–S53.
- Nicotera P, Lipton SA. 1999. Excitotoxins in neuronal apoptosis and necrosis. *J Cereb Blood Flow Metab* 19:583–591.
- Peterson RS, Lee DW, Fernando G, Schliner BA. 2004. Radial glia express aromatase in the injured zebra finch brain. *J Comp Neurol* 475:261–269.
- Peterson RS, Saldanha CJ, Schlinger BA. 2001. Rapid upregulation of aromatase mRNA and protein following neural injury in the zebra finch (*Taeniopygia guttata*). *J Neuroendocrinol* 13:317–323.

- Rau SW, Dubal DB, Bottner M, Gerhold LM, Wise PM. 2003a. Estradiol attenuates programmed cell death after stroke-like injury. *J Neurosci* 23:11420–11426.
- Rau SW, Dubal DB, Bottner M, Wise PM. 2003b. Estradiol differentially regulates c-Fos after focal cerebral ischemia. *J Neurosci* 23:10487–10494.
- Rusa R, Alkayed NJ, Crain BJ, Traystman RJ, Kimes AS, London ED, Klaus JA, Hurn PD. 1999. 17Beta-estradiol reduces stroke injury in estrogen-deficient female animals. *Stroke* 30:1665–1670.
- Saldanha CJ, Schlinger BA, Micevych PE, Horvath TL. 2004. Presynaptic N-methyl-D-aspartate receptor expression is increased by estrogen in an aromatase-rich area of the songbird hippocampus. *J Comp Neurol* 469:522–534.
- Saldanha CJ, Tuerk MJ, Kim YH, Fernandes AO, Arnold AP, Schlinger BA. 2000. Distribution and regulation of telencephalic aromatase expression in the zebra finch revealed with a specific antibody. *J Comp Neurol* 423:619–630.
- Schlinger BA, Amur-Umarjee S, Shen P, Campagnoni AT, Arnold AP. 1994. Neuronal and non-neuronal aromatase in primary cultures of developing zebra finch telencephalon. *J Neurosci* 14:7541–7552.
- Schlinger BA, Arnold AP. 1991. Brain is the major site of estrogen synthesis in a male songbird. *Proc Natl Acad Sci USA* 88:4191–4194.
- Schlinger BA, Arnold AP. 1992. Circulating estrogens in a male songbird originate in the brain. *Proc Natl Acad Sci USA* 89:7650–7653.
- Schmued LC, Hopkins KJ. 2000. Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res* 874:123–130.
- Shen P, Schlinger BA, Campagnoni AT, Arnold AP. 1995. An atlas of aromatase mRNA expression in the zebra finch brain. *J Comp Neurol* 360:172–184.
- Simpkins JW, Rajakumar G, Zhang YQ, Simpkins CE, Greenwald D, Yu CJ, Bodor N, Day AL. 1997. Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat. *J Neurosurg* 87:724–730.
- Stokes TM, Leonard CM, Nottebohm F. 1974. The telencephalon, diencephalon, and mesencephalon of the canary, *Serinus canaria*, in stereotaxic coordinates. *J Comp Neurol* 156:337–374.
- Tang DG, Porter AT. 1996. Apoptosis: a current molecular analysis. *Pathol Oncol Res* 2:117–131.
- Toung TJ, Traystman RJ, Hurn PD. 1998. Estrogen-mediated neuroprotection after experimental stroke in male rats. *Stroke* 29:1666–1670.
- Wade J, Schlinger BA, Hodges L, Arnold AP. 1994. Fadrozole: a potent and specific inhibitor of aromatase in the zebra finch brain. *Gen Comp Endocrinol* 94:53–61.
- Watson RE Jr, Wiegand SJ, Clough RW, Hoffman GE. 1986. Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides* 7:155–159.
- Wise PM. 2002. Estrogens and neuroprotection. *Trends Endocrinol Metab* 13:229–230.
- Wise PM. 2003. Estrogens: protective or risk factors in brain function? *Prog Neurobiol* 69:181–191.
- Wynne RD, Saldanha CJ. 2004. Glial aromatization decreases neural injury in the zebra finch (*Taeniopygia guttata*): influence on apoptosis. *J Neuroendocrinol* 16:676–683.
- Yune TY, Kim SJ, Lee SM, Lee YK, Oh YJ, Kim YC, Markelonis GJ, Oh TH. 2004. Systemic administration of 17beta-estradiol reduces apoptotic cell death and improves functional recovery following traumatic spinal cord injury in rats. *J Neurotrauma* 21:293–306.