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Inhibition of TrkB Limits Development of the Zebra finch Song System

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Abstract

Large sexual dimorphisms exist in the zebra finch song system. Masculinization may be mediated by both estradiol and expression of one or more Z-genes (males: ZZ; females: ZW). Roles of the Z-gene tyrosine kinase B (TrkB) in HVC in masculinizing both it and its target the robust nucleus of the arcopallium (RA) were tested using siRNA administration in juvenile males at two ages (post-hatching days 15-17 or 25-27). Birds were euthanized 10 days later. Potential interactions or additive effects with estradiol were evaluated by treating males with the estrogen synthesis inhibitor fadrozole. Females treated with estradiol were also exposed to the siRNA at the later age. Local inhibition of TrkB in males of both ages reduced the volume of HVC, an effect due to a change in cell number and not cell size. In the older males, in which the treatment spanned the period when the projection from HVC to RA grows, TrkB inhibition reduced the volume of RA and the relative number of cells within it. TrkB siRNA in HVC decreased the volume of and soma size in the RA of females, and the projection from HVC to RA in both sexes. Estradiol in females masculinized various aspects of the song system, and its effect in masculinizing the volume of RA was decreased by TrkB inhibition. However, effects of fadrozole in males were limited. The data indicate that TrkB is involved in masculinizing the song system, but for most measures it probably does not work in concert with E2.

Keywords

Z-chromosome; sexual differentiation; neurotrophin receptor; brain derived neurotrophic factor; estradiol

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Conflict of Interest

The authors declared no conflict of interest.

1. Introduction

Sex differences in brain and behavior are present across vertebrates, and those in zebra finches are particularly striking. In this species, only males produce song, and the brain areas that control the learning and production of this behavior are more developed in males than females. HVC (proper name; Reiner et al., 2004) and its target the robust nucleus of the arcopallium (RA) are involved in the motor production of song. Each of these regions contains more and larger cells in males compared to females (Wade and Arnold, 2004). Additionally, the projection from HVC into RA only exists in males (Konishi and Akutagawa, 1985). Area X of the striatum and the lateral magnocellular nucleus of the anterior nidopallium (LMAN) are regions critical for song development and plasticity. Area X cannot be detected in the brains of female zebra finches with any of a variety of markers, including nissl staining (Wade, 2001). The morphology of LMAN is relatively similar between the sexes, but its projections are decreased in adult females compared to males (Nordeen et al., 1992).

Steroid hormones modulate development of these sex differences. For example, data from *in vitro* samples indicate a critical role for estradiol (E2) in masculinization. Administration of this hormone to slice preparations of female zebra finch brains facilitates growth of the projection from HVC into RA, while treatment of male slices with the estrogen synthesis (aromatase) inhibitor fadrozole or the estrogen receptor antagonist tamoxifen prevents masculinization of this pathway (Holloway and Clayton, 2001). Treating hatchling females with E2 enhances this projection *in vivo* as well (Simpson and Vicario, 1991). This type of manipulation also masculinizes the morphology of HVC and RA, but only partially (Grisham and Arnold, 1995; Wade, 2001), suggesting that other factors also play important roles. In parallel, limiting E2 availability (Wade et al., 1999; Wade and Arnold, 1994) and action (Mathews et al., 1988; Mathews and Arnold, 1990) in males fail to prevent masculine development.

Sex chromosome genes are strong candidates for additional factors critical to sexual differentiation, as their expression differs in males and females. In birds, males are homogametic (ZZ; females: ZW), and dosage compensation is limited (Itoh et al., 2007). A growing body of work suggests the possibility that increased Z-gene expression is involved in masculinization of the song circuit (Agate et al., 2003; Chen et al., 2005; Tomaszewski et al., 2009). Consistent with this idea, we recently demonstrated that local inhibition of a Z-gene, tubulin specific chaperone protein A (TBCA), in the LMAN of developing songbirds demasculinizes morphology of both this region and its target RA (Beach and Wade, 2015).

Another Z-gene of particular interest is tyrosine kinase B (TrkB). It is the high affinity receptor for brain derived neurotrophic factor (BDNF); both the receptor and its ligand are present in the developing song system, with increased expression in males compared to females (Dittrich et al., 1999; Tang and Wade, 2013, 2012; Wade, 2000). E2 treatment of developing female zebra finches increases BDNF protein in HVC and RA (Tang and Wade, 2012), and its mRNA is sensitive to modulation by E2 during development in both sexes (Dittrich et al., 1999).

BDNF, acting at TrkB, is important for the survival and differentiation of neurons (Huang and Reichardt, 2001), as well as for synapse development and synaptic transmission (Deinhardt and Chao, 2014). For songbirds specifically, BDNF infusion prevents cell death in the RA of juvenile males following removal of pre-synaptic input from LMAN (Johnson et al., 1997). In adult white-crowned sparrows, BDNF in RA mediates seasonal changes in neural structure (Wissman and Brenowitz, 2009). In HVC, the neurotrophin also facilitates song learning in developing male zebra finches (Dittrich et al., 2013).

The present set of studies used siRNA designed against the zebra finch transcript for TrkB to test the hypothesis that TrkB signaling in HVC is involved in masculinization of the morphology of this brain region and of its target RA. In Experiment 1, the siRNA was infused directly into the HVC of males between post-hatching days 15 and 17, and morphology of HVC and RA was analyzed 10 days later, during a period of heightened sexual differentiation. Potential additive or interactive effects of TrkB and E2 were assessed by conducting these siRNA manipulations in males given systemic injections of the estrogen synthesis inhibitor fadrozole beginning on post-hatching day 3. Based on the results of that initial study, Experiment 2 investigated the effects of the same TrkB siRNA manipulation at a later age (days 25-27) in both (A) males and (B) females. As in Experiment 1, some of the males in Experiment 2 were exposed to fadrozole; and E2 was administered to a group of the females. The birds were euthanized 10 days following siRNA treatment. In addition to analyzing characteristics of the individual song control regions, the projection between HVC and RA was evaluated in the second study. Quantification of this pathway was possible in the older birds used for Experiment 2, because this projection grows in males at about 30 days of age (Konishi and Akutagawa, 1985).

2. Results

2.1 Validation of TrkB Antibody and siRNA Manipulation

Prior to conducting the main experiments, two pilot studies were done. In the first, we took additional measures compared to previous immunohistochemical work to validate the primary antibody for TrkB; a preadsorption control was used on a Western blot. We felt this was important for the second step, which was to confirm efficacy of the siRNA manipulation via Western analysis. On the blot used to confirm specificity of the TrkB primary antibody, three bands representing the 145kD full length TrkB receptor, the 95kD truncated TrkB receptor, and an unknown third band of 126kD were recognized. Detection of each of these bands was completely eliminated when the primary antibody was pre-adsorbed with the antigen against which it was raised (Figure 1).

Western blot analyses used to validate the treatment paradigm indicated that the TrkB-specific siRNA significantly decreased relative levels of this protein compared to the control treatment. The full length ($t_7 = 2.05$, $p = 0.040$; Figure 1) and truncated ($t_7 = 2.53$, $p = 0.020$) isoforms were reduced by 32% and 35%, respectively. Analysis of the loading control, actin, alone indicated no effect of the siRNA manipulation ($t_7 = 0.50$, $p = 0.316$), suggesting specificity of the manipulation to reduce TrkB.

2.2 Experiment 1: Males Evaluated at 25-27 Days Post-Hatching

TrkB siRNA significantly reduced cell number within HVC ($F_{1,17} = 6.25$, $p = 0.023$; Figure 2) compared to the control manipulation, which was administered to the other side of the brain. It also decreased the volume of the region ($F_{1,17} = 7.62$, $p = 0.013$). Main effects of fadrozole compared to a saline control were not detected on these two measures, and the two treatment variables (fadrozole and TrkB siRNA) did not interact (all $F_{1,17} < 3.15$, $p > 0.094$). HVC soma size was not affected by TrkB siRNA or fadrozole treatment, and no interaction existed between the variables (all $F_{1,22} < 0.90$, $p > 0.352$).

RA cell number was not affected by TrkB siRNA infusion into HVC or systemic fadrozole treatment, and no interaction existed between the variables (all $F_{1,18} < 3.11$, $p > 0.095$; Figure 3). Main effects of these manipulations also were not detected for the volume of RA (both $F_{1,18} < 1.34$, $p > 0.262$). An interaction between the TrkB siRNA and fadrozole did exist for RA volume ($F_{1,18} = 5.20$, $p = 0.035$). However, pairwise comparisons did not reveal significant effects of siRNA treatment in either the fadrozole- ($t_8 = 1.67$, $p = 0.134$) or saline-treated group ($t_{10} = 1.53$, $p = 0.157$). Similarly, fadrozole did not significantly affect the volume of either the siRNA or control side of the brain (both $t_{18} < 1.68$, $p > 0.110$). A trend for TrkB siRNA to increase soma size in RA was detected ($F_{1,18} = 4.42$, $p = 0.050$). However, fadrozole treatment had no effect on this measure, and no interaction existed between the variables (both $F_{1,17} < 0.21$, $p > 0.655$).

2.3.1 Experiment 2A: Males Evaluated at 35-37 Days Post-Hatching—HVC cell number could not be reliably quantified in a sufficient number of birds in this experiment. We believe this relates to the infusion of 1,1'-Diocadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI) administered following the siRNA or its control for quantification of the projection to RA. This additional microliter of liquid injected created changes in the morphology of HVC that made it difficult to be confident that all neuron-shaped cells of within a section were consistently counted.

The volume of HVC could be easily determined, however, as the borders of the region were clear in a sufficient number of birds to provide a reasonable estimate of relative differences. The size of this region was significantly decreased by local exposure to TrkB siRNA ($F_{1,8} = 6.97$, $p = 0.030$; Figure 4), but no effect of fadrozole ($F_{1,8} = 0.66$, $p = 0.443$) was detected, and the two variables did not interact ($F_{1,8} = 0.03$, $p = 0.874$). In contrast, the siRNA manipulation did not modify soma size within this brain region ($F_{1,8} = 4.67$, $p = 0.063$). There also was no effect of fadrozole ($F_{1,8} = 0.12$, $p = 0.735$) or interaction between the two manipulations ($F_{1,8} = 0.73$, $p = 0.419$).

RA cell number was decreased by TrkB siRNA infusion into HVC ($F_{1,15} = 6.23$, $p = 0.025$; Figure 5), but fadrozole had no effect ($F_{1,15} = 0.004$, $p = 0.947$), and the variables did not interact ($F_{1,15} = 2.04$, $p = 0.174$). In parallel, the volume of RA was decreased by the siRNA manipulation ($F_{1,15} = 18.21$, $p < 0.001$) but not affected by fadrozole ($F_{1,15} = 0.301$, $p = 0.592$), and there was no interaction ($F_{1,15} = 0.297$, $p = 0.594$). Average soma size in RA was not affected by either the siRNA or fadrozole manipulation (all $F_{1,15} < 1.55$, $p > 0.231$).

The projection between HVC and RA, as determined by the relative quantity of anterograde tracer transported, was quantified in two ways. One evaluated the percentage of area covered by DiI in a set of boxes matching those used for females, in which RA is far smaller than in male by this age. The other used larger units for analysis, designed to capture much of the cross-sectional area of the brain region in males. The results were basically the same. Using the first method, there was a main effect of the siRNA manipulation; it decreased labeling in RA ($F_{1,13} = 28.80$, $p < 0.001$; Figure 6). The effect of fadrozole treatment did not quite reach statistical significance ($F_{1,13} = 4.68$, $p = 0.050$), but the variables interacted ($F_{1,13} = 5.38$, $p = 0.037$). Pairwise comparisons indicated a significant decrease due to siRNA treatment within both the fadrozole- ($t_6 = 3.50$, $p = 0.013$) and saline-treated ($t_7 = 4.51$, $p = 0.003$) groups, but the effect was far larger in the control birds. Interestingly, fadrozole decreased the labeling on the control side of the brain ($t_{13} = 2.41$, $p = 0.032$), but not on the side in which TrkB siRNA was injected ($t_{13} = 0.65$, $p = 0.525$). In the analysis of a larger portion of RA, siRNA manipulation also decreased the labeling in RA ($F_{1,13} = 20.78$, $p < 0.001$; not shown). The effect of fadrozole treatment was not statistically significant ($F_{1,13} = 2.88$, $p = 0.114$), and the variables interacted ($F_{1,13} = 6.73$, $p = 0.022$). Pairwise comparisons indicated a significant decrease due to siRNA treatment within the saline-treated group ($t_7 = 4.24$, $p = 0.004$), but not the fadrozole-treated birds ($t_6 = 2.16$, $p = 0.074$). However, the mean difference within the fadrozole-treated birds was similar across the two types of measurements (small box: siRNA/control = 39.8%; large box: 46.8%). As in the analysis of the smaller region, fadrozole decreased the relative quantity of labeling on the control side of males ($t_{13} = 2.18$, $p = 0.048$), but not on the side of the brain that had received the TrkB siRNA ($t_{13} = 0.42$, $p = 0.681$).

2.3.2 Experiment 2B: Females Evaluated at 35-37 Days Post-Hatching—

Estimates of relative cell number in and the volume of HVC could not be determined in these females because the injection impinged substantially on this region, which is very small in females by this age. HVC soma size could be reliably estimated and was not affected by TrkB siRNA ($F_{1,11} = 0.49$, $p = 0.499$). However, E2 significantly increased this measure ($F_{1,11} = 88.72$, $p < 0.001$; not shown). No interaction existed between the variables ($F_{1,11} = 0.021$, $p = 0.889$).

RA cell number was not affected by TrkB siRNA infusion into HVC ($F_{1,13} = 1.71$, $p = 0.214$), but more cells were detected in E2-treated females compared to the control birds ($F_{1,13} = 5.28$, $p = 0.039$; Figures 7 and 8); no interaction existed between the variables ($F_{1,13} = 0.08$, $p = 0.789$). The volume of RA was both decreased by the siRNA manipulation ($F_{1,13} = 16.41$, $p < 0.001$) and increased by E2 treatment ($F_{1,13} = 81.87$, $p < 0.001$). An interaction between the hormone and TrkB manipulations was also detected ($F_{1,13} = 8.19$, $p = 0.013$). Pairwise comparisons indicated that within both the siRNA and its control groups, E2 increased the volume of RA compared to the blank implant (both $t_{13} < 5.36$, $p < 0.001$). More interesting is the result that TrkB siRNA decreased this measure in E2-treated ($t_7 = 3.54$, $p = 0.012$), but not control ($t_7 = 1.53$, $p = 0.170$), females. Average soma size in RA was decreased by the TrkB siRNA treatment ($F_{1,16} = 5.72$, $p = 0.029$) and increased by E2 ($F_{1,16} = 16.84$, $p = 0.001$); these variables did not interact ($F_{1,16} = 0.42$, $p = 0.525$).

Finally, the relative quantity of tracer transported from HVC to RA was significantly decreased by the TrkB siRNA manipulation ($F_{1,12} = 16.87$, $p = 0.001$; Figure 9), but was unaffected by E2-treatment ($F_{1,12} = 0.01$, $p = 0.914$), and the variables did not interact ($F_{1,12} = 0.06$, $p = 0.810$).

3. Discussion

3.1 Summary

Inhibiting TrkB in the HVC of zebra finches over a 10-day period in juvenile development had a variety of demasculinizing effects on both HVC and RA, some of which appeared to depend on the age of this manipulation. Specifically, limiting availability of TrkB in the HVC of males decreased the volume of the brain region, an effect due to a change in the number, but not the size, of the cells. This manipulation in males also had parallel effects on a target region, RA, but only in older birds that received the siRNA treatment during the period that projections grow from HVC to RA (Konishi and Akutagawa, 1985). As in males, volume of the female RA was decreased by exposing HVC to TrkB siRNA for 10 days beginning at 25-27 days of age. However, in this sex, the effect appeared to be due to a decrease in the size, rather than the number, of cells. Across both males and females the projection from HVC to RA was diminished by infusion of TrkB siRNA into HVC. Collectively, the results are consistent with a role for local expression of the Z-gene TrkB in HVC on masculinizing various aspects of song system morphology. This mechanism, along with potential relationships to E2, are discussed below.

3.2 TrkB Appears to Modulate Cell Survival in HVC and Has Downstream Effects on RA

The changes in HVC volume and cell number caused by local TrkB inhibition in males during the two developmental periods investigated here are likely due to effects on cell survival. While males add more cells than females in HVC from approximately day 25 to 45, cell numbers decline in females from day 15 to 30. Particularly relevant to the current set of experiments, cells die in females at rates greater than males at post-hatching days 20 and 35 (Kirn and DeVoogd, 1989). Thus, decreasing TrkB in the HVC of males by administration of siRNA may have demasculinized (or feminized) the pattern of cell survival during this period.

The fact that significant effects of inhibiting TrkB in the HVC of males were detected in RA only after the projection between them develops, indicates the specificity of the local manipulation and, importantly, suggests that activity of this neurotrophic receptor has secondary effects on masculinization of a target region. We were intrigued that administration of TrkB siRNA to the HVC of females also decreased some aspects of RA morphology, as well as the fact that the projection between from HVC to RA was reduced by limiting TrkB in the HVC of both males and females. Collectively these data suggest that, while certainly a large sex difference in innervation exists (Holloway and Clayton, 2001; Simpson and Vicario, 1991), at least some minimal projection is present in females as well as males after one month of age.

It is interesting that inhibiting TrkB in HVC produced somewhat different effects in the RA of males and females. While the volume decreased in both sexes, the change was due to a decrease in cell number in males and cell (soma) size in females. In contrast to HVC, cells are added to RA at similar rates in the two sexes through about post-hatching day 25, after which point they die at higher rates in females, creating a sexual dimorphism in neuron number. The current data suggest that one mechanism facilitating the survival of RA cells in males is TrkB activity in HVC. TrkB is expressed in RA as well during development (Dittrich et al., 1999; Wade, 2000), so certainly local effects are possible as well. These mechanisms warrant further investigation. In addition, the role of TrkB in maintaining cell size in females should be elucidated. As effects of the siRNA manipulation were not detected on RA soma size in males or soma size within the HVC of either sex, it is difficult to make predictions about the biological relevance of this result.

A few studies have investigated TrkB in the developing zebra finch brain, and the results are generally consistent with the possibilities proposed here – that this Z-gene is involved in masculinization of HVC and RA. That is, while studies of TrkB mRNA and protein were conducted at different ages, they suggest that both are expressed in the HVC and RA of developing zebra finches and in some cases to a greater degree in males compared to females (Chen et al., 2005; Dittrich et al., 1999; Johnson et al., 1997; Tang and Wade, 2012; Wade, 2000).

3.3 Effects of Modulating E2 Availability

Our initial hypothesis had been that E2 and TrkB work in concert to contribute to masculinization of the song system. To test this idea in males, we limited exposure to both of these molecules, with the prediction that masculinization would be decreased more by the combination of the treatments than by the TrkB siRNA alone. We tested the idea that TrkB enhances masculinizing effects of E2 by treating females with the hormone while limiting TrkB availability, predicting that inhibition of TrkB would reduce the masculinizing effects of E2.

The present data provide some, but limited, support for these types of mechanisms on basic morphology within HVC or RA. Only one interaction between hormone and TrkB siRNA manipulation was detected on these measures. In females, TrkB siRNA reduced the volume of RA only in E2-treated, but not control, birds. This result is consistent with the hypothesis that TrkB is important for E2's masculinization of RA. While an exciting possibility, caution is warranted, as this may represent a floor effect. The size of RA is already very small in female birds that have not received E2 (see Figure 8).

In males, an interaction between the fadrozole and TrkB siRNA manipulations was detected, such that limiting E2 reduced the projection from HVC to RA only on the control side of the brain and not where TrkB was inhibited in HVC. Similarly, the effect of the TrkB siRNA was far greater in control birds compared to those with lower E2. It therefore appears that the estrogen synthesis inhibitor and TrkB siRNA can each interfere with the effects of the other. The mechanisms associated with these influences remain to be investigated. Regardless, the pattern of the interactions is not consistent with the idea that TrkB and E2 have additive or synergistic effects on masculinization of the projection from HVC to RA.

While all of the anticipated effects of E2 on masculinizing morphology within HVC and RA were detected in females (Wade, 2001; Wade and Arnold, 2004), no main effects of fadrozole treatment were detected in males at either of the two ages investigated. These results parallel other data on fadrozole treatment after hatching, which also produced no significant effects on the morphology of HVC or RA (Wade and Arnold, 1994), although a small but significant *increase* in RA soma size due to the same fadrozole manipulation used here has been detected (Beach and Wade, 2015). The differences across these studies are interesting, and warrant further investigation. Specifically, Wade and Arnold (1994) used only peripheral injections of fadrozole, Beach and Wade (2015) combined fadrozole injections with TBCA siRNA infusion into LMAN, and the current work involved TrkB siRNA in HVC with the systemic fadrozole treatment. A variety of explanations for the differences seen in the TBCA study and the present one are possible. One intriguing idea is that damage to a particular brain area (via the cannulae used to provide siRNA) modifies the response to changes in E2 levels. Similarly, a variety of pieces of evidence suggest differing responses in male and female zebra finches to alterations in E2 availability (Wade, 2016). The mechanisms responsible should be investigated.

The one effect of E2 that we expected to see in females and did not was on increasing the projection from HVC to RA. Simpson and Vicario (1991) produced masculinized innervation of RA from HVC with E2 implants in hatchlings and juveniles. However, unlike the present study, the birds were not analyzed until adulthood, and removal of the E2 in some birds after about two weeks resulted in a lack of detectable innervation. Therefore, it is possible that longer exposure to the hormone is required to masculinize the projections in females. Holloway and Clayton (2001) observed increased innervation following E2 administration to female slice cultures, a treatment that presumably results in more direct exposure to the brain region than a systemic treatment. Our result from males is consistent with the Holloway and Clayton study, however. The data on the innervation of RA from HVC in their cultures of male brains provide one of the few examples of a substantial demasculinizing effect of inhibiting E2 exposure. The present study extended those results to an *in vivo* paradigm, showing a fadrozole-induced decrease in the relative size of this projection on the control size of the brain. While the relationship to the TrkB/BDNF system still remains to be worked out, it appears that E2 is important for the normal masculinization of this pathway in the zebra finch.

3.4 Conclusions

The present work is a first step in documenting a role for TrkB in masculinization of HVC morphology. It also suggests that interactions between this Z-gene and E2 are limited. Some new support for the importance of this hormone in development of the projection from HVC to RA is provided by the current study, but the role of E2 in masculinization of various measures within the two brain regions is still unclear. A variety of questions must now be answered, including the increasing set of inconsistencies in results of altering E2 exposure in males and females, as well as what the relevant ligand is for TrkB. It binds both BDNF and NT-4/5 with high affinity; work in the developing song system has concentrated to date on the former. Finally, it is important to keep in mind that, while a unique step forward, the data here probably represent a small piece of the puzzle on sexual differentiation of the song

system. It is quite likely that other genes, including those on the Z- and female-specific W-chromosome, are important for specific aspects of this process.

4. Experimental Procedures

4.1 Animals

Zebra finches were reared in walk-in aviaries, each of which contained 5-7 adult pairs with their offspring. The birds were kept in a 12:12 light: dark cycle, and provided seed, water, cuttlebone and gravel *ad libitum*. Each week, they were also given oranges, spinach, and bread mixed with hard-boiled chicken eggs. Nest boxes were checked daily, and the day a bird was found was considered post-hatching day 1. New hatchlings were toe-clipped for unique identification, and portions of the removed toes were used to identify the genetic sex of each bird by PCR (Agate et al., 2002).

All procedures were conducted in accordance with the National Institute of Health guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University.

4.2 TrkB Antibody Specificity

Specificity of the TrkB antibody was confirmed using the whole telencephalon of two 25-day-old zebra finches of each sex using Western blot analysis. Protein was extracted from homogenized samples using RIPA Lysis buffer (Santa-Cruz Biotechnology, Dallas TX) according to manufacturer instructions, and 25µg per sample was loaded in duplicate into a 4-20% mini-protean TGX gel (Bio-Rad Laboratories, Hercules CA) along with a Precision Plus Dual Color Standard (Bio-Rad Laboratories). Separated samples were transferred to PVDF membranes (Millipore, Billerica MA) at 4°C. Membranes were then cut vertically so that equivalent sets of samples could be exposed to the primary antibody alone, as well as following preadsorption with the peptide against which it was raised. In this case, a 10-fold excess of the antigen (sc-12 P Santa-Cruz Biotechnology) was incubated with the antibody at 4°C overnight, prior to application to the Western blots. Membranes were also cut horizontally so that the full length (145kD) and truncated (95kD) forms of TrkB, and the actin loading control (43kD), could be simultaneously probed. Detection methods for TrkB and actin were different and optimized for each protein of interest.

For TrkB detection, membranes were treated with the SuperSignal Western Blot Enhancer Kit (Life Technologies, Carlsbad, CA) per manufacturer's instructions. The membranes were first blocked with SuperBlock Blocking Buffer (with 0.1% Tween-20; Life Technologies) for 1 hour at room temperature to eliminate non-specific binding. The TrkB primary antibody (2µg/ml; sc-12 Santa-Cruz Biotechnology), suspended in primary antibody diluent from the SuperSignal Western Blot Enhancer kit, was applied to membranes overnight at 4°C (with or without preadsorption, see above). After PBS rinses, the HRP-linked secondary antibody (1:5000; #7074 Cell Signaling Technology Inc., Danvers, MA) was diluted in SuperBlock Blocking Buffer and applied to the membrane for 1 hour at room temperature. Immunoreactivity was detected by chemiluminescence (SuperSignal West Pico

Chemiluminescent Substrate, Life Technologies) followed by exposure to HyBlot CL autoradiography film (Denville Scientific Inc., Metuchen, NJ).

For actin detection, each membrane was incubated in 5% non-fat milk for 1 hour at room temperature to prevent non-specific binding. The actin primary antibody (1 μ g/ml; sc-1615 Santa-Cruz Biotechnology) was then applied overnight at 4°C. The blots were then incubated in HRP-linked secondary antibody (1 μ g/15ml; sc-2020 Santa-Cruz Biotechnology) for 1 hour at room temperature and chemiluminescence was visualized as above using Clarity Western ECL substrate (Bio-Rad Laboratories).

4.3 Validation of TrkB siRNA Manipulation

siRNAs were generated by Santa-Cruz (sc-270129, Santa Cruz Biotechnology) based on the TrkB mRNA sequence from GenBank (Accession number AY67950.1). This TrkB siRNA is a pool of 3 target-specific sequences composed of 19-25 nucleotides that were designed for maximum knockdown efficacy. Before surgery, 30pmol of the siRNA was transfected into a pellet containing the hemagglutinating virus of Japan envelope (HVJ-E; GenomeONE-Neo EX HVJ-E, Cosmo Bio Co., LTD, Koto-ku, Tokyo, Japan), a replication-incompetent vector developed from the Sendai virus (Lund et al., 2010) per manufacturer's instructions for treatment of laboratory animals. A control sequence (sc-44238, Santa Cruz Biotechnology) was prepared in an identical manner and transfected at the same time. Like other siRNAs, this control sequence activates the RISC complex, but does so without degradation of a target due to lack of complementarity to any known cellular mRNA. This method of siRNA transfection and delivery has been successfully used in developing zebra finches (Beach and Wade, 2015). The viral genome of HVJ-E is inactive, with only the cell membrane fusion properties intact, allowing effective delivery of siRNAs into cells without eliciting a cytotoxic response (Kato et al., 2013).

Juvenile males (n=8) were anaesthetized with isoflurane, and positioned into a stereotaxic instrument (Kopf Instruments, model 900, Tujunga, CA). Injections were unilateral, so that one hemisphere received TrkB siRNA while the contralateral side received the control sequence. Injections were 0.5mm anterior to the bifurcation of the midsagittal sinus (λ), 2.5mm lateral to the midline, and 0.7mm ventral from the surface of the skull. siRNA and the control (1 μ l each) were infused over 5 minutes, at a rate of 0.2 μ l per minute. All animals were returned to their home aviaries until euthanasia.

Animals were euthanized 10 days after the injection by rapid decapitation, and their brains were immediately frozen in cold methyl-butane and stored in -80°C until sectioning. Micropunches of HVC were obtained and processed as in (Tang and Wade, 2012). Briefly, brains were coronally sectioned at 300 μ m and thaw-mounted onto SuperFrost Plus slides. HVC from each side of the brain was removed using a stainless steel cannula and were pooled within side for each animal. Tissue was immediately suspended in cold RIPA lysis buffer (Santa-Cruz Biotechnology) and homogenized. Samples were then centrifuged at 10,000g for 10 minutes at 4°C. The protein supernatants were collected, and a small volume of each sample was used for concentration quantification using the Bio-Rad Protein Assay (Bio-Rad Laboratories). The remainder of each sample was stored at -20°C until Western blot analysis.

Individual samples (25µg) were processed as indicated above in section 4.2. Samples were divided among 3 gels, but the proteins from the two sides of the brain for each individual were always on the same gel so they could be directly compared. Optical densities of individual bands for TrkB (full length and truncated) and actin were quantified using Image J (National Institutes of Health). Background labeling immediately beneath each band of interest was subtracted, and then a ratio of each form of TrkB:actin was calculated for each side of the brain for each individual. One-tailed t-tests were used to evaluate differences between siRNA-treated and control samples.

4.4 Endocrine and siRNA Manipulations

4.4.1 Experiment 1: Males Euthanized at Days 25-27—Beginning on post-hatching day 3, and continuing until the day of euthanasia, each male received an injection of 20µg of fadrozole hydrochloride (n=11; Sigma-Aldrich) in 10µl of 0.75% saline into the breast muscle. This manipulation is highly effective in reducing brain aromatase in developing zebra finches (Wade et al., 1994). Control males received the same volume of 0.75% saline (n=13). Between days 15 and 17, each bird then received an infusion of TrkB siRNA or its control into HVC (details of the treatment paradigm are identical to those described above in section 4.3 for the validation of this manipulation). Birds were returned to their home aviaries after surgery, and rapidly decapitated 10 days later.

4.4.2 Experiment 2A: Males Euthanized at Days 35-37—Each bird received daily fadrozole or control injections (n=12 per treatment group) beginning on day 3 and continuing until euthanasia as described in the section above (4.4.1). However, in this study, the birds received unilateral infusions of the TrkB siRNA or its control into HVC between days 25 to 27 (same procedure as in Experiment 1). Ten minutes following these manipulations, the HVC on each side of the brain received 1 µl of 0.1% DiI (D-282, Molecular Probes, Inc., Eugene, OR) diluted in dimethylsulfoxide. Birds were returned to their home aviaries after surgery, and rapidly decapitated 10 days later.

4.4.3 Experiment 2B: Females Euthanized at Days 35-37—Females received a subcutaneous implant of either 17β-estradiol (Steraloids, Welton, NH) or a blank pellet (n=9 per treatment) on post-hatching day 3 as in Tang and Wade (2009). Hormone implants were produced using a 1:5 mixture E₂ and silicone sealant (Dow Corning, Midland, MI) that was expelled in a line through a 3-cc syringe onto wax paper and dried overnight. The mixture was cut into 1mm lengths and then quartered, so that each implant contained approximately 100 µg of E₂. Control blank pellets were produced identically, except that they did not contain the hormone. At post-hatching days 25-27, the females received the same intracranial infusions of the siRNA and DiI into HVC as the males in Experiment 2A. Birds were returned to their home aviaries after surgery, and rapidly decapitated 10 days later.

4.5 Tissue Collection and Processing for Experimental Animals

Brains were removed and immediately frozen in cold methyl-butane and stored in -80°C. The sex of each bird was confirmed by visual observation of the gonads. Brains were coronally sectioned at 20µm, and thaw-mounted in six series onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh PA). Tissue was stored at -80°C with desiccant until

processing. One series of slides were stained with thionin for confirmation of accurate injections adjacent to HVC and for analysis of morphology in both HVC and its target RA. Animals with injections that were not properly localized to HVC were deleted from the studies. In addition, it was occasionally the case that a variable could not be reliably estimated in some individuals due to tissue damage. Final sample sizes are indicated in the figures, and degrees of freedom are indicated in the text describing the results.

4.6 Quantification of Morphology

Photographs of nissl-stained sections of HVC and RA were captured via Image J (National Institutes of Health) in each section in which it was visible. The image files were coded and flipped as necessary so that hormone treatment and side of brain (siRNA manipulation) were blind to the observer. Cross-sectional areas of HVC and RA were determined by tracing the border of each region on both sides of the brain using Image J in every section that it was visible. Unilateral volumes of each brain region were estimated by multiplying the sum of these areas by the sampling interval (0.12mm). The cell counter plug-in in the software was then used to mark individual cells within the traces of HVC and RA, and these manually counted values across sections were summed to estimate relative cell numbers on each side of the brain within these regions. Cells could not be double-counted, as the sections were 120µm apart. To obtain an estimate of soma size in HVC, the borders of 20 randomly selected cells within three sections evenly spread across the rostro-caudal extent of this region were traced using Image J. Means were obtained from each of the three sections, and those numbers were averaged within side of the brain for each bird. In RA, the methods for estimation of soma size were the same, except that 20 randomly selected cells from every section containing RA were traced for calculation of soma size; this difference is due to the fact that RA spans approximately half the number of tissue sections as HVC.

For analysis of relative strength of the projection from HVC to RA in Experiment 2, DiI was photographed on each side of RA using a TRITC filter. These images were flipped as necessary and coded as described above, so that the experimenter would be not only blind to the endocrine manipulation but also the siRNA exposure on each side of the brain. The percent area covered by DiI labeling in RA was evaluated using Image J (Beach and Wade, 2015; Tang and Wade, 2014) in a square box designed to cover much of the cross-sectional area of the region of both males (296µm × 222µm) and females (100µm × 100µm). In both cases, the regions chosen were from the middle two sections (rostro-caudal) of RA and included the highest density of labeling within the brain region (typically the more dorsal portion). While comparisons cannot be made across the sexes due to differences in endocrine manipulations, we also analyzed the males with the box size used for females, as an additional way of assessing whether the general patterns of the results were parallel. In all cases, the “threshold” function was used to mark the DiI labeling and to quantify the area covered by it within RA. The mean percentage of RA area covered by DiI from each side of each bird was calculated.

4.7 Statistical Analyses

Mixed model ANOVAs were conducted within each experiment to analyze each variable as feasible. Experiment 1 evaluated effects of fadrozole (between individuals) and TrkB siRNA

(within individuals) on the morphology of HVC and RA in males euthanized at 25-27 days of age. Experiment 2A conducted parallel analyses in 35-37 day old males, and Experiment 2B tested the effects of E2 (between animals) and TrkB siRNA (within individuals) in females euthanized at 35-37 days of age.

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Highlights

- Local TrkB knockdown demasculinizes HVC morphology
- TrkB knockdown in HVC inhibits strength of the projection to RA
- TrkB inhibition in HVC demasculinizes RA once projection has grown
- Limited interactions between TrkB and estradiol exist on song system morphology

Figure 1.

Confirmation of TrkB antibody specificity and TrkB siRNA efficacy via Western blot analyses. The set of images in on the left shows the three bands labeled with the TrkB primary antibody in protein homogenized from the whole telencephalon of two individual 25-day-old zebra finches. The lanes in the middle are from duplicate samples on another portion of the same gel, and document their elimination following preadsorption of the antibody with the immunizing peptide. The set of images of the right are representative lanes from a different gel showing reduction in protein extracted from micropunches of the HVC from a 35-day-old male zebra finch. In this pilot study, ten days prior to tissue collection, the control sequence was targeted to HVC in one hemisphere and the TrkB siRNA was injected in the same location on the other side of the brain.

Figure 2.

Effects of TrkB siRNA in HVC and systemic fadrozole treatment in juvenile male zebra finches on morphology of HVC - Experiment 1. The photographs show the two sides of the brain in one nissl-stained coronal section from a saline-treated male. Asterisks mark the injection sites. The upward facing arrows indicate the ventral border of HVC in both images. The graphs show the effects of the manipulations on (A) relative number of cells, (B) volume and (C) soma size within HVC. All values represent the mean $\pm$ SE. Mixed-model ANOVAs were used to analyze the data (TrkB siRNA within subjects; fadrozole between subjects). *indicates a main effect of siRNA treatment; significant main effects of fadrozole and interactions between the two manipulations were not detected. Sample sizes for each treatment group are indicated within the bars. Scale bar = 200μm for both images.

Figure 3.

Effects of TrkB siRNA in HVC and systemic fadrozole treatment in juvenile male zebra finches on morphology of RA – Experiment 1. The graphs show the effects of the manipulations on (A) relative number of cells, (B) volume and (C) soma size within RA. All values represent the mean $\pm$ SE. Mixed-model ANOVAs were used to analyze the data (TrkB siRNA within subjects; fadrozole between subjects). + indicates a trend for a main effect of siRNA treatment; significant main effects of fadrozole and interactions between the two manipulations were not detected. Sample sizes for each treatment group are indicated within the bars.

Figure 4.

Effects of TrkB siRNA in HVC and systemic fadrozole treatment in juvenile male zebra finches on morphology of HVC – Experiment 2A. The histograms show the effects of the manipulations on (A) HVC volume and (B) soma size within the region. All values represent the mean $\pm$ SE. Mixed-model ANOVAs were used to analyze the data (TrkB siRNA within subjects; fadrozole between subjects). *indicates a main effect of siRNA treatment; significant main effects of fadrozole and interactions between the two manipulations were not detected. Sample sizes for each treatment group are indicated within the bars.

Figure 5.

Effects of TrkB siRNA in HVC and systemic fadrozole treatment in juvenile male zebra finches on morphology of RA – Experiment 2A. The photographs document the reduction in overall size of RA due to infusion of the siRNA in HVC on one side of the brain (right) compared to the control manipulation administered to the other side (left). The graphs show the effects of the manipulations on (A) relative number of cells counted, (B) volume and (C) soma size within RA. All values represent the mean $\pm$ SE. Mixed-model ANOVAs were used to analyze the data (TrkB siRNA within subjects; fadrozole between subjects).

*indicates a main effect of siRNA treatment; significant main effects of fadrozole and interactions between the two manipulations were not detected. Sample sizes for each treatment group are indicated within the bars. Scale bar = 200μm for both photographs.

Figure 6.

Effects of TrkB siRNA in HVC and systemic fadrozole treatment in juvenile male zebra finches on the projection from HVC to RA – Experiment 2A. The photographs depict DiI in RA on both sides of the brain of two males, one treated with saline and one who received the estrogen synthesis inhibitor. These images represent the portion of RA that was quantified in the analysis of the smaller areas, those that matched the quantification in females (Figure 9). In the histogram, all values represent the mean $\pm$ SE. Mixed-model ANOVAs were used to analyze the data (TrkB siRNA within subjects; fadrozole between subjects). *indicates a

main effect of siRNA treatment, and the # is intended to highlight that this effect is largely due to a decrease in saline-treated birds. While the effect of fadrozole did not quite reach statistical significance in this analysis ($p = 0.05$), an interaction was detected such that fadrozole only decreased the labeling on the control side of the brain and not where the TrkB siRNA was infused. Sample sizes for each treatment group are indicated within the bars.

Figure 7.

RA in juvenile females treated with either estradiol (E2) or a control capsule – Experiment 2B. Each of these two birds received TrkB siRNA in the HVC on one side of its brain, and the control infusion on the other. Insets show effects of these manipulations on soma size. Scale bars on the bottom right indicate 200 μ m for the larger photos and 20 μ m for the insets.

Figure 8.

Quantification of RA morphology in juvenile females that received TrkB siRNA in HVC and systemic estradiol – Experiment 2B. The graphs show data on the (A) relative number of cells counted, (B) volume and (C) soma size within RA. All values represent the mean $\pm$ SE. Mixed-model ANOVAs were used to analyze the data (TrkB siRNA within subjects; fadrozole between subjects). *indicates a main effect of siRNA treatment, and different letters (a vs. b) denote a main effect of hormone treatment. A significant interaction between the variables was detected for (B) volume, and the NS's indicate the one pairwise comparison that was not statistically significant. Sample sizes for each treatment group are indicated within the bars.

Figure 9.

Effects of TrkB siRNA in HVC and systemic estradiol treatment in juvenile female zebra finches on the projection from HVC to RA – Experiment 2B. The larger photographs are from the two sides of the brain of a female that received a blank implant. The images on the top are from a nissl-stained section and show where the cannulae penetrated HVC. The second set of large images depicts the Dil injections in an adjacent section from this bird. The small images below show the labeling detected in RA (scale bar here = 20μm) from an E2-treated individual. In the histogram, all values represent the mean ± SE. Mixed-model

ANOVAs were used to analyze the data (TrkB siRNA within subjects; fadrozole between subjects). * indicates a main effect of siRNA treatment. Sample sizes for each treatment group are indicated within the bars.

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