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Research report

Conserved role of *Drosophila melanogaster* FoxP in motor coordination and courtship song

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HIGHLIGHTS

- The *Drosophila* FoxP2 homolog (FoxP) is important for motor coordination.
- FoxP is expressed in the insect brain region homologous to the basal ganglia.
- FoxP exhibits sex-specific effects on behavior.

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ABSTRACT

FoxP2 is a highly conserved vertebrate transcription factor known for its importance in human speech and language production. Disruption of FoxP2 in several vertebrate models indicates a conserved functional role for this gene in both sound production and motor coordination. Although FoxP2 is known to be strongly expressed in brain regions important for motor coordination, little is known about FoxP2's role in the nervous system. The recent discovery of the well-conserved *Drosophila melanogaster* homolog, *FoxP*, provides an opportunity to study the role of this crucial gene in an invertebrate model. We hypothesized that, like FoxP2, *Drosophila* FoxP is important for behaviors requiring fine motor coordination. We used targeted RNA interference to reduce expression of FoxP and assayed the effects on a variety of adult behaviors. Male flies with reduced FoxP expression exhibit decreased levels of courtship behavior, altered pulse-song structure, and sex-specific motor impairments in walking and flight. Acute disruption of synaptic activity in FoxP expressing neurons using a temperature-sensitive *shibire* allele dramatically impaired motor coordination. Utilizing a GFP reporter to visualize FoxP in the fly brain reveals expression in relatively few neurons in distributed clusters within the larval and adult CNS, including distinct labeling of the adult protocerebral bridge – a section of the insect central complex known to be important for motor coordination and thought to be homologous to areas of the vertebrate basal ganglia. Our results establish the necessity of this gene in motor coordination in an invertebrate model and suggest a functional homology with vertebrate FoxP2.

1. Introduction

FoxP2 is a vertebrate transcription factor best known for its importance in speech and language production in humans. Its role in human behavior was originally discovered in a multigenerational family whose affected members have a severe speech and language disorder throughout life, and underlying this deficit is a single point mutation in the DNA binding domain of FOXP2 [1]. Since this discovery, independent mutations and truncations of FoxP2 have been linked to disorders with specific impairment in production of fluent speech [1–3]. Across vertebrate models, FoxP2 is remarkably well conserved, both in amino acid sequence and brain expression patterns [4–6].

FoxP2 effects on vocal production are not unique to humans. As a parallel to learned human speech, knockdown of FoxP2 in male zebra finch chicks during the critical song learning period significantly alters the structure of their crystallized adult song [7]. This result closely resembles impairments seen in humans, indicating that FoxP2 may play a conserved functional role in vocal production. In mice, a variety of *FoxP2* mutations and deletions have demonstrated effects on development and behavior. FoxP2

Abbreviations: CI, courtship index; CL, copulation latency; IPI, inter-pulse-interval; FI, flight index; DAMS, Drosophila automated monitoring system; CX, central complex; PB, protocerebral bridge.

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null mice are developmentally delayed and die within 3 weeks of birth, indicating a crucial role of FoxP2 in early postnatal life [8–10]. In contrast, mice heterozygous for functional FoxP2 were developmentally normal but exhibited a variety of other deficits, such as a reduction in the amplitude of ultrasonic vocalizations [11], abnormal synaptic plasticity, and deficits in motor skill learning [8,12,13]. From this variety of work in vertebrates, it is suggested that FoxP2 plays a role in fine motor control, which may have provided a neural substrate for development of complex vocalizations such as language [14]. Despite these insights into the potentially conserved role of FoxP2 in sound production and fine motor control, the precise function of this gene remains poorly understood.

Recently, a gene in the fruit fly *Drosophila melanogaster* was identified as a closely related homolog to the vertebrate FoxP subfamily [15]. This *Drosophila* FoxP is highly similar in sequence to the vertebrate FoxP2 [16], and is highly expressed in the nervous system [15,17,18]. The discovery of this invertebrate homolog in a genetically tractable organism such as the fly provides new possibilities for functional analysis and understanding of the evolutionary importance of the *FoxP2* gene. Sound production as a means of social communication is crucial to many species of invertebrates. Like many insects, male fruit flies produce an acoustic signal in the form of a courtship song. In the presence of a virgin female, a male will initiate a sequence of courtship behaviors including a unilateral wing vibration to produce a pulse song with a precise species-specific inter-pulse-interval (IPI) [19]. Both the courtship sequence and pulse song are highly stereotyped and easily quantifiable [19]. Based on evidence in vertebrates, we hypothesized that the *Drosophila FoxP* gene is also important for courtship song production and fine motor control in the fly. Therefore we sought to characterize the behavioral role and expression pattern of this gene in fruit flies in order to better understand the development of fine motor circuits in insects and ultimately identify the potentially conserved developmental and molecular roles of FoxP2 across organisms.

Using RNA interference (RNAi) to knockdown FoxP levels, we studied the behavioral effects of reduced FoxP in the context of courtship, locomotion, and flight behaviors. We found deficits in all of these behaviors in adults, with males more strongly affected than females in these assays. In parallel, we generated a FoxP antibody and a FoxP-Gal4 line which, when combined with two different UAS-GFP lines, allowed for visualization of the expression pattern of FoxP in the larval and adult CNS. These molecular tools revealed that FoxP is limited to a relatively small subset of neurons in the brain and ventral ganglion, which appear in several distinct clusters throughout. Particularly strong expression was evident in the protocerebral bridge, part of the central complex, which is thought to be involved in sensory-motor integration [21], and has been compared to the vertebrate basal ganglia [22]. When we used our *FoxP-Gal4* with a conditional temperature sensitive *UAS-shibire* line to transiently disrupt neurotransmission in FoxP expressing neurons in adults, we observed dramatic effects on motor coordination. Our results provide the first functional characterization of FoxP2 in invertebrates and suggest an intriguing homology with this crucial human speech and language gene.

2. Materials and methods

2.1. Animals

Drosophila melanogaster fruit flies were maintained at room temperature (23–25 °C) or in a 29 °C incubator on standard yeast and glucose media. We reduced FoxP mRNA expression by crossing GAL4 driver lines with a UAS-RNAi construct specific to FoxP: UAS-*FoxP*IR (15732) from the Vienna *Drosophila* Stock Center [23]. Two different GAL4 drivers were crossed to the UAS-RNAi line, including the pan-neural elav-GAL4 (FBst0000458) and the ubiquitously expressed Act5c-GAL4 (FBst0003954). The two GAL4 drivers were also crossed with the w^{1118}RNAi parental strain (6000, VDRC) as additional controls. For conditional knockdown experiments in adults we recombined UAS-*FoxP*IR (15732) with a temperature sensitive $GAL80^{ts}$ line (FBst0007108), which blocks Gal4 activity at room temperature. The resulting w;$GAL80^{ts}$;*FoxP*IR line was then crossed with either C155 or Act5c GAL4 drivers.

We also generated a GAL4 line driven by the putative FoxP promoter (FoxP-Gal4), and recombined it with UAS-CD8::GFP (FBst0005130) and UAS-nls::GFP (FBst0004776) for visualizing the pattern of FoxP protein expression in the CNS. The FoxP-Gal4 was also recombined with the temperature sensitive UAS-Shi^{ts1} line (FBst0044222) to selectively disrupt FoxP neuron function.

2.2. RNAi efficacy

RNAi knockdown was assessed using RT-PCR and FoxP antibody staining. RNA was extracted from 30 adult fly heads using Trizol (Invitrogen, Carlsbad, CA, USA), and 1 μg of RNA was reverse transcribed using Superscript III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. FoxP cDNA was amplified by PCR using the primers 5′-CCCATCCGACAAACAAATTC-3′ and 5′-TCACATTCTCAACCCGCATA-3′, Failsafe (Epicentre, Madison, WI, USA) with Buffer D, and the following program: 2 min at 94 °C for 1 cycle; 15 s at 94 °C, 15 s at 47 °C, 45 s at 72 °C for 35 cycles; 5 min at 72 °C for 1 cycle. The ribosomal marker Rp49 (Primers 5′-AAG-ATG-ACC-ATC-CGC-CCA-GCA-3′ and 5′-CCC-TTG-AAG-CGG-CGA-CGC-3′) was used as a control and all PCR products were separated by gel electrophoresis for identification.

2.3. Antibody production

The polyclonal FoxP antibody was raised against a Maltose Binding Protein-FoxP fusion. The fusion was generated by inserting a 462 bp (154aa) PCR product from the FoxP coding region into the pMAL-c5X vector (New England Biolabs, Ipswich, MA, USA). The fusion protein was injected into a guinea pig to produce the FoxP antibody (Pocono Rabbit Farm, Canadensis, PA, USA). The resulting antiserum was affinity purified as described in [24]. The region used is not conserved with vertebrate FoxP2, but is common to both A and B isoforms of FoxP. Primers used were 5′-atgcatcggatacatgacgacgagtattc-3′ and 5′-gagttcgccatgcggaagTactat-3′.

2.4. Transgenic fly generation

For creation of the FoxP-Gal4 line, a 1.5 kb fragment of genomic DNA upstream of the FoxP gene (Primers: 5′-CCGGATCCTGT-TTTTAAAACTGAAATTTATAATCATTACCATTG-3′ and 5′-CCGGTACC-GCCTTAGGATGCATTTTGAAAC-3′ containing the BamHI and KpnI restriction sites, respectively) was used as the putative promoter region and amplified by PCR using DyNAzyme EXT DNA polymerase (New England Biolabs, Ipswich, MA, USA), which was then cloned into the pGaTB GAL4 vector. The resulting construct of the FoxP promoter and GAL4 was subcloned into the pCaSpeR2 P-element transformation vector. This construct was injected into embryos (Bestgene, Chino Hills, CA, USA), and transformants were selected based upon CNS fluorescence expression when combined with UAS-CD8::GFP.

2.5. Behavior

For all adult behavior experiments, flies were anesthetized with CO_2 and separated by genotype and gender within a few hours