

Glial aromatization increases the expression of bone morphogenetic protein-2 in the injured zebra finch brain

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Abstract

In songbirds, brain injury upregulates glial aromatase. The resulting local estrogen synthesis mitigates apoptosis and enhances cytogenesis by poorly understood mechanisms. Bone morphogenetic proteins (BMPs), long studied for their role in neural development, are also neuroprotective and cytogenic in the adult brain. BMPs remain uncharacterized in songbirds, as do the mechanisms regulating their post-injury expression. We first established the expression of BMPs 2, 4, 6, and 7 in the adult zebra finch brain using RT-PCR. Next, we determined the effect of neural insult on BMP expression, by comparing BMP transcripts between injured and uninjured telencephalic hemispheres using semi-quantitative PCR. The expression of BMPs 2 and 4, but not 6 and 7, increased 24 h

post-injury. To determine the influence of aromatase on BMP expression, we compared BMP expression following delivery of the aromatase inhibitor Fadrozole or vehicle into contralateral hemispheres. Fadrozole decreased BMP2, but not BMP4, expression, suggesting that aromatization may induce BMP2 expression following injury. Since BMPs are gliogenic and neurotrophic, future studies will test if the neuroprotective and cytogenic effects of aromatase upregulation are mediated by BMP2. Songbirds may be excellent models towards understanding the role of local estrogen synthesis and its downstream mechanisms on neuroprotection and repair.

Keywords: estrogen, gliosis, growth factors, neuroprotection, stroke.

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Following brain injury, numerous changes occur in the homeotherm brain including the upregulation of the enzyme aromatase (*estrogen synthase*) by reactive astrocytes around the area of injury (Garcia-Segura *et al.* 1999; Peterson *et al.* 2001). This induction of aromatase decreases the area of gliosis (Hoyk *et al.* 2004; Wynne *et al.* 2008a), and apoptotic degeneration (Azcoitia *et al.* 2003; Garcia-Segura *et al.* 2003; Wynne and Saldanha 2004), increases injury-induced cytogenesis and may enhance neural recovery (Garcia-Segura *et al.* 1999; Peterson *et al.* 2007).

Songbirds provide excellent models for studies of aromatase, brain damage, and recovery. First, aromatase is abundantly expressed in several brain regions of every songbird species investigated (Shen *et al.* 1994, 1995; Balthazart *et al.* 1996; Saldanha and Schlinger 1997; Foidart *et al.* 1998; Saldanha *et al.* 1998, 1999, 2000; Soma *et al.* 2003; Peterson *et al.* 2005). Second, many songbird species exhibit high levels of steroid-mediated neuroplasticity throughout life (Nottebohm 2002; Brenowitz 2004; Meitzen *et al.* 2007). Finally, the role of induced aromatase following brain injury is well studied in the zebra finch, *Taeniopygia guttata* (Peterson *et al.* 2001, 2004, 2007; Wynne and Saldanha 2004; Wynne *et al.* 2008a)

In this species, aromatase protein is upregulated as early as 6 h post-insult, appears to be maximal at 24 h, and persists for at least 6 weeks post-injury, (Wynne *et al.* 2008a). The neuroprotective effects of this local aromatase upregulation are profound. Injury induced aromatase decreases the areas of reactive gliosis (Wynne *et al.* 2008a), and degeneration (Wynne and Saldanha 2004; Saldanha *et al.* 2005), and increases cellular proliferation (Lee *et al.* 2007; Peterson *et al.* 2007). Despite these effects however, we do not completely comprehend the precise mechanisms whereby aromatase regulates degeneration and proliferation in any vertebrate.

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Abbreviations used: BMP, bone morphogenetic protein; DEPC, diethylpyrocarbonate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IACUC, institutional animal care and use committee; MCAO, middle cerebral artery occlusion; NCBI, national center for biotechnology information; PBS, phosphate buffered saline; sqPCR, semi-quantitative polymerase chain reaction.

Bone morphogenetic proteins (BMPs) are diffusible factors critical for the development of the nervous system in vertebrates (Liu and Niswander 2005). They are also expressed in the adult brain (Charytoniuk *et al.* 2000; Chen *et al.* 2003; Peretto *et al.* 2004) where they influence cell proliferation, survival, and fate, particularly following brain injury (Wang *et al.* 2001; Chang *et al.* 2002; Brederlau *et al.* 2004; Chou *et al.* 2006; Hampton *et al.* 2007; Colak *et al.* 2008). Specifically, BMPs 2, 4, 6, and 7, are upregulated following brain damage in rodents (Chang *et al.* 2003; Hampton *et al.* 2007; Liu *et al.* 2007), and exogenous administration of either BMP 6 or BMP 7 limits degeneration and increases recovery of behavioral function following middle cerebral artery occlusion (MCAO) (Liu *et al.* 2001; Wang *et al.* 2001; Chang *et al.* 2003; Chou *et al.* 2006). In addition to being neuroprotective, BMPs may also enhance gliogenesis and neurogenesis in the post-injury environment (Chou *et al.* 2006; Xin *et al.* 2006). Despite their potential benefits and their endogenous response to insult, however, the mechanisms that upregulate BMPs after brain injury have yet to be elucidated.

In non-neural tissues, several BMPs have been shown to be responsive to estrogens as well as selective estrogen receptor modulators. Estrogen administration increases the expression of BMPs 2 and 6 (Plant and Tobias 2002; Zhou *et al.* 2003; Zhang *et al.* 2005, 2007; Hertrampf *et al.* 2007) but decrease the expression of BMPs 4 and 7 (Ozkaynak *et al.* 1997; Monroe *et al.* 2000; Min *et al.* 2007). However, we do not as of yet know if local synthesis via aromatization in the brain is capable of regulating BMPs in any vertebrate.

In the zebra finch, injury induced aromatase expression and cytogenesis appear maximal at 24 h post-insult (Lee *et al.* 2007; Wynne *et al.* 2008a). This upregulation is neuroprotective and cytogenic. However, no experiment, to our knowledge, has directly compared injury-induced changes in the zebra finch brain between sexes. Thus, we asked if aromatase influences BMPs 2, 4, 6, or 7 at 24 h after injury in adult males and females. In order to accomplish this we first addressed the question of whether or not these BMPs are expressed in the adult zebra finch brain using RT-PCR. Next, we compared levels of BMP expression between injured and uninjured conditions at 24 h post-insult using semi-quantitative polymerase chain reactions (sqPCR). Lastly, we tested the role of injury induced aromatization on the expression of those BMPs affected by brain damage.

Materials and methods

Subjects

All subjects were adult (> 90 days) zebra finches. All birds were housed and cared for in the Biological Sciences Animal Facility at Lehigh University in accordance with Institutional Animal Care and Use Committee rules and guidelines. Birds were housed in same sex pairs, in wire metal cages (18"×18"×16"), under a 14 : 10

(light:dark) cycle, at a temperature of 20 ± 2°C. Food, grit, water, and cuttlebones were provided *ad libitum*.

Experiment 1. Expression of BMPs in adult zebra finch brain

To determine if BMPs 2, 4, 6, or 7 are expressed in the zebra finch, we performed RT-PCR on total RNA extracted from the brains adult birds, sequenced the resultant cDNAs and compared the obtained sequences to those of BMPs from other species (see Supplemental Fig. S1).

RNA isolation

Birds were decapitated, each telencephalic lobe rapidly dissected out into 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA, USA) and completely homogenized. Total RNA was isolated from the samples using a variant of the method suggested by the manufacturer. Briefly: samples were equilibrated for 10 min following homogenization, and then centrifuged at < 12 000 *g* for 10 min at 4°C. The upper phase (RNA) was collected and treated with 500 µL isopropanol for 15–20 min at room temperature to precipitate RNA which was subsequently pelleted by a < 12 000 *g* spin for 15 min at 4°C. The pellet was then washed with 70% ethanol and reconstituted with diethylpyrocarbonate (DEPC) treated water pre-equilibrated at 50°C.

Three approaches were used to test the quality of the extracted RNA. First, each sample was run on a 1% agarose gel and stained with ethidium bromide to reveal only the 28S and 18S ribosomal RNA bands. Next, we analyzed 1.5 µL of each sample on a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA), and used only those samples that had a 260/280 ratio that exceeded 1.85. Finally, some RNA samples were treated with either RQ1 DNase (Promega, Madison, WI, USA) or RNase A (Qiagen, Valencia, CA, USA) for 30 min prior to reverse transcription and amplification by polymerase (RT-PCR). In all cases, an amplicon was clearly visible in the DNase treated samples, but not in the RNase treated samples, suggesting that the isolation of RNA was effective, with minimal, if any, genomic DNA contamination.

Reverse transcription (RT)

In a 200 µL, thin-walled tube on ice, 1 µg of total RNA from the sample was added to 0.5 µg of oligo(dT)₂₀ (Invitrogen) and DEPC treated water combining for a total volume of 5 µL. The tube was incubated for 5 min at 70°C, 5 min at 4°C, and then put back on ice. The following were then added to the tube: 4 µL of ImProm II 5X RT buffer, 2.5 µL of 25 mM MgCl₂, and 1 µL of ImPromII RT enzyme (Promega) with 1 µL of 10 mM dNTPs and 1 µL of RNase OUT RNase inhibitor (Invitrogen) and 5.5 µL of DEPC treated water. The tube was then incubated at 25°C for 5 min, 42°C for 60 min, and 70°C for 20 min. The sample was cooled to 37°C, at which point we added five units of RNase H (Ambion, Austin, TX, USA) to the tube, and allowed the reaction to continue at 37°C for 20 min.

Amplification

The resulting cDNA was then amplified by polymerase chain reaction (PCR) using the method and reagents outlined in the GoTaq Green Master Mix data sheet (Promega). The primers that were used for these amplifications are listed in Table 1, under the subheading 'Sequencing: PCR'. The primer pairs used to amplify

Table 1 Primer sequences used for amplification and sequencing of bone morphogenetic proteins in the zebra finch

Sequencing: PCR			
Transcript	Forward primer	Reverse primer	
BMP 2	'718F': CGTTAGGATTAGCAGGTCTTT	'1163R': CCTCCACAACCATATCTTGAT	
BMP 4	'4F': GGTAACCGAATGCTGATGGTC	'441R': GCTGCTGAGTTGAAGACGAA	
BMP 6	'845F': GACATGGTCATGAGCTTTGT	'1299R': TTGAAGAAGGCCACCATGAA	
BMP 7	'811F': AACAGCAGCCCTCACGGT	'1121R': AGCACTGAGATGGCATTGAG	
Sequencing: 5' RACE			
Primer name	Primer sequence	Primer name	Primer sequence
BMP4-441R	GCTGCTGAGTTGAAGACGAA	BMP4-275R	GACTGGAGCCGGTAGAGATCC
BMP4-122R	GGGATTAGGCTAGCATGGTTAG		
Semi-quantitative PCR			
Transcript	Forward primer	Reverse primer	Amplicon length
BMP 2	'953F': CTTTACTGCCATGGGGAATG	'1085R': AGTTCTGTCGGCACACAGCA	132 bp
BMP 4	'4F': GGTAACCGAATGCTGATGGTC	'58R': TCCCAGCAGGACTTGGCATA	55 bp
BMP 6	'962F': CAGCTGCTGAGTTTCGGATCT	'1037R': AGATGCTGATAAGGAAGGTTTGGT	76 bp
BMP 7	'884F': AAGCCTGCAAGAAGCACGA	'963R': ATAGCCCTCTGGAGCGATGAT	80 bp

Primers were named for their corresponding locations within known sequences of other species: chicken BMP2 (NM_204358.1), chicken BMP4 (NM_205237.1), mouse BMP6 (NM_007556.2), chicken BMP7 (AF205877.1).

portions of BMPs 4, 6, and 7 correspond to locations that span intron-exon borders in the chicken and mouse genomes (NC_006092, NC_000079, NC_006107, respectively). The BMP 2 primers correspond to areas within the same exon when compared to sequence from the chicken genome (NC_006090). An additional portion of the BMP4 transcript was amplified using a 5'RACE (rapid amplification of cDNA ends) kit (Invitrogen). The primers used for the 5'RACE reactions are also listed in Table 1, under the subheading 'Sequencing: 5'RACE'.

Gel extraction and sequencing

All of the amplified products were run on 1% agarose gels, stained with ethidium bromide, and visualized by ultraviolet light. The products were extracted from the gels using the microcentrifuge protocol and the materials provided with the QIAquick gel extraction kit (Qiagen). The gel extracted products were then sequenced using the ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). These sequences were queried using NCBI basic local alignment search tool and resulting similarities to known cDNA homologues were obtained (Supplemental Fig. S1).

Experiment 2. Effect of injury on BMP expression in adult birds

Surgery

Eight adult birds (four of each sex) were used in these studies. Five to ten minutes prior to surgery, we anesthetized each bird with 0.03 cc of nembutal (sodium pentobarbital 25 mg/mL in 55% ethanol, 25% dH₂O, and 20% propylene glycol, via intramuscular injection with a 0.3 mL syringe/insulin needle; Becton-Dickinson, Franklin Lakes, NJ, USA). We then positioned each subject in a stereotaxic apparatus, with the head angled at 45 degrees, and

exposed the skullcap with a midline incision approximately 1 cm in length. We marked positions at 2 mm anterior and 3 mm lateral to the pineal, where bilateral craniotomies were made sequentially using an 18G needle (Becton-Dickinson). For our treatment, we made a unilateral, penetrating injury by lowering a 22S Hamilton Syringe (Hamilton, Reno, NV, USA) 3 mm ventrally (at a 45° angle) into the entopallium (Stokes *et al.* 1974; Nixdorf and Bischof, unpublished) The needle was maintained in this position for 120 s before being removed, after which the scalp was sealed with Collodion Flexible (EM Science, Gibbstown, NJ, USA), and the birds were allowed to recover under a heat lamp before being returned to the housing conditions described above. We injured right and left hemispheres in equal proportion within each test group, and all surgeries were performed and completed between 1300 and 1600 h to control for photoperiodic effects. 24 h following surgery, we quickly decapitated the birds and dissected out the telencephalic lobes. RNA was isolated and reverse transcribed as described above.

Semi-quantitative PCR

One microlitre of each cDNA sample was then loaded into each of five corresponding wells in a 96 well plate for sqPCR analysis using the ABI 7300 real-time PCR system (Applied Biosystems) with the SYBR green power master mix reagent (Applied Biosystems). Averaged Ct values obtained for the BMP products, each run in triplicate, were normalized against the expression values for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was run in duplicate for each sample. Primers for the sqPCR experiments were designed either manually or by using Primer Express software (Applied Biosystems) and are listed in Table 1 under the subheading 'Semi-quantitative PCR'.

Experiment 3. Effect of aromatase inhibition on BMPs

Surgery

Fourteen adult zebra finches, seven males and seven females, were anesthetized and prepared for surgery as described above, a 22s Hamilton syringe was then lowered into one hemisphere where the needle was allowed to equilibrate for 60 s. Subsequently, 5 μ L of Fadrozole (20 mg/mL in 0.1 M PBS, a gift from Novartis, Summit, NJ, USA) was injected over a period of 2 min, and then the needle was kept in place for 60 more seconds before it was retracted. The needle was then moved to the contralateral hemisphere and lowered similarly for an injection of vehicle (0.1 M PBS). Half of the subjects within each test group received Fadrozole first, the other half received vehicle injection first. Similarly, we counterbalanced which hemisphere received Fadrozole across all subjects. All incisions were sealed with Collodion flexible, and all surgeries were completed between 1300 and 1600 h.

At 24 h post-treatment, we decapitated the birds, and dissected out the telencephalic lobes. RNA was isolated and reverse transcribed as described above. Since only BMP2 and BMP4 were responsive to injury, we chose not to examine BMPs 6 and 7 in this study. The primers used for GAPDH, BMP 2, and BMP 4 were the same for this experiment as those used in the injured versus uninjured experiment (see Table 1, subheading: 'Semi-quantitative PCR' and Wynne *et al.* 2008b).

Statistical analysis

Data from the sqPCR experiments (Experiments 2 and 3) were generated and analyzed in the form of mean delta Ct values. Mean delta Cts were obtained by subtracting the averaged Ct values for the housekeeping gene (GAPDH) from the averaged Ct values for the gene of interest. The mean delta Cts within each experiment were then compared using a two-factor, repeated measures ANOVA to compare the effects of treatment (within subjects variable) and sex on the expression of each BMP. On rare occasions, samples from individual subjects yielded measurements for GAPDH expression that suggested experimental error. As an *a priori* criterion, any subject whose GAPDH Ct values differed by more than 1 cycle between treatments was excluded from the statistical analysis. A total of two subjects were dropped from the experiments. All statistical analyses were conducted using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Experiment 1. BMP expression in adult zebra finch brain

Partial sequences for the mRNA of BMPs 2, 4, 6, and 7 were compiled via shotgun sequencing of cDNA amplified by RT-PCR from total RNA that was extracted from adult zebra finch brain tissue. For each gene product, sequence was generated for both strands of the cDNA using the forward and the reverse primers. Only contiguous stretches of sequence that were 100 per cent complimentary between the two reactions were submitted to GenBank. (Accession numbers: BMP 2, EU420009; BMP 4, EU420010; BMP 6, EU420011; BMP 7, EU420012) To verify that our zebra

finch sequences did, in fact, represent homologues of the BMPs, we next compared our sequences to known or predicted BMP sequences in more commonly used models for vertebrate development: the chicken (*Gallus gallus*), and the mouse (*Mus musculus*) (see Supplemental Fig. S1) Percent similarity scores obtained for partial sequences using NCBI's basic local alignment search tool (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) strongly suggest that BMPs 2, 4, 6, and 7 are conserved into passerine songbirds. Specifically, the BMP2 sequence from zebra finch tissue was 96% similar to that in chicken (NM_204358.1) and 83% similar to that in the mouse (NM_007553.2). BMP4 sequence in the zebra finch was 91% similar to that in chicken (NM_205237.1), and 77% similar to that in the mouse (NM_007554.2). The zebra finch sequence for BMP6 was 91% similar to the predicted sequence for chicken (XM_418956.2), and 79% similar to the known sequence in the mouse (NM_007556.2). For BMP7, the zebra finch sequence was 91% similar to chicken (AF205877.1), and 81% similar to mouse (NM_007557.2). Due to the high similarity for each sequence generated, we conclude that the samples amplified reliably demonstrate the presence of detectable amounts of transcripts for highly conserved homologues to BMPs 2, 4, 6, and 7 in the adult zebra finch brain.

Experiment 2. BMP expression following neural injury

Using semi-quantitative PCR, we compared the relative levels of mRNA expression for BMPs 2, 4, 6, and 7 between injured and uninjured hemispheres within individuals, as well as between sexes. All of the samples demonstrated a single melting curve that corresponded to the predicted melting temperature for the amplicon of interest. Measures of fluorescence (Ct values) for the BMPs were normalized against measures for GAPDH expression, and these delta Ct values were analyzed using a 2-factor repeated measures ANOVA for each BMP examined. None of these comparisons revealed a main effect of sex on BMP expression (BMP 2: $F_{(1,6)} = 0.221$, $p = 0.655$; BMP 4: $F_{(1,5)} = 0.645$, $p = 0.458$; BMP 6: $F_{(1,5)} = 0.558$, $p = 0.478$, and BMP 7: $F_{(1,5)} = 0.229$, $p = 0.652$). There were no significant differences in the expression of BMPs 6 and 7 between injured and uninjured samples at 24 h post-treatment, $F_{(1,5)} = 0.008$, $p = 0.932$ (BMP 6), and $F_{(1,5)} = 1.495$, $p = 0.276$ (BMP 7). There were, however, significant differences between injured and uninjured expression levels for BMP 2, $F_{(1,6)} = 9.063$, $p = 0.024$; 7.046 ± 0.321 (injured) vs. 7.946 ± 0.175 (uninjured), and BMP 4, $F_{(1,5)} = 10.272$, $p = 0.024$; 10.574 ± 0.371 (injured) vs. 12.122 ± 0.224 (uninjured), at the 24 h time point (see Fig. 1). Lastly, there were no significant interactions between injury and sex (BMP 4: $F_{(1,5)} = 1.367$, $p = 0.295$; BMP 6: $F_{(1,5)} = 0.414$, $p = 0.548$; BMP 7: $F_{(1,5)} = 1.267$, $p = 0.311$). However, in the case of BMP 2 there was a trend towards an interaction, $F_{(1,6)} = 0.511$, and

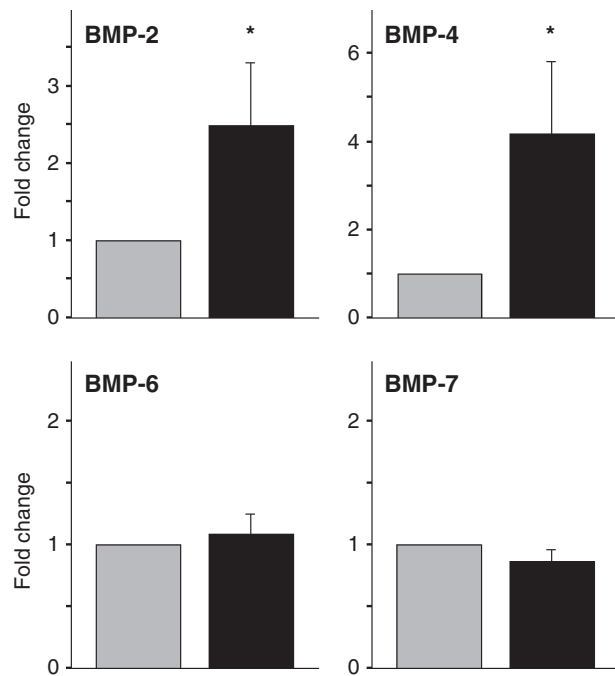


Fig. 1 Transcription of bone morphogenetic proteins (BMPs) 2 and 4 was increased in the injured (black bars) hemisphere 24 h following injury. Expression of BMPs 6 and 7 was not significantly different between injured hemispheres and uninjured (grey) controls at this timepoint. Transcript levels are collapsed across sex resulting in $n = 8$ for BMP-2 and $n = 7$ for BMPs-4, 6, and 7. Fold change in expression was calculated using the double delta Ct method assuming 100% efficiency. The asterisk denotes $p < 0.03$ from the ANOVA conducted on the delta Ct measures (see Results section).

$p = 0.057$, and a qualitative examination of the data suggests that females may show a more dramatic increase in BMP2 expression after injury than males.

Experiment 3. Aromatase inhibition and post-injury BMP expression

Since BMP 2 and 4 transcripts increased in abundance 24 h following injury, a timepoint when aromatase mRNA and protein are also increased (Wynne *et al.* 2008a), we next measured BMP expression in subjects that had received bilateral injuries and a unilateral injection of the aromatase inhibitor Fadrozole. A 2-way, repeated measures ANOVA revealed that BMP 2 expression is decreased in response to Fadrozole treatment in the injured brain of adult zebra finches, $F_{(1,11)} = 10.068, p = 0.009; 7.298 \pm 1.225$ (PBS) vs. 7.809 ± 0.849 (Fadrozole). In contrast, there did not appear to be a difference in the levels of BMP 4 expression as an effect of Fadrozole treatment, $F_{(1,12)} = 0.003, p = 0.957$ (see Fig. 2). Again, there was no effect of sex on BMP 2 expression ($F_{(1,11)} = 0.09, p = 0.769$), nor was there an effect on BMP 4 expression ($F_{(1,12)} = 0.135, p = 0.720$). Also, there was no interaction between sex and Fadrozole

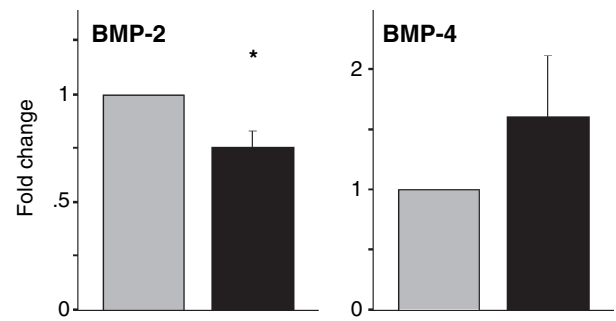


Fig. 2 Inhibition of aromatase by Fadrozole (black bars) significantly decreased post-injury BMP-2 expression, but had no significant effect on BMP-4 expression. Transcript levels are collapsed across sex resulting in $n = 13$ for BMP-2, and $n = 14$ for BMP-4. Fold change in expression was calculated using the double delta Ct method assuming 100% efficiency. The asterisk denotes $p < 0.01$ from the ANOVA conducted on the delta Ct measures (see Results section).

treatment for BMP 4, $F_{(1,12)} = 1.802, p = 0.204$, however, there was a trend in the BMP 2 data and a qualitative examination of the data suggests that females responded more dramatically to the Fadrozole treatment than males, $F_{(1,11)} = 3.827, p = 0.076$.

Discussion

Our results indicate that BMPs 2, 4, 6, and 7 are constitutively synthesized in the brains of both male and female adult zebra finches. We were unable to detect any sex differences in the constitutive or injury-induced levels of any of these BMPs, nor any significant interactions of sex with injury or injury-induced aromatization. BMPs 2 and 4, but not BMPs 6 or 7, were upregulated by injury 24 h post-damage. Though the expression of both BMPs 2 and 4 was increased in response to injury, only BMP 2 expression was significantly affected by the inhibition of induced aromatase around the site of damage.

Comparing the sequences obtained from the zebra finch to known or predicted sequences from other species demonstrated a high degree of similarity for each transcript, suggesting that BMPs 2, 4, 6, and 7 are highly conserved into songbirds (Supplemental Fig. S1). This highly conserved nature implies that these BMPs may be regulated by similar mechanisms and may serve similar functions in the zebra finch as in other species.

In mammalian models, neural injury increases the expression of BMPs 2, 4, 6, and 7. Recently, Hampton *et al.* (2007) reported increases in BMP 2 and/or 4 immunoreactive protein 4 days after a penetrating brain injury in mice. The current findings extend this work to show more specifically that mRNAs for both BMPs 2 and 4 are upregulated in the passerine brain in response to injury, and that this upregulation can occur as early as 24 h post-insult. Liu *et al.* (2007)

have reported an upregulation of BMP 6 mRNA at 7 days following MCAO, while Chang *et al.* (2003) reported BMP 7 mRNA upregulated as early as 8 h. Significant changes in expression levels of these two factors were undetectable in the zebra finch at the 24 h time point. While, it is possible that BMPs 6 and 7 are not responsive to injury in the songbird as they are in mammals, the more parsimonious explanation may be that the effect of injury on BMP 6 and 7 expression is only revealed at time points other than the one examined here. An examination of the extended time course of injury induced BMP expression in the zebra finch would further our ability to address these concerns.

The upregulation of BMPs 2 and 4 at 24 h post-insult in the zebra finch suggests that they may be involved in other processes occurring at this timepoint, namely, gliosis, degeneration and proliferation (Lee *et al.* 2007; Wynne *et al.* 2008a). Findings in rodents support a role for BMPs in these processes, as BMPs 2 and 4 have been shown to promote neuroprotection (Chang *et al.* 2002; Zhang *et al.* 2006), gliosis, and gliogenesis (Xin *et al.* 2006; Fuller *et al.* 2007) in response to mechanical injury or ischemic conditions.

The upregulation of BMPs 2 and 4 at 24 h also suggests that aromatase, which is maximally upregulated in the zebra finch at this time, may be associated with and perhaps influence the post-injury expression of these BMPs. In order to better understand the mechanisms responsible for BMP2 and 4 induction following injury, we examined the response of these factors to treatment with the aromatase inhibitor Fadrozole (Wade *et al.* 1994). Our results indicate that BMP2 expression is dampened by aromatase inhibition following mechanical injury, while an effect on BMP4 was undetectable. These findings suggest that transcription of BMP2 mRNA following injury is upregulated, at least in part, as a response to increases in local aromatization, and possibly estrogen synthesis. The lack of a significant response for BMP 4 may suggest that something other than aromatase, in the post-injury environment, is regulating BMP 4 expression. Since there are many factors that are upregulated following neural injury, as well as several regulational sites in known BMP 2 and 4 promoter sequences (Shore *et al.* 1998; Sugiura 1999; Helvering *et al.* 2000; Kawai and Sugiura 2001; Xu and Rogers 2007), it is reasonable to hypothesize that the expression of these factors following neural injury may be regulated, both positively and negatively, by multiple factors.

Notably, the decrease in BMP 2 expression following Fadrozole treatment was modest in comparison to its upregulation by injury. While injury caused an approximately 2.5-fold increase in BMP 2 mRNA, Fadrozole treatment decreased this amount by only ~ 25%. We suspect that BMP 2 transcription may be regulated by multiple factors in the injured brain, of which, aromatase is only one. However, it is possible that the dose of Fadrozole used, while enough to cause measurable and significant effects, may not

have inhibited all of the induced aromatase activity in the injured telencephalic lobe. In order to better determine the extent to which injury induced aromatase is regulating BMP 2 expression, our lab is presently testing a dose-response curve for Fadrozole treatment and BMP 2 expression. In the zebra finch, aromatase is predominantly upregulated in glia adjacent to the site of injury, while the distribution of BMP 2 expression following injury is still unknown. It is therefore possible that BMP 2 expression immediately around the injury could be aromatase induced, while BMP 2 upregulation at sites distal to the injury by something other than aromatase. Our lab is currently investigating the distribution of BMP 2 expression in the intact and injured songbird brain in order to better understand where and how this regulation may be occurring.

Given that the primary action of aromatase is to convert androgens to estrogens, and that BMP 2 transcription is classically regulated by estrogen in mesenchymal stem cells (Zhou *et al.* 2003), it is likely that the actions of injury induced aromatase on BMP 2 expression are mediated by local estrogen synthesis. However, it is also possible that the inhibition of aromatase may be increasing the local concentration of androgens while it is preventing estrogen synthesis, and, therefore, androgens rather than estrogens may be causing the observed effect. For this reason, future studies in our lab will be aimed at a conclusive determination of whether or not estrogens regulate BMP expression in the adult brain under normal conditions as well as during the response to injury.

As previously mentioned, both neuroprotection and cyto-genesis are occurring in the zebra finch brain at 24 h following injury, but the mechanisms behind these processes are poorly understood. BMP2 may be involved in these mechanisms, as it promotes the survival of embryonic striatal neurons (Hattori *et al.* 1999), and enhances the neuroprotective effects of fetal stem cell grafts in lesioned rats (Espejo *et al.* 1999). Additionally, *in vitro*, BMP 2 promotes both neurogenesis and gliogenesis in cultures of ventral mesencephalic cells (Reiriz *et al.* 1999) and gliogenesis in oxygen and glucose deprived astrocytic cultures (Xin *et al.* 2006). Finally, pre-treatment with noggin, a potent inhibitor of BMP2 may reduce neuroprotection in rats that have undergone MCAO (Chang *et al.* 2002). Whether or not BMP2 is neuroprotective and/or cytogenic in the injured vertebrate brain remains to be tested. Our laboratory is studying the timecourse of BMP 2 expression following brain damage towards understanding the role(s) of BMP2 in neuroprotection and/or neural repair. Additionally, we intend to study the effects of BMP 2 inhibition and over-expression on neuronal survival and cyto-genesis in the injured CNS.

In summary, in the adult zebra finch brain, secondary degeneration around sites of injury is decreased and cyto-genesis is increased by the rapid and robust upregulation of aromatase (Wynne and Saldanha 2004; Peterson *et al.*

2007; Wynne *et al.* 2008a). Taken together, the current findings suggest that several BMPs are expressed in the adult zebra finch brain and that BMPs 2 and 4 are increased following injury. The available data suggest that BMP2 may mediate the effect of injury-induced glial aromatase on neuronal damage, survival, and perhaps repair in the zebra finch.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1 Alignment for Partial Sequence of Bone Morphogenetic Proteins.

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1471-4159.2008.05352.x> (This link will take you to the article abstract).

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