The *Drosophila FoxP* gene is necessary for operant self-learning: Implications for the evolutionary origins of language

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1. Abstract

In humans, mutations of the transcription factor Forkhead box protein P2 (FoxP2) cause a severe speech and language disorder. Downregulating the Zebrafinch FoxP2 orthologue in development results in incomplete and inaccurate song imitation. These forms of vocal learning exhibit two common characteristics: 1. Spontaneous initiation of behavior ('trying out'); 2. Evaluation of sensory feedback shaping behavior. Using a torque learning essay in which both characteristics have been realized, we investigated the involvement of the fly orthologue, FoxP, in operant self-learning in the fruit fly *Drosophila*. The experiments were performed using stationary flying *Drosophila* at the torque compensator with heat as punishment. Both a P-Element insertion and RNAi-mediated knockdown of the isoform B of the *Drosophila* FoxP gene did not lead to alterations of the gross brain anatomy, nor to an impairment in operant world-learning, i.e., color-learning, compared to control flies. However, both fly strains were impaired in operant self-learning, i.e., yaw-torque learning without any environmental predictors. Neither the FoxP intron retention isoform nor isoform A appear to be involved in this form of learning. These results suggest a specific involvement of isoform B of the *Drosophila* FoxP gene in the neural plasticity underlying operant self-learning but not in other forms of learning. To investigate the effects of RNAi knockdown and P-Element insertion on FoxP abundance and localization in the fly central nervous system, we have generated polyclonal chicken antibodies against four different regions of the putative FoxP protein.

Perhaps not surprisingly, these results are consistent with the hypothesis that one of the evolutionary roots oflanguage is the ability to directly modify the neural circuits controlling behavior. It is noteworthy that these roots can apparently be traced back to the Ur-bilaterian, the last common ancestor of vertebrates and invertebrates.

2. The FoxP gene family tree

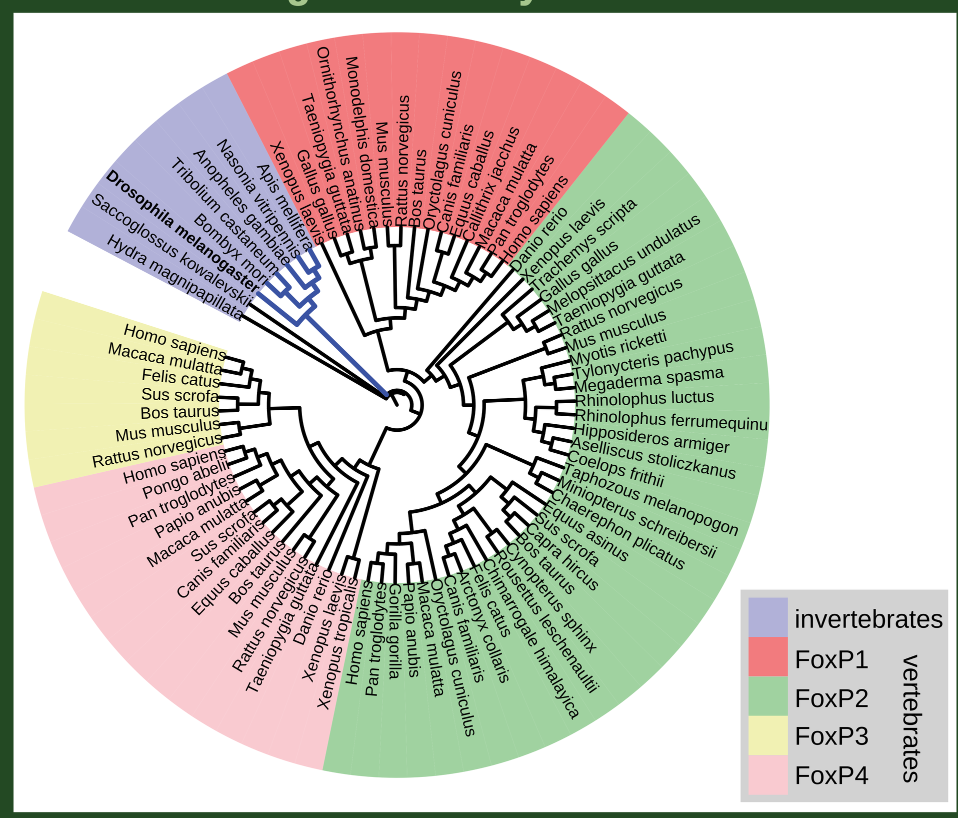

Fig. 1: ***The insect FoxP orthologues suggest the ancestral form***
The bilaterian FoxP gene family arose from a single FoxP gene. The ancestral variant, conserved in the invertebrate lineage, later underwent two subsequent duplications, leading to the four vertebrate genes, FoxP1, FoxP2, FoxP3 and FoxP4.

3. The *Drosophila* FoxP gene locus

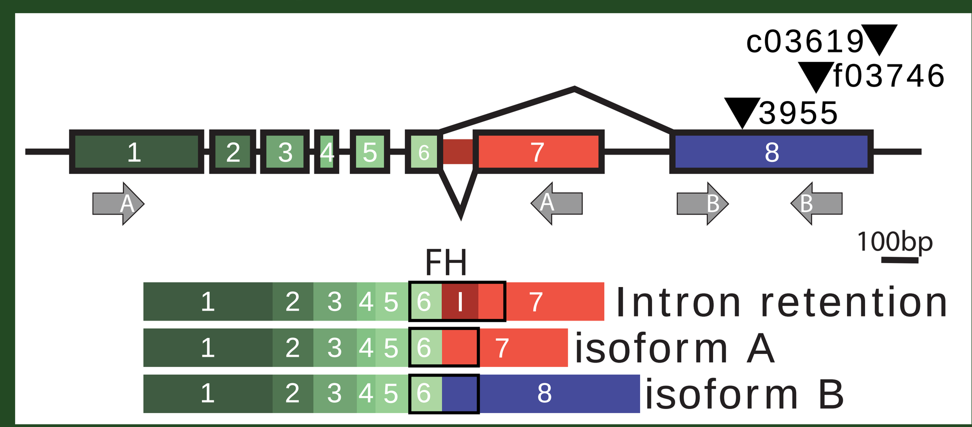

Fig. 2: ***The Drosophila FoxP gene locus and putative isoform mRNA structure.***
Triangles indicate insertions, grey arrows indicate the two (A, B) primer pairs used in our rtPCR. FH - Forkhead Box.

4. Characterizing three insertion lines

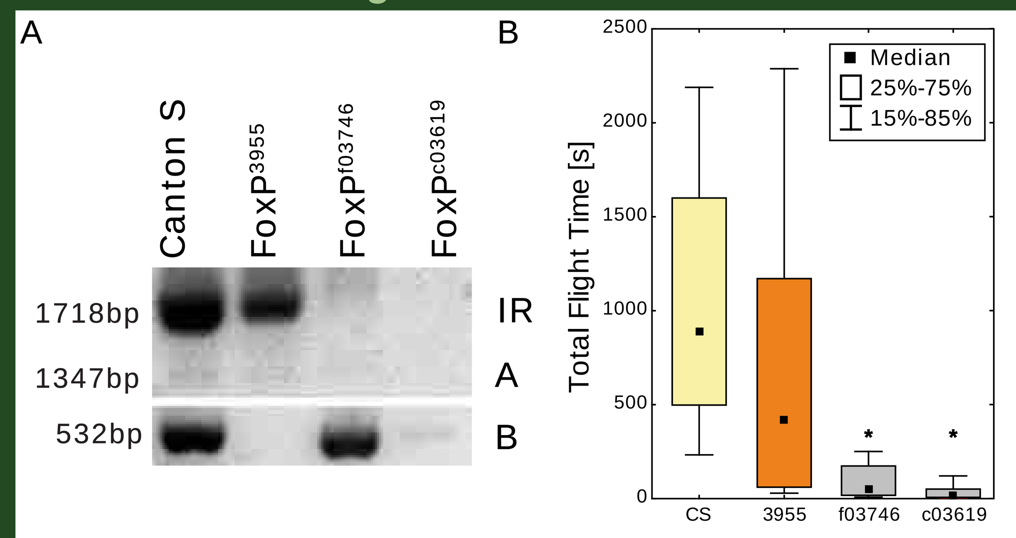

Fig. 3: ***The three insertion mutants differ in isoform expression patterns and only one insetion line shows normal flight performance.***
A - rtPCR results using the primers as described in Fig. 2. The three lines show marked differences in the expression patterns of the three isoforms. B - Flight performace tests show that only line 3955 is suitable for behavioral experiments at the torque meter. Number of animals: CS: 18, 3955: 30, f03746: 34, c03619: 37

5. PKC activity is required specifically for self-learning

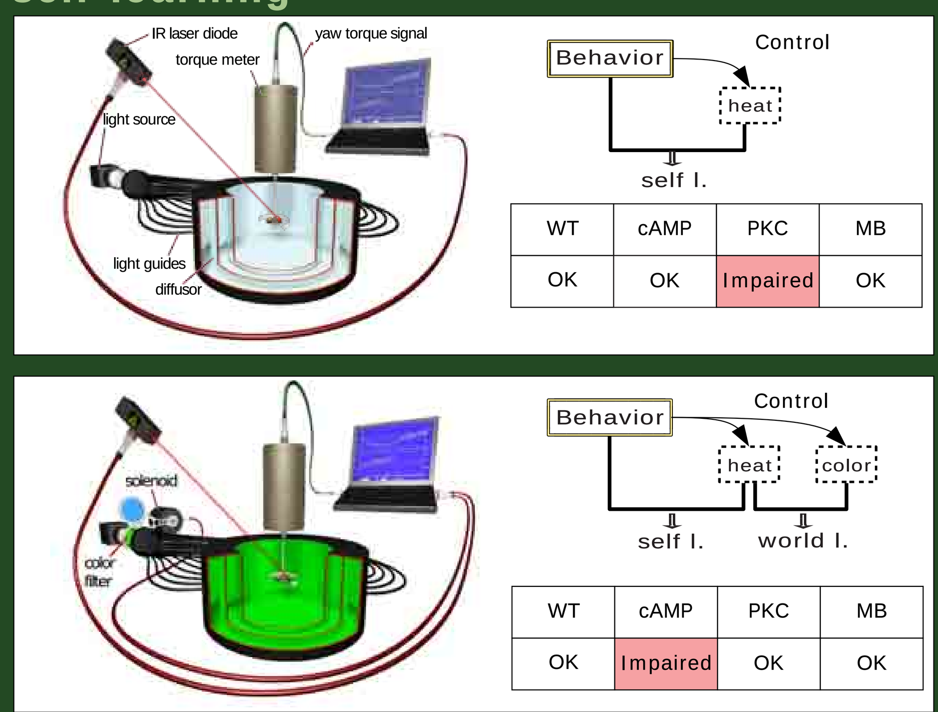

Fig. 4: ***Two operant conditioning experiments, distinguished by the presence or absence of predictive stimuli.***
Above: Flies learn to avoid the heat by trying out different behavioral programs and evaluating the resulting sensory feedback. No sensory predictors are present. Manipulating PKC activity, but not cAMP levels abolishes learning in this task. Below: Adding predictive color stimuli allows the animal to also learn which colors are predicting the heat punishment. Manipulating cAMP levels abolishes learning in this task, while reducing PKC activity has no effect. *Brembs & Plendl, Curr. Biol. 2008*

6. Insertion 3955 in the FoxP gene affects self-learning

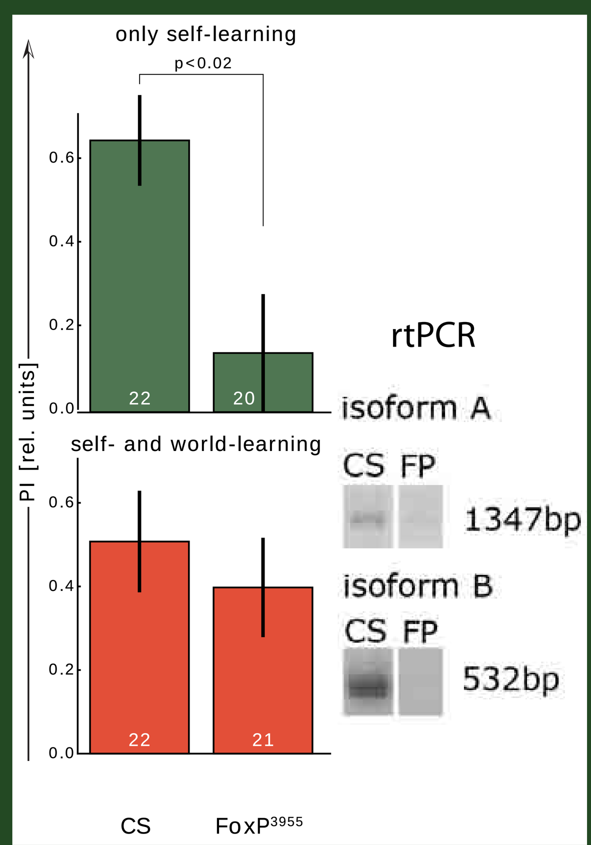

Fig. 5: ***FoxP function dissociates between self- and world-learning.***
Canton S wild-type flies perform well in both learning situations, whereas a FoxP insertion mutant line (3955) how significantly reduced learning scores specifically in the self-learning task.
Reverse transcriptase PCR shows that the insertion affects both FoxP isoforms, but while small amounts of isoform A can still be detected, isoform B appears to be entirely absent

7. *Drosophila* FoxP isoform B is required for self-learning

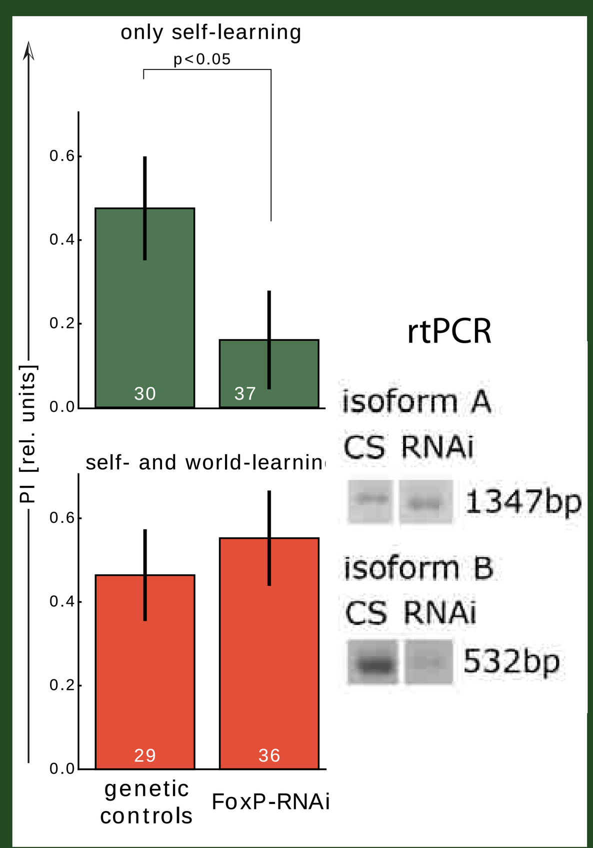

Fig. 6: ***Self-learning requires isoform B.***
Targeting isoform B with with an RNAi construct directed against the last exon of the FoxP gene yields a phenocopy of the FoxP[3955] insertion: self-learning is abolished, while world-learning is unaffected.

8. FoxP protein expression

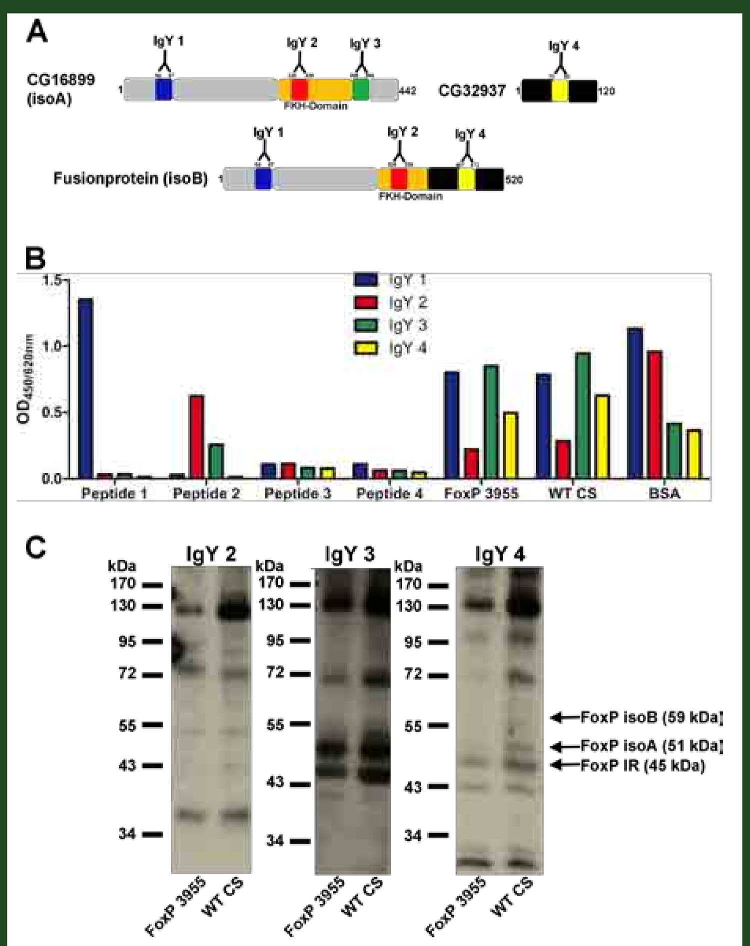

Fig. 7: ***Raising polyclonal chicken IgY antibodies against Drosophila FoxP protein.***
A Peptide regions used for BSA-conjugates to immunize chicken. Peptide 1 (IgY1), peptide 2 (IgY2) and peptide 3 (IgY) are sequences of CG16899 (isoform A) and peptide 4 is located in CG32937. All IgY except IgY 3 could bind to a putative fusionprotein of CG16899 and CG32937 (isoform B). **B** Indirect ELISA-Titer after eight boosts. Only IgY 1 and IgY 2 specficially detect their peptide. All IgY bind to extracts of *Drosophila* heads from FoxP[3955] or wildtype Canton S. The detection of BSA is shown as a positive control. **C** Immunoblot using IgY2, IgY3 and IgY4 binding to head extracts from FoxP[3955] or wildtype Canton S. Different polyclonal antibodies show different positive protein bands.

9. No obvious brain defects in FoxP[3955] mutants

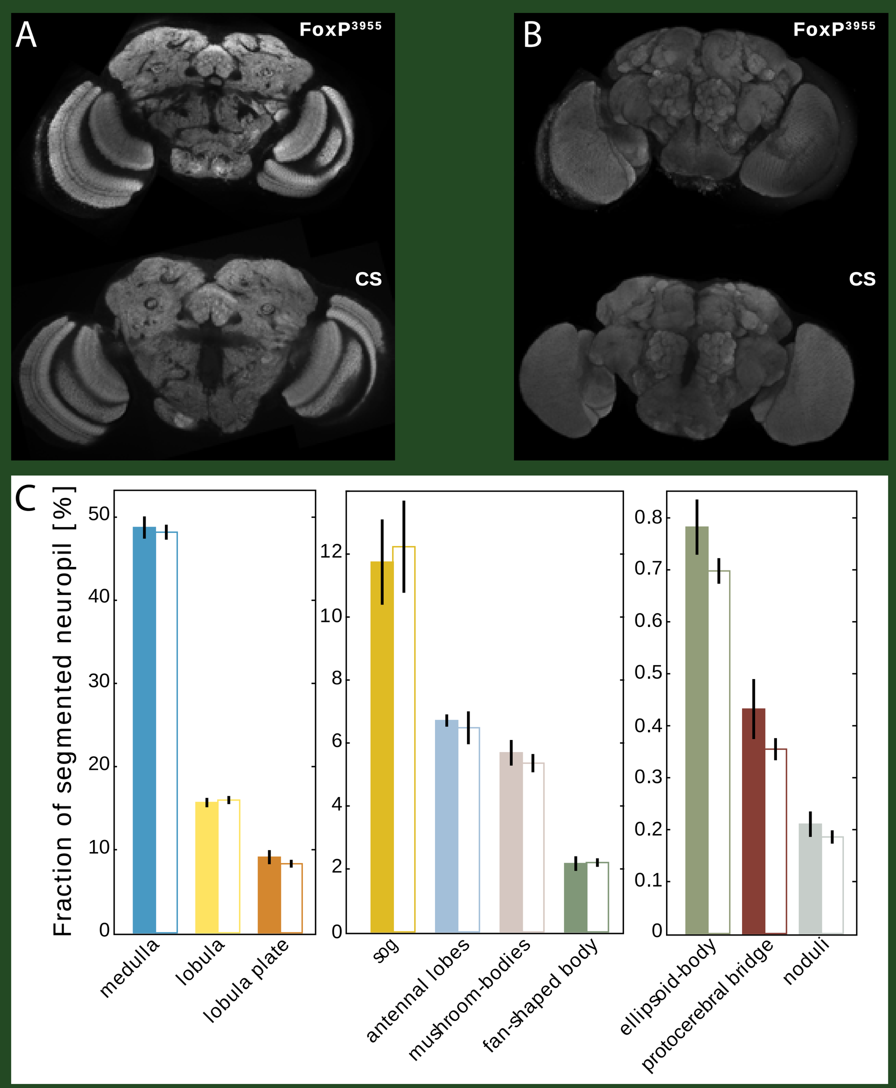

Fig. 8: *Neither qualitative nor quantitative anatomical comparison reveals nany major differences between wildtype CS and FoxP mutant brains.*
A - Frontal sections of one typical wildtype and mutant fly brain, respectively. B - Volume rendering of a wildtype and a mutant fly brain. C - Quantitative study comparing the relative volumes of ten registered neuropil regions. Number of animals: FoxP: 7; CS: 5.

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