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Molecular characterization of the injury-induced aromatase transcript in the adult zebra finch brain

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Abstract

In the zebra finch (*Taeniopygia guttata*), the aromatase gene is transcribed from one of two promoters resulting in two transcripts constitutively expressed in brain or ovary. These transcripts differ only in Exon 1 which lies in the 5′ un-translated region (UTR). An inducible form of aromatase is expressed following brain injury in glia. Towards characterizing this transcript, we (a) examined the up-regulation of amplicons within the aromatase transcript using quantitative PCR (qPCR), (b) performed 5′ and 3′ rapid amplification of cDNA ends (RACE) on injured brain RNA and (c) sequenced the injury-induced aromatase transcript. qPCR suggested that inducible aromatase may contain a novel 3′UTR. However, neither 3′ nor 5′ RACE revealed novel UTRs in the injured telencephalon. We then sequenced aromatase from injured entopallium, a region that lacks detectable constitutive aromatase. Inducible aromatase was identical in sequence to the known neural aromatase transcript. These data suggest that injury-induced aromatase differs from ovarian, but is indistinguishable from neuronal aromatase. We suggest that an injury-specific signal in glia may modulate aromatase transcription. Alternatively, injury-induced aromatase transcription may be silenced under constitutive conditions. To the best of our knowledge, this is the first report that documents the sequence of inducible aromatase in any vertebrate.

Keywords: apoptosis, estrogen, glia, neuron, neuroprotection, stroke.

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Sex steroids such as estrogens (Es) exert multiple and diverse actions on the developing and adult vertebrate brain. In the developing brain, Es play a critical role in the sexual differentiation and organization of several neural circuits critical for reproductive behaviors in mammals (MacLusky and Naftolin 1981; Dohler *et al.* 1982; Arnold and Gorski 1984) and birds (Gurney and Konishi 1980; Ottinger *et al.* 1984; DeVoogd 1986; Harding 1986; Harding *et al.* 1988; Balthazart *et al.* 1992, 1996). In the adult brain, Es affect the length of neurites, the density of dendritic spines and the number of synapses on dendritic spines in birds and mammals (DeVoogd and Nottebohm 1981; Gould *et al.* 1990; Leranth *et al.* 2000, 2002; Dominguez *et al.* 2004). Es also affect aspects of learning and memory, fine motor skills, mood, pain sensitivity (McEwen 2001; Maggi *et al.* 2004) and are critical in the protection of neural circuits from degeneration associated with injury (Garcia-Segura *et al.* 2001; Wise 2002; Cho *et al.* 2003; Merchenthaler *et al.* 2003; Rau *et al.* 2003a,b; Wynne and Saldanha 2004; Saldanha *et al.* 2005; Wise *et al.* 2005; Suzuki *et al.* 2006; Wynne *et al.* 2008).

The synthesis of estradiol (E_2) from testosterone is catalyzed by the enzyme aromatase. In most vertebrate species, the major organs that synthesize E_2 via aromatase are the gonads, placenta and the brain (Simpson *et al.* 1994; Vanselow *et al.* 1999), but also include the liver, skin fibroblasts and adipose tissue (Harada 1993). In humans, rabbits, cattle, sheep, zebra finches and mice, tissue-specific expression of aromatase is regulated by different promoter regions and alternative splicing (Simpson *et al.* 1993; Yamada *et al.* 1995; Honda *et al.* 1996; Yamada-Mouri *et al.* 1996; Ramachandran *et al.* 1999; Vanselow *et al.* 2001; Golovine *et al.* 2003). Alternative promoters generate variants of the aromatase transcript that differ in the 5′

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Abbreviations used: RACE, rapid amplification of cDNA ends; UTR, un-translated region.

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un-translated region (UTR) but are identical in their coding regions and 3′UTRs. Translation of the aromatase coding region thus yields the same protein product in all tissues where the transcript is expressed. Unlike the aforementioned species where a single copy gene gives rise to different aromatase transcripts, the existence of more than one functional copy of the aromatase gene has been reported in the pig (Graddy *et al.* 2000), goldfish (Tchoudakova and Callard 1998) and zebrafish (Kishida and Callard 2001). This differential tissue-specific expression of aromatase may be critical in the regulation of E_2 biosynthesis.

Aromatase expression seems to be confined to neuronal populations in the intact homeotherm brain during development and adulthood. Aromatase activity (Hutchison and Schumacher 1986; Sharp *et al.* 1986; Foidart *et al.* 1998; Soma *et al.* 1999, 2003; Riters *et al.* 2001) and expression (Riters *et al.* 2000) increase in the spring in several avian species, an increase that represents an up-regulation of aromatase in neurons and not an additional cell type. Correspondingly, the lower levels of aromatase observed in the adult relative to the embryonic brain reflects a decrease of aromatase in neurons and not the loss of aromatization in non-neuronal cells (Shinoda 1994; Jacobs *et al.* 1999; Perlman and Arnold 2003; Zhao *et al.* 2007). Therefore, in the intact avian brain, aromatase is constitutively expressed in neurons (Balthazart *et al.* 1990; Negri Cesi *et al.* 1992; Schlinger 1997).

Aromatase is expressed by glial cells *in vitro* (Schlinger *et al.* 1994; Zwain and Yen 1999; Yague *et al.* 2004, 2006) and following brain injury to the rodent (Garcia-Segura *et al.* 1999; Garcia-Segura *et al.* 2001; Azcoitia *et al.* 2001, 2003) and zebra finch brain (Peterson *et al.* 2001, 2004; Wynne and Saldanha 2004; Saldanha *et al.* 2005; Wynne *et al.* 2008). Glial aromatization limits the extent of brain damage following injury in mammals and birds (Garcia-Segura *et al.* 2001; Garcia-Segura *et al.*, 2003; Wynne and Saldanha 2004; Saldanha *et al.* 2005; Wynne *et al.* 2008). However, questions remain as to how glial aromatase transcription is regulated following injury. In other words, how is the expression of aromatase in glia restricted to times of brain damage?

Songbirds may be good models for studies involving the regulation of injury-induced gene expression. The aromatase transcript is alternatively spliced in the zebra finch, yielding two different transcripts that differ in their 5′UTRs encoded by either exon 1a or exon 1b. These aromatase transcripts are found in neurons of the brain (exon 1a) or thecal and granulosa cells of the ovary (exon 1b; Ramachandran *et al.* 1999). Downstream exons are identical and the product of both tissue-specific promoters generates the same functional protein in the brain and ovary (Schlinger and Arnold 1991, 1992). Neuronal aromatase, relative to mammals, is more abundant yet constrained to several telencephalic regions in the zebra finch (Schlinger 1997; Saldanha *et al.* 2000; Goodson *et al.* 2005). Additionally, glial aromatase is only detectable *in vivo* following neural insult to the zebra finch brain (Peterson *et al.* 2001, 2004; Wynne and Saldanha 2004; Saldanha *et al.* 2005; Wynne *et al.* 2008) and in primary dissociated cell cultures of the developing zebra finch telencephalon (Schlinger *et al.* 1994). Further, local E_2 synthesis via glial aromatase reduces injury size and decreases the injury-induced wave of secondary degeneration through decreases in apoptotic pathways (Wynne and Saldanha 2004; Saldanha *et al.* 2005; Wynne *et al.* 2008). Thus, songbirds are excellent models for studying the regulation of E synthesis following injury to the brain.

Since aromatase is inducible in glia but constitutively expressed in neurons, we tested the hypothesis that injury-induced up-regulated aromatase represents a novel aromatase transcript in the zebra finch brain. We show that the identity of the aromatase transcript up-regulated following brain injury differs from that expressed in ovarian cells but is indistinguishable from that expressed constitutively in neurons.

Materials and methods

Subjects and housing

Adult male zebra finches (>90 days post-hatching) were obtained from our breeding colony at the Biological Sciences Animal Facility, Lehigh University. Birds were placed into same-sex cages (18″ × 18″ × 16″; up to four per cage) under a 14 : 10 LD cycle (lights on at 06:00 h) at a room temperature of 20 ± 2°C. Food, water, grit, and cuttlebones were available *ad libitum*. All housing and experimental protocols used in this study were approved by the Lehigh University Institutional Animal Care and Use Committee (IACUC).

Experimental design

To determine the sequence of injury-induced up-regulated aromatase in the zebra finch brain, we used a combination of semi-quantitative real time polymerase chain reaction (qPCR), 5′ RACE and 3′ RACE on RNA extracted from injured and non-injured brain. Detailed descriptions of each approach appear below. We first asked if the abundance of particular exons was affected by neural insult using qPCR (Experiment 1). Next, we compared 5′ and 3′ RACE products from RNA extracted from injured and non-injured brain (Experiment 2). Finally, we used PCR to sequence the entire open reading frame of injury-induced aromatase from a region of the zebra finch brain that lacks detectable constitutive aromatase (Experiment 3).

Experiment 1: qPCR

Neural injury A

Subjects were anesthetized with 0.08 mL of 16 mg/mL ketamine and 0.3 mg/mL xylazine in 0.9% saline. Birds were placed in a stereotaxic apparatus with the head angled at 45°. The cranium was exposed by incision and an 18G needle was used to create a unilateral craniotomy 2 mm anterior to the pineal gland and 3 mm lateral to the midline into the right hemisphere of the brain. Injury was targeted toward the entopallial nucleus 3 mm ventral to the brain surface (Stokes *et al.* 1974) because the entopallium lacks

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constitutively expressed aromatase based upon *in situ* hybridization (Shen *et al.* 1994, 1995; Jacobs *et al.* 1999) and immunocytochemistry (Shen *et al.* 1995; Saldanha *et al.* 2000, 2005; Wynne and Saldanha 2004). A 50 μL 22 s Hamilton syringe (Hamilton Company, Reno, NV, USA) was placed at the surface of the brain at an angle of 45° and lowered to the target where it resided for 120 s and was then retracted. The scalp was repositioned over the cranium and sealed with Collodion Flexible (EM Science, Gibbstown, NJ, USA). Following surgery, the birds recovered from anesthesia under a heat lamp and were killed 24 h following injury.

Tissue collection and RNA preparation A

Birds (n = 5) were decapitated and the injured telencephalic hemisphere was separated from the non-injured telencephalic hemisphere in each bird and weighed. Total RNA preparations were performed by using the RNeasy mini kit (Qiagen, Valencia, CA, USA) as recommended by the supplier. The amount of total RNA was determined by spectrophotometry (Nanodrop Technologies, Wilmington, DE, USA) and the integrity of the RNA was determined by visualization of the 28S and 18S ribosomal RNA bands after separation on a 1% agarose gel stained with ethidium bromide.

qPCR

To determine which exons in the known transcripts of aromatase were up-regulated following brain injury, we used qPCR with aromatase-specific primers. For every qPCR experiment, 800 ng of total RNA was reverse transcribed with an oligo$(dT)_{20}$ using the Superscript III first strand synthesis kit for reverse transcription polymerase chain reaction (RT-PCR; Invitrogen, Carlsbad, CA, USA). For qPCR, 1 μL (or 5% of the total first strand synthesis reaction) of the resulting cDNA was amplified with Power SYBR Green PCR master mix (Applied Biosystems, University Park, IL, USA) in 25 μL of total reaction volume. To test the efficiency of the qPCR reaction, 1 μL of a 1 : 10 dilution of the cDNA from the first strand synthesis reaction (or 0.5% of the total first strand synthesis reaction) was amplified. Runs between these concentrations generated similar results, thus, we present data from reactions where 1% of the total first strand synthesis reaction was amplified. Assays were done in 96-well optical plates and each sample was amplified in triplicate. In every run, wells without the RT product were included in order to detect any external contamination. Amplicons were generated against exons 1a, 1b, 2, 8, 9 and 10 of the zebra finch aromatase transcript as well as the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; see Table 1). As an additional method to decrease variability, product lengths (74–78 bp) and the predicted melting temperature of each amplicon (63–67°C) were similar across amplicons generated. Primers were designed using zebra finch aromatase and GAPDH-specific sequences found in the GenBank database (GenBank accession numbers AF170274, AF170273, S75898 and AF255390).

Table 1 Zebra finch specific primer sequences used for real time PCR, 5′ RACE, 3′ RACE and RT-PCR experiments

Real time PCR			
Amplicon	Forward primer	Reverse primer	Amplicon length
Aro exon 1a	CTCCACCGACAAAAATCCAC	GGGTTTCCAGGACCATCTTT	78 bp
Aro exon 1b	ACAGCCCCTGCCTCTCTC	GGGTTTCCAGGACCATCTTT	78 bp
Aro exon 8	GAGAAAAATGTICCTTCCCGC	GCAATGAACTTCCCCACACAG	74 bp
Aro exon 9	CCCAGAAGAAATGCAAACGA	TAGTGGTTGAGAAAATCCTGTAACTTTA	76 bp
Aro exon 10	CCTCCTCACCTGACTGACTATAGAAA	AGCTTAGAGGGAATTTGACCTTCTT	76 bp
GAPDH	TGCTGCTCAGAACATTATCCCA	TTTCCCACAGCCTTAGCAGCT	54 bp
5′ RACE		**3′ RACE**	
Primer name	Primer sequence	Primer name	Primer sequence
Aro485R	TGTGCTGGGTTGTTGTTAAAT	Aro1617F	AATCAGGGTGATGGAATGGA
Aro381R	CCAAACTCTCACAAAGTCTCC	Aro2540F	CAGGTTCCCACAGCACAGCAG
Aro162R	GCTGGTGATGTTGTAGTGCAG	Aro2953F	GGAGTTGCCAACTTGGTCAAG
Aro131R	GGGTTTCCAGGACCATCTTT		
Aro64R	GATTTTTGTCGGTGGAGAGTG		
RT-PCR			
Transcript	Forward primer	Reverse primer	Annealing temperatures
Aro 44F-1003R	CACTCTCCACCGACAAAAATC	ACATTCTCAGCAGTCAGATC	53.0°C
Aro 591F-1274R	CGGATGATCGCGATTTGT	GTAGCCGTCAATGACATCGTC	53.0°C
Aro 984F-1643R	GATCTGACTGCTGAGAATGT	TGCTGATCCATTCCATCACC	57.3°C
Aro 1542F-2294R	GACTTATCCATGCACCCCATAGA	CTGCAGCAAGAGACTGGGTTT	63.0°C
Aro 1884F-2839R	CCCTTTCAAACACTGCCAGTTCAG	ACCCCAGCCTGGTTCATAGTT	57.3°C
Aro 2540F-2973R	CAGGTTCCCACAGCACAGCAG	CTTGACCAAGTTGGCAACTCC	63.0°C
NR1	CACAAGCCCAACGCCATCCAGATG	AAGAGCGTCACTGATGTGGGCTGA	63.0°C

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Amplification and quantification were performed in an Applied Biosystems 7300 qPCR instrument under the following cycling conditions: an activational step at 50°C for 2 min, pre-incubation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Following PCR amplification, a final dissociation step of 95°C for 15 s, renaturation at 60°C for 1 min, denaturation at 95°C for 15 s and renaturation at 60°C for 15 s was performed to generate dissociation curves used to ascertain that only the expected products had been generated. Fluorescence of SYBR green was measured and subsequently analyzed using Sequence Detection Software SDS v1.4 (Applied Biosystems) and threshold cycle values (Ct) were generated.

Statistical analysis

The Ct values for all GAPDH samples were statistically indistinguishable between telencephalic hemisphere and across qPCR runs. We therefore collapsed the data across the two qPCR runs. For each bird, triplicate Cts were averaged for GAPDH and aromatase. To determine relative changes in the abundance of each aromatase transcript generated, ΔCts were calculated by subtracting the Ct value of the housekeeping gene GAPDH from the aromatase Ct. ΔCt values were then analyzed via a repeated measures two-way ANOVA with telencephalic hemisphere (injured versus non-injured; within subject) and amplicon as the main variables. Least square means tests were used to determine the source of significant variation.

As an additional statistical analysis, we divided ΔCt values from the injured telencephalon by ΔCt values from the non-injured telencephalon to generate the ratio between treatments for each amplicon generated. Finally, 99% confidence intervals were computed from these group means and errors [mean ± (2.58 × standard error of the mean (SEM)] to determine if the ratio between treatments was significantly different from one.

Experiment 2: Rapid amplification of 5′ and 3′ cDNA ends

5′ RACE

To determine if additional 5′ aromatase mRNA splice variants are present following brain injury, 5′ RACE was carried out with the 5′ RACE system for rapid amplification of cDNA ends kit (Invitrogen) as recommended by the supplier with slight modifications. Five micrograms of total RNA from injured, non-injured and intact zebra finch telencephalon were reverse transcribed with a gene-specific primer ZFAROM485R (see Table 1) for 90 min at 42°C. DNA was recovered via centrifugation at 13 000 *g* for 30 min at 20°C and terminal deoxynucleotidyl transferase tailing of cDNA was performed for 1 h at 37°C.

A nested gene-specific primer that hybridizes to sequence found in Exon 3 and is shared by all known aromatase transcripts in the zebra finch (ZFAROM381R; Table 1) and an abridged anchor primer (Invitrogen) were used for the first amplification. An additional nested gene-specific primer that was either specific for sequence found in the form of aromatase preferentially expressed in the brain (ZFAROM64R; Table 1) or exon 2 (ZFAROM162R; Ramachandran *et al.* 1999) and an abridged universal amplification primer (Invitrogen) were used for the second amplification. Amplification of products were performed in a PTC-100 Programmable Thermal Controller (MJ Research, Waltham, MA, USA) under the following cycling conditions: pre-incubation at 94°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 7 min. PCR products were visualized on a 1% ethidium bromide stained agarose gel. The resulting products were gel extracted using the QIAquick Gel Extraction kit (Qiagen) and subsequently sequenced with the corresponding forward or reverse primer on an ABI 310 Genetic Analyzer with the BigDye Terminator reagent v3.1 (Applied Biosystems). The obtained sequences were analyzed with DNA sequencing analysis software v5.1 (Applied Biosystems).

3′ RACE

To determine if additional 3′ aromatase mRNA splice variants are transcribed following brain injury, 3′ RACE was carried out with the 3′ RACE system for rapid amplification of cDNA ends kit (Invitrogen) as recommended by the supplier. Briefly, 5 μg of total RNA from injured and non-injured zebra finch telencephalon were reverse transcribed with an adapter primer (Invitrogen) at 42°C for 50 min. The gene-specific primers ZFAROM1617F, which hybridizes to sequence found in the aromatase coding region and is shared by all known aromatase transcripts in the zebra finch, or ZFAROM2540F, which hybridizes to sequence found in Exon 10 of the 3′UTR and may not be present in the injury-induced aromatase transcript (Table 1), were used in conjunction with an abridged universal amplification primer or a universal amplification primer (Invitrogen) for PCR amplification. Products generated by ZFAROM1617F and the universal amplification primer were amplified using AccuPrime DNA *Taq* Polymerase High Fidelity (Invitrogen) as recommended by the supplier. Products synthesized from ZFAROM2540F to the abridged universal amplification primer were amplified using protocols previously described above. Visualization, gel extraction and sequencing of products were performed as described above under subheading 5′ RACE.

Experiment 3: Sequencing of injury-induced aromatase

Neural injury B

Lesions of the entopallial nucleus were similar to those described previously with a few modifications. The cranium was exposed by incision and an 18G needle was used to create a bilateral craniotomy 3.2 mm anterior to the pineal gland and 3.7 mm lateral to the midline. These coordinates were used in order to minimize disruption of already existing pools of neuronal aromatase (Saldanha *et al.* 2000). Injury was targeted toward the entopallial nucleus 3.5 mm ventral to the brain surface. A 50 μL 22 s Hamilton syringe was positioned at the surface of the brain at an angle of 0º and lowered to the target where it remained for 120 s and was then retracted. The scalp was repositioned over the cranium, sealed with Collodion Flexible and birds recovered under a heat lamp. All birds were then killed 24 h following injury.

Tissue collection and RNA preparation B

Birds (n = 7 injured; n = 5 intact) were decapitated and the brains were rapidly dissected on ice. The injured and intact entopallium were microdissected under a dissection scope and samples were pooled into their respective group and weighed. Whole intact zebra finch telencephalon (n = 2) was rapidly dissected out on ice and weighed. Only those samples observed to have visible needle tract terminating in the entopallial nucleus were used for these experiments. Total RNA was isolated from each sample using TRIZOL reagent (Invitrogen) as

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described by the supplier with slight modifications. For RNA precipitation, samples were incubated in isopropyl alcohol for 20 min at 20°C and then centrifuged at 12 000 *g* for 30 min at 4°C. The amount of total RNA as well as the quality was assessed as previously described under the subheading Experiment 1: Tissue Collection and RNA Preparation A.

RT-PCR

To isolate the aromatase transcript up-regulated in the injured entopallium, we used gene-specific primers targeted to different sequences of the aromatase transcript and RT-PCR. One microgram of total RNA from injured and intact entopallium as well as intact telencephalon was reverse transcribed with a random hexamer using the Superscript III first strand synthesis kit for RT-PCR (Invitrogen) in a 21 μL reaction. For PCR, 2 μL of the resulting cDNA was amplified in a 50 μL reaction using forward and reverse primer pairs that generated amplicons targeted against different portions of known aromatase sequence (see Table 1). The 50 μL amplification reaction contained 20 mM Tris–HCl (pH = 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 400 nM of forward and reverse gene-specific primer, 200 μM each dATP, dCTP, dGTP and dTTP, cDNA, and 2.5 units of *Taq* DNA polymerase. Cycling parameters were similar to those found under subheading 5′ RACE with few modifications. Twenty-six, twenty-seven, and twenty-eight cycles of PCR amplification was performed at annealing temperatures indicated in Table 1 for each primer pair. Visualization, gel extraction and sequencing of products were performed as described previously under subheading Experiment 2: 5′ RACE.

Subcloning and sequencing

As an additional method for sequencing PCR products specific for the injury-induced aromatase transcript, we subcloned individual PCR products using the pEXP5-CT/TOPO expression kit according to the protocol supplied by the manufacturer (Invitrogen). Five colonies from each Luria-Bertani agar plate were further cultured in 5 mL LB medium containing 100 μg/mL ampicillin, shaken overnight at 37°C. DNA was isolated from cultures by the Wizard Plus Miniprep DNA Purification system (Promega, Madison, WI, USA) and the amount of total DNA was determined by a Nanodrop ND-1000 instrument. Recombinant plasmids were sequenced using the pEXP5-CT/TOPO vector-specific T7 promoter primer (Invitrogen) as described under the subheading Experiment 2: 5′ RACE.

5′ RACE and 3′ RACE

5′ RACE and 3′ RACE were performed as described above with slight modifications. For these reactions, 2.5 μg of total RNA from injured and intact entopallium were reversed transcribed. Amplification, visualization, subcloning and sequencing of products were performed as described above.

Results

qPCR: 2-way ANOVA analysis of aromatase expression following injury

qPCR revealed that amplicons within exons 1a (5′UTR of brain-specific transcript) and 8 (coding region of both known zebra finch aromatase transcripts) increased following insult to the brain as indicated by decreases in the respective ΔCt values (see Fig. 1b).

However, amplicons within exons 1b, 9 and 10 were not different between the injured and the non-injured telencephalic hemisphere (Fig. 1b). More specifically, when ΔCt values from qPCR were analyzed, we found a statistically significant interaction between amplicon and telencephalic hemisphere ($F_{(4,24)} = 4.431$; $p = 0.0080$) and this term was due to lower ΔCt values in the injured relative to the non-injured telencephalon for amplicons generated against exon 1a and exon 8 ($p < 0.05$; see Fig. 1). We were able to detect an effect on aromatase amplicons ($F_{(4,24)} = 147.198$; $p = 0.0001$), but no detectable effect of injury on overall expression levels of aromatase amplicons generated ($F_{(1,24)} = 3.847$; $p = 0.0975$). The effect on aromatase amplicon was due to larger ΔCt values for the amplicon generated against exon 1b versus all other amplicons studied ($p < 0.05$; Fig. 1b).

qPCR: 99% confidence-interval analysis of aromatase expression following injury

When 99% confidence intervals were computed from group means generated by division of ΔCt values from the injured telencephalic hemisphere by ΔCt values from the non-injured telencephalic hemisphere, we found that amplicons in Exon 1A (brain), 8 and 9 yielded values that were less than the value of one. Conversely, amplicons in Exons 1B (ovarian) and 10 generated values equal to the value of one. For this analysis, all values that are < 1 represent that the aromatase transcript is more abundant in the injured relative to the non-injured telencephalic hemisphere (Fig. 1c).

5′ and 3′ RACE

Examination of 5′ and 3′ RACE products confirmed that no novel aromatase transcripts were present in the injured telencephalic hemisphere in relation to the non-injured telencephalic hemisphere (see Fig. 2).

Sequences generated with the aromatase gene-specific reverse primer ZFAROM64R in the injured telencephalon showed 100% sequence homology to aromatase sequences from both the non-injured telencephalon and known aromatase sequences expressing exon 1a, which is typically found in the zebra finch brain (basepairs 357–391; GenBank accession number AF170274). Sequences generated with the aromatase gene-specific reverse primer ZFAROM162R in the injured telencephalon demonstrated 99% homology to sequences generated from the intact telencephalon (basepairs 357–449; for reference see GenBank accession number AF170274). We were unable to detect in either telencephalic hemisphere aromatase transcripts containing exon 1b, which are typically expressed in the ovary (GenBank accession

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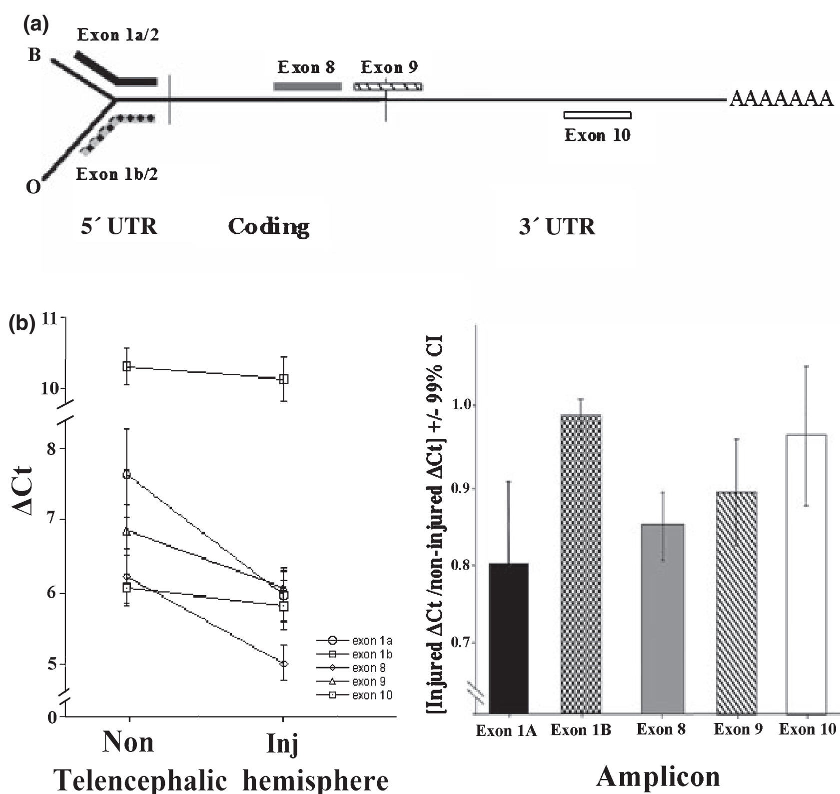

Fig. 1 (a) Schematic representing the different 5′ UTR sequences of the brain (B) and ovarian (O) aromatase transcripts in the adult zebra finch. Amplicons generated against different portions of the aromatase transcript (5′UTR: exons 1a, 1b, and 2; Coding region: exon 8 and 9; 3′UTR: exons 9 and 10) for qPCR are shown. (b) Line graph showing the change in ΔCt values (normalized against GAPDH) between non-injured versus injured telencephalon for each amplicon. (c) 99% confidence intervals calculated by dividing injured ΔCt values by non-injured ΔCt values for each amplicon.

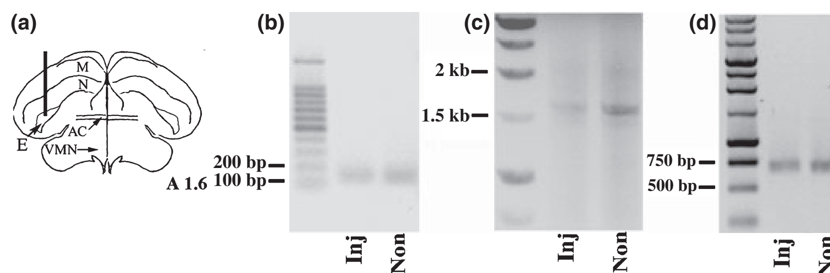

Fig. 2 (a) Schematic representing mechanical injury targeted towards the entopallium (E) through a coronal section of the zebra finch brain. M = mesopallium, N = nidopallium, AC = anterior commissure, VMN = ventromedial nucleus of the hypothalamus. The anteroposterior position of the sections is depicted. (b) Gel electrophoresis showing 5′ RACE products (generated with ZFAROM64R) from the injured (INJ) and non-injured (NON) telencephalon run in conjunction with a 100 bp DNA step ladder. (c and d) 3′ RACE products from the INJ and NON telencephalon run along side a 1kB DNA step ladder. The coding region specific primer ZFAROM1617F (C) and the 3′ UTR specific primer ZFAROM2540F (d) were used to generate the 3′ RACE products shown.

number AF170273). Sequences for the 3′UTR of aromatase from the injured telencephalon using the aromatase gene-specific primer ZFAROM1617F and ZFAROM2540F demonstrated 99% homology to sequences of aromatase found in the non-injured telencephalon and 98% homology to known aromatase sequences (basepairs 1641–3101; GenBank accession number S75898).

RT-PCR and RACE of injury-induced aromatase

Following microdissection of injured and intact entopallium, we amplified aromatase sequences containing a portion of the 5′ UTR and the coding region using 26, 27 and 28 cycles in a RT-PCR reaction.

We were unable to detect aromatase in the intact entopallium. We were, however, able to detect the requisite *N*-methyl-*D*-aspartate-type glutamate receptor subunit in the intact entopallium were it has been previously shown to be expressed at high levels in the adult zebra finch (Accession Number AY642587; Saldanha *et al.* 2004; Fig. 3). Aromatase was readily detectable in the injured entopallium and the size of the amplicon matched that obtained from whole telencephalon (see Fig. 3b).

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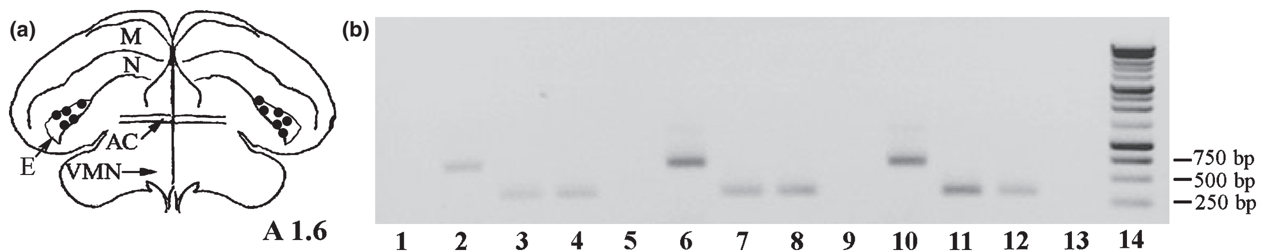

Fig. 3 (a) Diagram depicting tissue samples microdissected from the adult male zebra finch brain when the tip of the needle (·) terminated in the entopallium nucleus. (b) Gel electrophoresis demonstrating the lack of aromatase in the intact entopallium (lanes 1, 5 and 9), the presence of aromatase in the injured entopallium (3, 7 and 11) and whole zebra finch telencephalon (4, 8 and 12) following 26 (1–4), 27 (5–8) and 28 (9–13) cycles of PCR. The presence of the *N*-methyl-*D*-aspartate-type glutamate receptor subunit in intact entopallium (2, 6 and 10) is shown. A no template control and a 1 kb DNA step ladder are shown in lanes 13 and 14 respectively.

Since aromatase was undetectable in the intact entopallium following 28 cycles of RT-PCR using 5′UTR and coding region-specific sequences, we used aromatase-specific primers targeted against the terminal portion of the coding region and the beginning of the 3′UTR to further validate the low to undetectable levels of aromatase in the intact entopallium. For these experiments, we again observed that aromatase was only detectable in the injured entopallium and not in the intact entopallium (data not shown).

Once we demonstrated that different portions of the aromatase transcript were absent in the intact entopallium but present in the injured entopallium following 28 cycles of PCR, we sequenced the aromatase transcript in the injured entopallium using several aromatase-specific primer pairs (Table 1) as well as 5′ and 3′ RACE (Fig. 4). PCR primer-based and vector-based sequencing protocols generated sequences that were 99% homologous to each other. We report sequences from the PCR primer-based protocol because these sequences were replicated by use of both the forward and reverse primer used to generate the original PCR product.

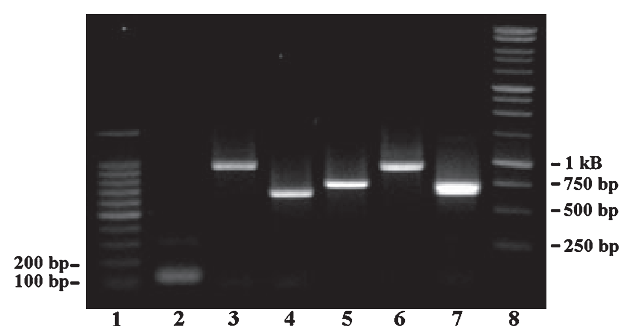

Fig. 4 Gel representing the injury-induced aromatase transcript up-regulated in the injured entopallium. 100 bp DNA step ladder (lane 1), 5′ RACE product using ZFAROM64R (2); 44F-1003R (3), 984F-1643R (4), 1542F-2274R (5), 1884F-2839R (6), 3′ RACE product using ZFAROM2540F (7) and a 1 kB DNA step ladder (8).

Once the entire sequence of the injury-induced aromatase transcript was determined, we used the computer program NCBI BLAST to determine the percent similarity between known aromatase and injury-induced aromatase. We found that the injury-induced aromatase sequence was 99% similar to aromatase cDNA sequences generated from a zebra finch ovarian library containing exon 2–10 of the aromatase transcript (Shen *et al.* 1994; Genbank accession number L81143). Putative Exon 1 in the injury–up-regulated sequence was 99% similar to the clone ZA12/28A for the zebra finch brain-specific exon (Fig. 1 in Ramachandran *et al.* 1999) and 99% similar to a portion of genomic zebra finch DNA contained in BAC TG_Ba-58N4 (Zebra finch genome; accession number AC192095). Interestingly, we were able to find 95% homology to sequences containing Exon 1A from the zebra finch brain (AF170274) and this percentage was the result of a four nucleotide difference between the two sequences. Our sequence did not match with sequence against Exon 1B of the ovarian-specific aromatase transcript (Shen *et al.* 1994; Genebank accession number L81143; Ramachandran *et al.* 1999; Genebank accession number AF170273; see Fig. 5). Taken together these data strongly suggest that the identity of injury–up-regulated aromatase mRNA is highly similar to that expressed in neurons (Ramachandran *et al.* 1999), but distinct from that expressed in the ovary (Shen *et al.* 1994; Ramachandran *et al.* 1999).

Discussion

In the present study, we characterized the aromatase transcript that is induced following injury to the brain in the adult zebra finch. We show that the injury-induced aromatase transcript differs from that constitutively expressed in the ovary. Additionally, we demonstrate that the injury-induced aromatase transcript is the same as aromatase expressed constitutively in neurons of the brain. These data suggest the injury-induced aromatase transcript up-regulated in reactive astrocytes following neural injury does not

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Injury-induced              ------------AACCTATCCAGACCTGGAAGAGGAGCACACCAGGAGCCAGGACAGCAC
Exon 1A ZA12/ZA28A          ---------------------------GTAAGAGGAGCACACCAGGAGCCAGGACAGCAC
TG_Ba-58N4 AC192095         ------------AACCTATCCAGACCTGTAAGAGGAGCACACCAGGAGCCAGGACAGCAC 12206
Exon 1A Genbank AF170274    -----------CAACCTATCCAGACCTGTAAGAGGAGCACACCAGGAGCCAGGA---CAC 402
Exon 1B Genbank AF170273    ATTTGTAGGAACTACAAAGGGGGTGAGAGGTACCAAGCAAAGCTGAACACATCTGAGCTT 703
                                                               **** * * * *  **      *

Injury-induced              TCTCCACCGACAAAAATCCACAAGTAACGAGCAGAC-ACAG
Exon 1A ZA12/ZA28A          TCTCCACCGACAAAAATCCACAAGTAACGAGCAGAC-ACAG
TG_Ba-58N4 AC192095         TCTCCACCGGCAAAAATCCACAAGTAACGAGCAGAC-ACAG 12246
Exon 1A Genbank AF170274    TCTCCACCGACAAAAATCCACAAGTAACGAGCAGAC-ACAG 442
Exon 1B Genbank AF170273    TGACAGCCCCTGCCTCTCTCCCCTCTTCTCTCTCTGAAG 744
                            *  *  **        **  *      *   *   *   **
```

Fig. 5 Clustal alignment of Exon 1 specific sequences from injury-induced aromatase, brain aromatase from the cDNA clones ZA12/ZA28A, genomic DNA contained in the BAC clone TG_Ba-58N4, brain aromatase reported in Genbank and ovarian aromatase. All data presented are zebra finch specific sequences.

represent a novel aromatase transcript in the injured zebra finch brain.

The current data suggest that the injury-induced aromatase transcript up-regulated following neural insult does not contain Exon 1b as found in aromatase sequences that are typically expressed in the ovary (Ramachandran *et al.* 1999). qPCR experiments were unable to detect an increase in the expression of ovarian-specific sequences in the injured relative to the non-injured telencephalon. Results from both 5′ RACE experiments and sequencing of the up-regulated aromatase transcript from injured entopallium confirmed the absence of Exon 1b-containing transcripts in the injured zebra finch brain. Although a low level of Exon 1b is detectable in the zebra finch brain (Ramachandran *et al.* 1999), the abundance of the aromatase transcript containing this particular exon does not increase following brain injury. This is in good agreement with studies demonstrating low to undetectable levels of ovarian-specific aromatase sequences in the intact brain (Honda *et al.* 1996; Tchoudakova and Callard 1998; Ramachandran *et al.* 1999; Vanselow *et al.* 2001; Golovine *et al.* 2003). These data also suggest that the regulation of aromatase gene expression in the ovary and the brain is highly conserved across different vertebrate species.

We hypothesized that the injury-induced aromatase transcript represents a novel mRNA species up-regulated following insult to the brain. This hypothesis is plausible since the aromatase gene is regulated in a cell and tissue-specific manner in several vertebrate species. For instance, the expression of different Exon 1 containing aromatase transcripts has been reported in glioblastoma and neuroblastoma cell lines (Yague *et al.* 2006). Additionally, tissue-specific expression of the aromatase gene is regulated by at least 10 distinct promoters in humans (Simpson *et al.* 1994; Kamat *et al.* 2002; Yague *et al.* 2006) and upon activation, a different 5′ untranslated Exon 1 is spliced to a common Exon 2 (Simpson *et al.* 1993). Six distinct promoters have been identified in the sheep (Vanselow *et al.* 1999) and seven have been discovered in the cow (Furbass *et al.* 1997; Vanselow *et al.* 2001). Multiple promoters have also been reported in the mouse (Honda *et al.* 1996), rabbit (Bouraima *et al.* 2001), monkey (Yamada *et al.* 1995), zebra finch (Ramachandran *et al.* 1999) and rat (Yamada-Mouri *et al.* 1996). Studies in rodents also suggest the presence of alternative splicing of the aromatase transcript in somatic cells, spermatocytes and round spermatids in the 3′UTR (Carreau *et al.* 2004).

Data from our qPCR experiments suggested that the injury-induced aromatase transcript may differ from known aromatase-specific sequences in the 3′UTR, specifically Exon 10. However, these data were not supported by 3′ RACE experiments where no additional aromatase transcript variants were detected in the injured telencephalon. More specifically, when a primer specific to a portion of the coding region (ZFAROM1617F) was used to generate a 3′ RACE product, no additional transcripts were detectable in the injured relative to the non-injured telencephalon. Similar results were found when the 3′ RACE was performed using a primer specific to a portion of the 3′UTR (ZFAROM2540F). Additionally, we did not detect novel sequences in the 3′ UTR of aromatase isolated from the injured entopallium. The results of our qPCR studies are in conflict with those from our 3′RACE studies. However, we are confident of our 3′RACE data and interpretations and suggest that the potentially misleading patterns of data from the qPCR studies may be attributed to high variability associated with the qPCR reaction, small numbers of subjects and/or variability in the molecular characteristics of the primers used (e.g., Tm).

In future experiments that seek to use qPCR methodologies, we suggest slight modifications to the experimental design. In an attempt to constrain the total contribution of total aromatase to total RNA isolated from each sample, we suggest creating a neural injury targeted to an area of the zebra finch brain that contains constitutively expressed neuronal aromatase, perhaps the hippocampus, caudomedial nidopallium or nucleus taeniae (Saldanha *et al.* 2000). Following insult, the injured area would need to be microdissected out as to minimize the variability associated

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with neural insult in each bird studied and then compared to the same area of the brain that has been left intact. This is in contrast to our methodology which extracted total RNA from the entire telencephalic hemisphere. We predict that variability associated with qPCR experiments would decrease and hence, qPCR data may allow us to conclude that the 3′UTR of the aromatase transcript up-regulated following insult is identical to known neural aromatase sequences.

We were unable to detect novel aromatase transcripts up-regulated following injury to the zebra finch brain. Our data suggest that the injury-induced aromatase transcripts are indistinguishable from the aromatase transcripts typically found constitutively expressed in neurons of the brain (Exon 1a; Ramachandran *et al.* 1999). We were unable to detect an aromatase transcript in the intact zebra finch entopallium following 26, 27 and 28 cycles of PCR. These same conditions were used to detect an aromatase transcript in the injured entopallium as well as the requisite subunit for the *N*-methyl-*D*-aspartate-type glutamate receptor in the intact entopallium where it is expressed at high levels in the adult zebra finch (Saldanha *et al.* 2004). We did not detect any additional aromatase transcript species in the injured relative to the non-injured telencephalon when performing 5′ RACE and 3′ RACE experiments. Furthermore, results from qPCR experiments reveal that aromatase sequences harboring Exon 1a increased following insult to the zebra finch brain and may account fully for the observed increase in expression of aromatase. To the best of our knowledge, this is the first report that documents the sequence of inducible aromatase in any vertebrate.

Aromatase expression increases in reactive glia following neural injury in the rodent (Garcia-Segura *et al.* 1999, 2001; Azcoitia *et al.* 2001, 2003) and zebra finch (Peterson *et al.* 2001, 2004; Wynne and Saldanha 2004; Saldanha *et al.* 2005; Wynne *et al.*, 2008). Aromatase expression in glia has also been reported *in vitro* (Schlinger *et al.* 1994; Zwain and Yen 1999; Yague *et al.* 2004, 2006). In the present study, we were unable to identify the specific cell type that up-regulated the aromatase transcript following neural injury. Pure astrocytic cell cultures would be needed to unequivocally determine how similar the sequence of injury-inducible and glial aromatase is in the adult zebra finch brain. To the best our knowledge, techniques to generate pure astrocytic cultures from the adult songbird brain are currently unavailable. Techniques to generate pure astrocytic cultures and thus, the sequence of glial aromatase are currently being explored in our laboratory.

Our data suggest that alternative splicing does not explain the induction of glial aromatase in the zebra finch. Glial aromatase transcription following brain injury may be modulated by an alternative promoter regulated by an injury-specific signal in glia. Alternatively, the transcription of injury-induced glial aromatase may be actively silenced in the intact homeotherm brain. Several silencer elements have been characterized in the human aromatase gene (Wang and Chen 1992; Zhou and Chen 1998; Jin *et al.* 2000) indicating a possibility for such factors to regulate aromatase gene expression in the brain. Future studies will determine the presence or absence of silencing elements or an alternative promoter immediately upstream of the aromatase gene in the zebra finch genome. If sequences representing silencing elements exist, the characterization of the zebra finch protein actively binding and silencing aromatase gene expression in glia would be further characterized. If an alternative promoter regulates the expression of aromatase in glia following neural insult, the cellular factors activating this promoter would be further investigated to determine what it is about neural injury that induces the expression of aromatase in glia following disruption of the neuropil. Whether or not an alternative promoter or such a silencer element exists in the zebra finch aromatase gene remains untested.

The up-regulation of glial aromatase limits the wave of secondary degeneration following insult (Wynne *et al.* 2008) and limits cellular degeneration through decreases in apoptosis in the songbird brain (Wynne and Saldanha 2004). These decreases in neural injury size and apoptosis are modulated in an estrogen dependent manner (Saldanha *et al.* 2005). The ability to activate glial aromatase gene expression immediately following insult to the brain may be critical in supplying damaged neural circuits with high levels of estrogen in a local and highly specific manner without affecting peripheral tissues. This estrogen may function in the repair of damaged circuits and the restoration of lost neural function. Whether glial aromatization functions in repairing damaged neural circuits or in the recovery of function following brain injury remains untested.

In summary, the present data strongly suggest that the transcript up-regulated following neural injury to the zebra finch brain differs from the aromatase transcript constitutively expressed in the ovary. The injury-induced aromatase transcript is identical to constitutively expressed neuronal aromatase in the adult zebra finch brain. We suspect that injury-induced aromatase may be the product of the activation of an alternative promoter that gives rise to a transcript identical in sequence to the form expressed constitutively in neurons. Alternatively, injury-induced aromatase may result from de-repression of the aromatase gene in glia cells mediated by a silencer element that prevents glial aromatase expression in the intact brain. Questions remain as to how physiological changes following neural injury induce the expression of the aromatase gene in glia.

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