Identification of FoxP circuits involved in locomotion and object fixation in Drosophila

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The FoxP family of transcription factors is necessary for operant self-learning, an evolutionary conserved form of motor learning. The expression pattern, molecular function and mechanisms of action of the Drosophila FoxP orthologue remain to be elucidated. By editing the genomic locus of FoxP with CRISPR/Cas9, we find that the three different FoxP isoforms are expressed in neurons, but not in glia and that not all neurons express all isoforms. Furthermore, we detect FoxP expression in, e.g. the protocerebral bridge, the fan-shaped body and in motor neurons, but not in the mushroom bodies. Finally, we discover that FoxP expression during development, but not adulthood, is required for normal locomotion and landmark fixation in walking flies. While FoxP expression in the protocerebral bridge and motor neurons is involved in locomotion and landmark fixation, the FoxP gene can be excised from dorsal cluster neurons and mushroom-body Kenyon cells without affecting these behaviours.

1. Introduction

The family of Forkhead Box (Fox) genes comprises a large number of transcription factors that share the evolutionary conserved forkhead/winged-helix DNA-binding domain [1]. In mammals, the FoxP subfamily (FoxP1–4) members [2] are abundantly expressed during the development of multiple cell types, such as cardiomyocytes, neurons, lung epithelial secretory cells and T-cells [3]. In particular, FoxP1 and FoxP2 have generated interest because of their roles in regulating the development of cognitive processes such as speech and language acquisition [4–13].

Humans with FOXP1 deletions present with mild mental retardation, delayed onset of walking, gross motor impairments and significant language and speech deficits [8]. Mutations in FOXP2 cause a severe speech and language disorder characterized by deficits in language processing, verbal dyspraxia and impaired grammatical skills, without affecting other traits severely [4,5]. The function of FoxP genes in vocal learning appears to be evolutionary conserved as knock-outs of the zebra finch orthologue of human FOXP2 during the critical song learning period alters the structure of the crystallized song in the adults [14]. Such vocal learning is a form of motor learning that proceeds slowly from highly variable ‘babbling’ (in humans) and ‘subsong’ (in zebra finches) towards more stereotypic language and crystallized song, respectively. This specific kind of learning has been classified as a form of operant learning [15–17]. It was recently shown that, as in humans and zebra finches, also in flies, FoxP is involved in such operant learning [18].

The original forkhead (fkh) gene was identified in the fruit fly Drosophila melanogaster [19], where mutations cause defects in head fold involution during embryogenesis, causing the characteristic ‘fork head’. In contrast to chordates with four FoxP family members, only one orthologue of the FoxP subfamily is present in flies (dFoxP). The dFoxP gene gives rise to three different
transcripts by alternative splicing [2,18,20]; FoxP-isoform A (FoxP-IA), FoxP-isoform B (FoxP-iB) and FoxP-isoform IR (Intron Retention; FoxP-iIR) (figure 1a). The currently available reports as to the expression pattern of the FoxP gene have been contradictory and nothing is known as to whether the different isoforms are differentially expressed in different cell types. To resolve these issues we have tagged the endogenous FoxP gene, analysed the isoform-specific expression patterns and compared them with the expression of a selection of cell-type-specific markers.

Flies with a mutated FoxP gene not only show impairments in operant learning, but also in motor coordination and performance of inborn behaviours [18,20–22]. While isoform-specific alleles did show different phenotypes as well as different degrees of severity of these impairments, it remains unknown which neurons require specific alleles did show different phenotypes as well as performance of inborn behaviours [18,20].

2. Material and methods

2.1. Fly strains

Fly stocks were maintained at 18°C (table 1). Before experimental use, flies were kept at 25°C, in a 12/12 h light/dark regime at 60% relative humidity for at least one generation. All crosses were raised at 25°C (except for the ones involving the temperature-sensitive Gal4 inhibitor Gal80ts [23,24] that were raised at 18°C using four to six females and two to four males. For expression pattern visualizations, the FoxP-iB-Gal4 and FoxP-LexA driver line, respectively, were crossed with the appropriate effector lines containing different GFP or RFP variants (table 1). For behavioural analysis involving the UAS-tgRNA(4xFoxP), this effector line was always first crossed with a UAS-Cas9 line, and the resulting double-effector offspring with the appropriate driver line for each experiment (ELAV-Gal4, D42-Gal4, C380-Gal4, cnup-Gal4, ato-Gal4 and ELAV-Gal4; Tub-Gal80ts).

For local knock-out experiments, two genetic constructs need to be brought together for the method to work effectively. The endonuclease Cas9 needs to be present as well as the guide RNA (gRNA) to provide a target for the nuclease. Hence, the appropriate control groups express only one component of the CRISPR/Cas9 combination. One line drives expression only of the Cas9 endonuclease (i.e. xxx-Gal4 > UAS-Cas9, without gRNAs) and the other drives expression only of the gRNAs (i.e. xxx-Gal4 > UAS-tgRNA(4xFoxP) without Cas9). In this fashion, each strain not only controls for potential insertion effects of the transgenes used, but also for potential detrimental effects of expressing the components alone, irrespective of the excision of the target gene.

For the behavioural analysis involving the FoxP-KO mutant and the FoxP-iB-Gal4 driver line we crossed the lines back into Wild-Type Berlin genetic background for at least six generations in order to get the same genetic background as the WTB control.

2.2. In-silico sequences alignment

The transcript and protein sequences of the different FoxP isoforms were downloaded from https://flybase.org and aligned with Clustal Omega for multiple sequence alignment. The protein domains were analysed with the NCBI Conserved Domain Search tool, and the stop codons were identified with ExPaSy Translate tool (figure 1a).

2.3. Transgenics

We used CRISPR/Cas9 Homology-Directed Repair (HDR) to edit the FoxP locus [25] and generated t-RNA based vectors for producing multiple clustered regularly interspaced (CRISPR) gRNAs from a single transcript [26]. We created a total of two driver lines (FoxP-iB-Gal4 and FoxP-LexA), one mutant line (FoxP-KO) and one effector line (UAS-tgRNA(4xFoxP)).

2.3.1. FoxP-iB-Gal4

To create an isoform-specific driver line, we inserted a Gal4 sequence into exon 8, which is specific to isoform B. Two kb homology fragments were PCR-amplified (primers Hom1: 5’-GGGGGCGGCCGGCGTGAAGTTAAATG CCCCATATAATG-3’, 5’-GGGGGCGGCCCCCTCTCGT GTAAGGAAAGTTTCCGACCAGAAGTTA-3’; primers Hom2: 5’-GGGGGCGGCCGGCCACAAAGTGGCTTTGTACGTTAATG AAG-3’, 5’-GGGGGTTACCGTGACTGATCTGTTAATGAC G-3’) and digested with the appropriate restriction enzymes (Hom1: NotI and SacII, Hom2: Ascl and KpnI) to be ligated in the pT-GEM(0) (Addgene plasmid no. 62891; RRID:Addgene_62891) vector [27] which contained a Gal4 sequence and a 3xP3-RFP-SV40 sequence for selection of transformants. The gRNA sequences used are: sense 5’-CTTCGAGGCTAAACAGCCTTGTAG TGA-3’, and asense 5’-AAACTACACAAGTTTCTTGTAGC TC-3’. They were annealed and cloned inside a pU6-gRNA (Addgene plasmid no. 53062; RRID:Addgene_53062) vector [28], previously digested with BbsI restriction enzyme.

2.3.2. FoxP-LexA

To create a driver line that reflects expression of all FoxP isoforms, we inserted a LexA sequence into exon 3. Two kb homology fragments were PCR-amplified (primers Hom1: 5’-GGGGGCGGCCGGCGAAGTTAAATG CCCCATATAATG-3’, 5’-GGGGGCGGCCCCCTCTCGT GTAAGGAAAGTTTCCGACCAGAAGTTA-3’; primers Hom2: 5’-GGGGGCGGCCGGCCACAAAGTGGCTTTGTACGTTAATG AAG-3’, 5’-GGGGGTTACCGTGACTGATCTGTTAATGAC G-3’) and digested with the appropriate restriction enzymes (Hom1: NotI and SacII, Hom2: Kpnl and SpeI) to be ligated in the pT-GEM(0) vector which contained a LexA sequence and a 3xP3-RFP-SV40 sequence for selection of transformants. The gRNA sequences used are: sense 5’-CTTCGAGGCTAAACAGCCTTGTAG TGA-3’, and asense 5’-AAACTACACAAGTTTCTTGTAGC TC-3’. They were annealed and cloned inside a pU6-gRNA (Addgene plasmid no. 53062; RRID:Addgene_53062) vector [28], previously digested with BbsI restriction enzyme.

2.3.3. FoxP-KO

To prevent expression of any isoform of the FoxP gene, we removed part of exon 1, the complete exon 2 and part of exon 3. Two kb homology fragments were PCR-amplified (primers Hom1: 5’-GGGGGCGGCCGGCGAAGTTAAATG CCCCATATAATG-3’, 5’-GGGGGCGGCCCCCTCTCGT GTAAGGAAAGTTTCCGACCAGAAGTTA-3’; primers Hom2: 5’-GGGGGTTACCGTGACTGATCTGTTAATGAC G-3’, 5’-GGGGGTTACCGTGACTGATCTGTTAATGAC G-3’) and digested with the appropriate restriction enzymes (Hom1: NotI and SacII, Hom2: KpnI and SpeI) to be ligated in the pT-GEM(0) vector which contained a LexA sequence and a 3xP3-RFP-SV40 sequence for selection of transformants. The gRNA sequences used are: sense 5’-CTTCGAGGCTAAACAGCCTTGTAG TGA-3’, and asense 5’-AAACTACACAAGTTTCTTGTAGC TC-3’. They were annealed and cloned inside a pU6-gRNA (Addgene plasmid no. 53062; RRID:Addgene_53062) vector [28], previously digested with BbsI restriction enzyme.
Figure 1. FoxP-IB expression in the Drosophila nervous system. (a) Schematic of the FoxP gene locus before (above) and after (below) insertion of a Gal4 sequence into exon 8. (b) FoxP-IB-Gal4 > CD8-GFP expression pattern costained with nc82 in 3rd instar larvae, adult brain and adult VNC. (c) Driver line costained with a polyclonal FoxP antibody in larval and adult brain. The yellow arrowheads indicate colocalization, while the red ones indicate cells only positive for the antibody staining. (d) RT-qPCR for FoxP-iA, iB and IR on controls and hetero and homozygous FoxP-IB-Gal4 mutant. (d’) Primers used for the RT-qPCR. Data are expressed as means ± s.e.m. *p < 0.005. Scale bars: 50 µm.
GACAGATTGTGCCGG-3' and digested with the appropriate restriction enzymes (Hom1: NheI and SacII, Hom2: SpeI, PstI) to be ligated in the pHD-DsRed-attP (Addgene plasmid no. 51019; RRID: Addgene_51019) vector which contains a phosphorylated and annealed three sets of oligos (1. fw 5'-GCTCGGA TATGAACTCGGGCTGCACCAGCCGGGAA TCGAACC-3', 2. rev 5'-GCCCGAGTTCATATCCGAGCGTTTCA-3', 3. rev 5'-AAACTCTCTTCCGGGCACGTC-3'). They were annealed and cloned inside a pU6-gRNA vector previously digested with BbsI restriction enzyme.

### 2.3.4. UAS-t:gRNA(4xFoxP)

To create an isoform-unspecific conditional effector line we used the gRNA sequences used are: gRNA1 sense 5'-GCAATGCACCAGCCGGGAATCGAACC-3', gRNA2 sense 5'-TGTTCCGTA TTCAGA TGCACCAGCCGGGAA TCGAACC-3' and PstI) to be ligated in the pHD-DsRed-attP (Addgene plasmid no. 51019; RRID: Addgene_51019) vector which contains a phosphorylated and annealed three sets of oligos (1. fw 5'-GCTCGGA TATGAACTCGGGCTGCACCAGCCGGGAA TCGAACC-3', 2. rev 5'-GCCCGAGTTCATATCCGAGCGTTTCA-3', 3. rev 5'-AAACTCTCTTCCGGGCACGTC-3'). They were annealed and cloned inside a pU6-gRNA vector previously digested with BbsI restriction enzyme.

**Table 1.** Complete list of the fly lines used in this study.

<table>
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<th>genotype</th>
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<th>RRID</th>
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</tr>
<tr>
<td>cato-Gal4</td>
<td>driver line</td>
<td>BDSC_50422</td>
</tr>
<tr>
<td>cmyp-Gal4</td>
<td>driver line</td>
<td>BDSC_8816</td>
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<td>FoxP±/−</td>
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<td>/</td>
</tr>
<tr>
<td>FoxP-Ib-Gal4</td>
<td>driver line</td>
<td>/</td>
</tr>
<tr>
<td>FoxP-LexA</td>
<td>driver line</td>
<td>/</td>
</tr>
<tr>
<td>UAS-t:gRNA(4xFoxP)</td>
<td>effector line</td>
<td>/</td>
</tr>
<tr>
<td>ELAV-Gal4</td>
<td>driver line</td>
<td>/</td>
</tr>
<tr>
<td>LexAop-mCD8-RFP-UAS-mCD8-GFP</td>
<td>effector line</td>
<td>/</td>
</tr>
<tr>
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<td>/</td>
</tr>
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<td>driver line</td>
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<td>/</td>
</tr>
<tr>
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<tr>
<td>WTB</td>
<td>wild-type strain</td>
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</tr>
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The flies strains marked are the ones that we created in the present work.

**2.4. Immunohistochemistry**

Three to 6 day-old adults were fixed in 4% paraformaldehyde (PFA) at 4°C for 2 h and dissected in 0.01% phosphate-buffered saline with Triton® X-100 detergent (PBST). For larval staining, third instar larvae were selected, dissected in 0.01% PBST and fixed in 4% PFA at room temperature (RT) for 30 min. Clean brains were washed three times in 0.01% PBST for a total time of 45 min and then blocked with 10% normal goat serum (NGS) for 1 h. Subsequently, the brains were incubated with the appropriate primary antibody for one to two nights at 4°C (table 2). After three washing steps of 15 min each, the brains were incubated with the secondary antibody (table 3) for 5 h at RT. After an additional 15 min washing step, the brains were placed on glass microscope slides and mounted with the antifade mounting medium Vectashield® (Vector Laboratories, Burlingame, CA).

**2.5. Image acquisition and analysis**

All images were acquired with a Leica SP8 confocal microscope (RRID: SCR_018169), images were scanned at a frame size of 1024 x 1024 pixels at 200 or 100 Hz. The objectives were 20× dry and 20×/40×/60× oil immersion. Images were processed with ImageJ software (National Institutes of Health, Maryland, USA; RRID: SCR_003070) [29], only general adjustments to contrast, colour and brightness were made. Cell counting was performed with IMARIS 9.0 (Oxford instruments, UK) software on UAS-Stinger-GFP stacks, using the tool for spots counting. For the FoxP-Ib-Gal4/FoxP-LexA count (figure 4b), five brains were counted for each genotype at both larval (3rd instar) and adult (2–3 days old) stages. The colocalization analysis was performed with the ImageJ Colocalization Threshold tool (Tony Collins and Daniel James White) (figure 5b). The three-dimensional rendering (figure 12b) was performed with IMARIS 9.0 software on the Drosophila standard brain from https://www.virtualflybrain.org.

**2.6. RT-qPCR**

The knock-out efficiency was assessed using RT-qPCR (figures 1d and 4e). We extracted RNA from 20 flies for each genotype (white−, heterozygous mutant and homozygous mutant, both with a white− background), following the TriFast™ protocol from peqlab (a VWR company) (catalogue no. 30–2010). The RNA was subsequently transcribed into cDNA using the OneStep RT-PCR Kit from Qiagen (catalogue no. 210212) with the following thermocycler programme: 42°C for 2 min, 4°C pause until manual restart at 42°C for 30 min, 95°C for 3 min and finally 10°C oo. Subsequently, we performed the qPCR. Primer sequences were identical to those used by Mendoza et al [18]. For the qPCR reaction, we used a Bio-Rad CFX Connect Real-Time PCR
Detection System thermocycler and the Bio-Rad CFX manager software to store and analyse the data. Every sample was run in triplicate in a 96-well plate to a total volume of 10 µl. The mixture contained 5 µl sybrGreen master mix (ORATM qPCR Green ROX H Mix, 2X, from highQu, catalogue no. QF00201), 0.5 µl from each primer, 1 µl of 1 : 10 diluted cDNA and 3 µl H2O. As reference, we used the housekeeping gene rp49 (ribosomal protein 49), while as a negative control we used the same reaction mix without cDNA. The qPCR reaction programme used was: 95°C for 2 min, 95°C for 10 s, 60°C for 10 s, 65°C for 30 s (from step 2 to 4 × 39 rounds), 95°C for 10 s, and finally from 65°C to 95°C for +0.5°C/5 s. The experiments were repeated two to four times.

### 2.7. Behaviour

All behavioural experiments were performed in Buridan’s paradigm (RRID: SCR_006331) [30]. In this experiment, we analysed both temporal components of walking behaviour (often subsumed under ‘general locomotion’) and spatial components such as fixation of landmarks or the straightness of the walking trajectory. Buridan’s paradigm (figure 6a) consists of a round platform with a diameter of 117 mm which is surrounded by a water-filled moat. The platform is situated at the bottom of a uniformly illuminated white cylinder, 313 mm in height and 293 mm in diameter [31]. Two black stripes are placed on the inside of the cylinder, opposite each other, serving as the only visual cues for the flies. Two day-old female flies were collected and their wings were clipped under CO2 anesthesia. After one night recovery at 25°C, they were tested in Buridan’s paradigm for 15 min (doi:10.17504/protocols.io.c7vnS5). The position of the fly is recorded by a camera (Logitech Quickcam Pro 9000) connected to a computer running our BuriTrack software (http://buridan.sourceforge.net/).

The analysis software CeTrAn [31] (https://github.com/jcorlomb/CeTrAn) extracts a variety of parameters from the scored trajectories. From the parameters extracted by CeTrAn, we used the temporal parameters median speed (the median of all instantaneous speed data points measured when the fly is walking), distance travelled, number of walks (sections of the trajectory which connect the platform areas closest to the two stripes) and activity time (fraction of time spent walking), as well as the spatial parameters stripe deviation (angular deviation of the fly’s heading from the centre of the stripe in the frontal visual field) and meander (a measure of the tortuosity of the fly’s trajectory). Transition plots visualize the areas on the platform that the flies most frequently visited. More details in [31].

For the experiment involving Tub–Gal4eW (figure 8a–h and 11a,b), flies were raised at 18°C, moved to 30°C for 12 h (embryos) or 48 h (pupae and adults) and subsequently left at 25°C for the rest of the development (embryos and pupae) or overnight for recovery (adults) before testing.

### 2.8. Statistical analysis

All graphs were created and statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., California, USA; RRID: SCR_002798) software. Sample variances were compared with an F-test. In the absence of significantly different variances, we used Student’s t-tests (two-tailed) or one way ANOVAs followed by Tukey’s post hoc test for multiple comparisons. If the F-test was significant at 

\[ p < 0.005 \] (see below), we used a Mann–Whitney U-test or a Kruskal–Wallis ANOVA followed by Dunn’s post hoc test for multiple comparisons. Alpha values were set to 0.5%, in order to reduce the chances of false-positives, following the arguments detailed in [32], where BB is an author. Whenever null hypothesis significance testing was performed using the non-parametric tests, it is indicated in the figure legends. All other tests were performed using parametric tests.
3. Results

3.1. FoxP-iB expression in the Drosophila brain

The FoxP transcription factor binds DNA with the forkhead (FH) box domain (figure 1a, yellow boxes; raw data deposited at doi:10.6084/m9.figshare.12607700). The gene consists of seven introns and eight exons. The FH box is split into two different segments spanning exons 6, 7 and 8. The last two of seven introns and eight exons. The FH box is split into different segments spanning exons 6, 7 and 8. The last two exons (7 and 8) are subjected to alternative splicing, leading to two different protein isoforms: isoform A (FoxP-iA), which results from splicing exon 6 to exon 7, and isoform B (FoxP-iB) where instead exon 8 is spliced to exon 6. A third, intron-retention isoform is transcribed by failing to splice the intron between exon 6 and 7 out (FoxP-iIR). While the first two isoforms contain a complete and putatively functioning FH box (with 10 amino acids different between the two; figure 1a, dashed box), the putative FoxP-iIR FH box appears to be truncated due to a stop codon in the intron sequence (figure 1a, red line), putting the transcription factor function of this isoform in doubt. Of the three FoxP isoforms, FoxP-iB was most directly associated with the learning phenotype discovered by Mendoza et al. [18]. Therefore, we inserted the yeast transcription factor Gal4 into exon 8, the exon which is exclusive to FoxP-iB (figure 1a). This insertion leads to the expression of the Gal4 transcription factor only in FoxP-iB positive cells. At the same time, the insertion also disrupts the FH box DNA-binding domain of the FoxP gene, putatively preventing the FoxP protein to act as a transcription factor, effectively mutating the gene for this function.

Observing Gal4 expression with different green fluorescent proteins (GFPs) under control of the UAS promoter (to which Gal4 binds), revealed that FoxP-iB is expressed throughout the whole development of the fly, from embryo (data deposited at doi:10.6084/m9.figshare.12607652) to adult, in both brain and ventral nerve cord (VNC) (figure 1b). In 3rd instar larvae, we observe expression in the central brain (but not in the optic lobes) and in the anterior portion of the VNC. In the adult nervous system, the main neuropil expression domains comprise protocerebral bridge, gnathal ganglia (suboesophageal zone), vest, saddle, noduli and superior medial protocerebrum. GFP-positive cell body clusters could be found in the cortex of the central brain and around the optic lobes (figure 1b).

We next validated the expression pattern of our iB-specific driver line to the staining of an available isoform-unspecific polyclonal antibody [21]. We observed complete colocalization of the driver line with the antibody staining in both larvae and adults, i.e. there were no GFP-positive cells that were not also labelled by the FoxP antibody (figure 1c). The cells only stained for the FoxP antibody and not for GFP are presumably cells expressing the other FoxP isoforms (FoxP-iA and FoxP-iIR, figure 4). Notably, in contrast to previous reports [34,35] but consistent with [20], we did not detect any FoxP expression in mushroom body (MB) neurons, neither with our driver line, nor with the antibody.

Postulating that our transgene disrupted expression of the FoxP gene, we measured mRNA levels of all three isoforms with RT-qPCR (figure 1d). With one of the primers placed over the Gal4 insertion site, we observed approximately half the wild-type FoxP-iB expression levels in heterozygous animals, while wild-type FoxP-iB expression was essentially abolished in the homozygous transgenes. We did not observe any significant changes in the other two isoforms. However, this disruption of the FoxP-iB isoform did not have an effect on the development of the FoxP-positive neurons, as both heterozygous and homozygous FoxP-iB-Gal4 mutants showed the same number of GFP-labelled cell nuclei (data deposited at doi:10.6084/m9.figshare.12932705).

3.2. FoxP-iB is expressed in a variety of neurons, but not glia

With FoxP involved in learning and expression patterns suggesting neuronal expression (figure 1), we investigated whether the observed expression was exclusively neuronal, or if there were also FoxP-iB expressing glial cells. Therefore, we stained 3rd instar larva and adult brains with antibodies against ELAV (neuronal marker) and REPO (glial marker). At both developmental stages, the two stainings reveal exclusive FoxP-iB-mediated GFP colocalization with ELAV without any colocalization with REPO (figure 2a,b; raw data deposited at doi:10.6084/m9.figshare.12607706), suggesting that FoxP-iB is expressed exclusively in neurons. These data are consistent with results published previously [20,36], validating the methods employed here.

We next investigated in more detail the types of neurons in which FoxP-iB is expressed. Using a variety of antibodies (figure 3; raw data deposited at 10.6084/m9.figshare.12607712) used as markers for different neuronal cell types we detected FoxP-iB expression in most of the cell types investigated. For technical reasons, we stained adult brains only with the anti-TH antibody, while the remaining markers were used on larval nervous systems. Extensive colocalization was observed with p-SMAD1/5 (a motor neuron marker) in the VNC but not in the central brain (CB) (figure 3e). Some FoxP-iB neurons were positive for ChAT (cholinergic) or GABA (inhibitory) both in the VNC and in the CB (figure 3e). Finally, a few FoxP-iB-positive neurons were found to colocalize with tyrosine hydroxylase (dopaminergic neurons) in the CB only (figure 3d). These data are consistent with the study performed by Schatton et al. [37] in honeybees where they found colocalization between AmFoxP-positive neurons and GABAergic, cholinergic and monoaminergic markers. No colocalization was instead found in photoreceptor cells stained with Chaoptin (data deposited at doi:10.6084/m9.figshare.12607643), a marker for photoreceptor cells [38].
ably, the FoxP protein is involved in its own regulation such as amplifying all transcripts, with or without the insertion. Poss-

\[ \text{FoxP-iB} \]

In heterozygotes, the missing gene copy leads to a compensatory increase in transcription rate, but in homozygous mutant animals without any FoxP protein, only basal expression levels remain.

In order to directly compare the expression patterns of our two driver lines, we used them to drive reporter genes fluores-

\[ \text{Stinger-GFP} \]

centrating at different wavelengths (i.e. \( \text{LexAop-RFP;UAS-CD8-GFP} \)) and analysed their patterns in adults (figure 5; raw data depos-

\[ \text{REPO (glia, a) and ELAV (neurons, b) markers. Note the lack of colocalization of FoxP-iB driven GFP with the glial marker in both 3rd instar larvae and adult brains (a). By contrast, exclusive colocalization of FoxP-driven GFP with the neuronal marker was observed in both developmental stages (white arrowheads indicate typical examples). Scale bars: 50 µm.} \]

### 3.3. FoxP-iB is expressed in a subset of FoxP-expressing neurons

As the antibody staining against the FoxP protein indicated more cells expressing FoxP than our FoxP-iB-specific driver line was reporting (figure 1b), we created a second driver line, designed to drive expression in all FoxP cells, irrespective of isoform. We inserted a sequence for the bacterial LexA [39] transcription factor into exon 3 (figure 4a; raw data deposited at doi:10.6084/m9.figshare.12607730). Comparing Stinger-GFP expression from each driver line revealed a more expansive pattern for the isoform-unspecific driver (figure 4b), one that matches very well with the FoxP antibody staining (figure 1b). This visual impression was corroborated by a quantification of stained nuclei comparing between both drivers (figure 4c). This quantification allowed us to trace the proliferation of FoxP cells from around 500 in 3rd instar larvae to around 1800 in 3 day-old adults. By contrast, there are only about 300 cells expressing FoxP-iB in the 3rd larval instar and only around 1300 in 3 day-old adults. We noticed that the largest differences in terms of cell number between FoxP-LexA and FoxP-iB-Gal4 flies (both larvae and adults) were found in the CB, while the VNC numbers differed considerably less. Taken together, in 3rd instar larvae and in 3 day-old adults, 66% and 65%, respectively, of the total number of FoxP neurons in the Drosophila nervous system express FoxP-iB. As with our previous insertion, also this one was expected to disrupt expression of the FoxP gene. To investigate the extent of this disruption on the mRNA level, we again performed RT-qPCR. As expected, this insertion affected all isoforms. In heterozygous flies, the expression level was increased, while in homozygous flies it was decreased (figure 4e). It is important to note that here, in contrast to the FoxP-iB insertion, all primers are binding sequences down-

\[ \text{a, d} \]

stream of the insertion site (figure 1a,d) and so are amplifying all transcripts, with or without the insertion. Possibly, the FoxP protein is involved in its own regulation such that in heterozygotes, the missing gene copy leads to a compensatory increase in transcription rate, but in homozygous mutant animals without any FoxP protein, only basal expression levels remain.

We also crossed this FoxP-LexA line with LexAop-RFP;UAS-CD8-GFP and Tdc2-Gal4 to investigate any potential tyraminergic or octopaminergic FoxP neurons, but despite a close proximity between the two cell types, no colocalization was found (data deposited at doi:10.6084/m9.figshare.12607670).

### 3.4. FoxP-iB knock-out flies show a multitude of behavioural abnormalities

Mutations in the FoxP gene do not only affect operant self-learning, for instance, different alleles also affect flight performance and other locomotion behaviours to different degrees [18,20,21]. Because of the FoxP pleiotropy affecting various innate motor behaviours independently from motor learning, we turned to Buridan’s paradigm [30,31] as a powerful tool to measure several locomotor variables. Buridan’s paradigm allows us to test a broad panel of behavioural parameters covering temporal parameters such as speed or general activity...
time and spatial parameters such as the straightness of a fly’s trajectory (meander) or the degree to which the animal is heading towards one of the two vertical landmarks (stripe fixation; figure 6). With our insertions constituting novel alleles impairing FoxP expression (figures 1 and 4), we started by testing the heterozygous and homozygous driver strains without any effectors.

Consistent with previous findings of impaired locomotor behaviour in FoxP manipulated flies [18,20,21] and the qPCR results showing reduced FoxP expression (figure 1d), our FoxP-iB-Gal4 insertion shows abnormalities in Buridan’s paradigm both in temporal and in spatial parameters (figure 6; raw data deposited at doi:10.6084/m9.figshare.12607769). While the homozygous flies walked more slowly, spent more time at rest and fixed the stripes less strongly than wild-type control flies, heterozygous flies only differed from wild-type flies in their stripe fixation (figure 6c). With different effect sizes in each parameter, we selected two representative parameters each for the temporal and the spatial domain, respectively, for comparison of all subsequent lines: walking speed, activity time, meander and stripe fixation.

**Figure 3.** FoxP-iB is expressed in various types of neurons. Immunohistochemistry on FoxP-iB-Gal4 > CD8-GFP larvae and adults using different antibodies. (a) Some of the FoxP-iB positive neurons colocalize with p-SMAD1/5 in the VNC but not in the central brain. (b,c) FoxP-iB neurons positive for ChAT or GABA have been found in both the VNC and CB. (d) Only few FoxP-iB neurons colocalize with TH and only in the CB. White arrowheads indicate examples of colocalization. Scale bars, 25 μm. (a–c) larvae; (d) adult flies.
Because our insertion is located in the same exon as the insertion in the FoxP<i>955</i> mutant, we tested the FoxP<i>955</i> mutant flies in Buridan’s paradigm and found changes in several temporal parameters, similar to those observed in our driver line (figure 6d). However, for the spatial parameters (stripe deviation and meander) only stripe fixation appears normal in these flies, the increased meander indicates that the flies have problems walking straight, despite clearly walking towards the stripes. Thus, in addition to the deficits in operant self-learning and flight performance as reported previously [18], the FoxP<i>955</i> mutant flies are also deficient in several temporal and one spatial parameter of walking behaviour in Buridan’s paradigm. This walking phenotype is consistent with previous findings of walking deficits associated with FoxP manipulations [20,21], but was not detected in a previous publication where tests for walking deficits had been performed [35].

4. Knocking out all FoxP isoforms has similar effects as FoxP-<i>iB</i> knock-out

With such dramatic motor alterations when only FoxP-<i>iB</i>, which is expressed in about 65% of all FoxP-positive neurons (figure 4), is removed (figure 6), it is interesting to study the effects of removing the remaining isoforms for a complete FoxP knock-out. To avoid unwanted potential side-effects of expressing a different protein in its stead, we created a third fly line where the entire second exon is removed together with parts of exons 1 and 3. We validated this mutant with the polyclonal antibody we used before (figure 1). While the antibody detected the FoxP gene product in control flies, there was no signal in our homozygous knock-out flies (figure 7a,b; raw data deposited at doi:10.6084/m9.figshare.12607796).
Analogous to the behavioural characterization in the FoxP-iB insertion line, we tested both heterozygous and homozygous FoxP-KO deletion mutants in Buridan’s paradigm (figure 7c–f). The results of this experiment closely resemble the ones from the FoxP-iB-Gal4 insertion line, with homozygous mutants being both significantly less active (figure 7c) and fixating the stripes less strongly than the heterozygous mutants and the wild-type controls (figure 7d,e). For this allele, the heterozygous FoxP-KO mutants show higher values for all temporal parameters compared to the wild-type controls, while there is no difference in stripe deviation. This trend was already observed in FoxP-iB mutants but failed to reach statistical significance. Thus, the FoxP-KO allele exhibits differential dominance, with recessive modes of inheritance in some traits and, e.g. overdominance in others. A direct comparison of the data from the two homozygous alleles (FoxP-iB and FoxP-KO) showed no significant differences (figure 7f). Thus, removing the other FoxP isoforms

Figure 5. Comparison of FoxP-isoform expression patterns. (a) Confocal images of adult brains expressing FoxP-iB-Gal4 > CD8-GFP (green) and FoxP-LexA > CD8-RFP (red). (b) Difference rendering (blue) indicating areas expressing only non-iB isoforms. AL, antennal lobe; PVP, posterior ventrolateral protocerebrum; FSB, fan-shaped body; Lo, lobula; cec, cell cluster; GG, gnathal ganglion; PB, protocerebral bridge. Scale bars, 50 µm.
camera fluorescent bulbs
platform: \( \phi = 117 \text{ mm} \)
arena: \( \phi = 293 \text{ mm} \)

computer running the BuriTrack software

\[ a \]

\[ (b) \]

\[ (c) \]

\[ (d) \]

transition plots

\[ (e) \]

median speed (mm s\(^{-1}\))
distance travelled (m)
activity time (s)
meander (º*s mm\(^{-1}\))
stripe deviation (º)

relative transition frequency
0 – 95% quantile

Figure 6. (Caption continued.)
had few effects beyond the consequences of removing only FoxP-iB alone.

4.1. Local and conditional FoxP knock-out (FoxP-cKO)

Given the patchy expression pattern of FoxP in the fly’s nervous system (figures 1–5) and the grave consequences for behaviour in Buridan’s paradigm if it is manipulated (figures 6 and 7), we sought to investigate when and where FoxP is required for normal walking behaviour. To this end, we designed a fourth fly strain which carries a UAS-controlled effector (figure 8a; raw data deposited at doi:10.6084/m9.figshare.12607805). The four guide RNAs (gRNA) each target a different section of the FoxP gene (see Material and methods). If expressed together with the endonuclease Cas9, this effector efficiently mutates the targeted gene [26,40]. We validated this approach by driving both our gRNAs as well as Cas9 using the pan-neuronal elav-Gal4 driver and monitoring FoxP expression with the FoxP antibody used before (figure 8b). Flies with this pan-neuronal excision of the FoxP gene (FoxP-cKO) were also tested in Buridan’s paradigm and showed even more severe impairments than flies homozygous for a constitutive deletion of the gene (figure 8c). In fact, the mutated flies walked so little, that analysis of spatial parameters was not meaningful (figure 8d). To allow for temporal control of transgene expression, we also validated the use of the temperature-sensitive suppressor of Gal4, Gal80° (figure 8e). The constitutively expressed Gal80° prevents Gal4 from activating transcription of the UAS-controlled transgenes until the temperature is shifted from 18°C to 30°C, at which point the repressor becomes inactivated and Gal4-mediated transcription commences [23,24]. Using this system to drive gRNA/Cas9 expression for 12 h in the embryo phenocopies transcription commences [23,24]. Using this system to drive gRNA/Cas9 expression for 12 h in the embryo phenocopies CrossRef initial transcription 

4.2. Local FoxP-KO: brain regions and neuron types

Recently, Linneweber et al. [41] described the consequences of silencing dorsal cluster neurons (DCNs) on stripe fixation behaviour in Buridan’s paradigm. The FoxP-iB expression pattern suggests that at least some of these DCNs express FoxP (figure 1). Comparing our isoform-unspecific FoxP-LexA expression pattern with that of the atonal-Gal4 line used to drive expression in DCNs [42], we observed substantial overlap (figure 9a; raw data deposited at doi:10.6084/m9.figshare.12607811). Therefore, we used ato-Gal4 to excise the FoxP gene specifically in DCNs. Remarkably, this manipulation did not have any effect on the flies’ behaviour in Buridan’s paradigm (figure 9b).

The insect MBs are not only known as a centre for olfactory learning and memory [e.g. 43–50], they are also involved in the temporal and spatial control of locomotor activity [e.g. 51–60]. In addition, Castells-Nobau et al. [20] found a subtle structural phenotype in a sub-section of the MBs without detectable FoxP expression in the MB Kenyon cells themselves. Finally, there are two reports that expressing anti-FoxP RNAi constructs exclusively in the MBs can have behavioural effects [34,35]. For these reasons, despite neither [20] nor us being able to detect any FoxP expression in the MBs (and current RNAi constructs fail to knock down FoxP mRNA, see below), we deleted the FoxP gene from MB Kenyon cells using the ok107-Gal4 driver and tested the flies in Buridan’s paradigm. We did not detect any differences to control flies in these experiments (figure 9c).

There are two reasons for knocking out FoxP in motor neurons, besides FoxP expression there (figure 3); first, networks of motor neurons in the VNC control movement patterns and walking is directly affected by our manipulations so far (figures 6 and 7). Second, motor neurons were shown to be important for the type of operant self-learning that also requires FoxP [61]. Driving expression of gRNA/Cas9 with either of two motor neuron-specific driver lines (D42-Gal4 and C380-Gal4) led to a significant alteration in locomotor activity in Buridan’s paradigm, both for spatial and for temporal parameters (figure 10a,b; raw data deposited at doi:10.6084/m9.figshare.12607823).

Perhaps the most prevalent FoxP expression can be observed in the protocerebral bridge (figure 1). The driver line cmpr-Gal4 targets the protocerebral bridge specifically and drives expression in FoxP-positive neurons (figure 10c). Removing the FoxP gene exclusively in these neurons led to a significant reduction of locomotor activity (figure 10d) as well as a reduction in stripe fixation and to more tortuous trajectories (figure 10e). In fact, the stripe deviation increased to an extent that it can no longer be distinguished statistically from a random walk at the 0.5% level (Wilcoxon signed rank test against 45°, V = 33, p = 0.02).

4.3. Conditional FoxP-KO: developmental stages

With FoxP being a transcription factor active throughout development and particularly important during pupal development [20,62], we knocked out FoxP in all neurons by adding the Gal4 repressor Gal80° to our pan-neuronal FoxP-cKO (figure 11a; raw data deposited at doi:10.6084/m9.figshare.12607838) and treating the flies with a 48 h 30°C heat treatment during the early pupal stage. This regime did not affect walking behaviour in Buridan’s paradigm (figure 11b). Shifting the temperature treatment to immediately after eclosion also did not affect the flies’
 behaviour in Buridan’s paradigm (figure 11c). Taken together, these data indicate that FoxP is required for the proper development of, for instance, motor neurons and protocerebral bridge neurons, but once these circuitries are in place, FoxP expression does not appear to have any immediate mechanistic role in locomotion anymore.

5. Discussion

We have edited the genomic locus of the Drosophila FoxP gene in order to better understand the expression patterns of the FoxP isoforms and their involvement in behaviour. We have discovered that the isoforms differ with respect to
their expression in neuronal tissue. For instance, we found isoform B (FoxP-iB) expression in neuropil areas such as the superior medial protocerebrum, the protocerebral bridge, the noduli, the vest, the saddle, the gnathal ganglia and the medulla, while areas such as the antennal lobes, the fan-shaped body, the lobula and a glomerulus of the posterior ventrolateral protocerebrum contain other FoxP isoforms but not isoform B (summarized in figure 12) (raw data).
We also corroborated previous results [20] that FoxP is expressed in a large variety of neuronal cell types (figure 3). Our genomic manipulations created several new alleles of the FoxP gene which had a number of behavioural consequences that mimicked other, previously published alleles [20]. Specifically, we found that constitutive knock-out of either FoxP-IB alone or of all FoxP isoforms affects several parameters of locomotor behaviour, such as walking speed, the straightness of walking trajectories or landmark fixation (figures 6 and 7). We discovered that mutating the FoxP gene only in particular neurons can have different effects. For instance, knocking FoxP out in neurons of the dorsal cluster (where FoxP is expressed) or in MB Kenyon cells (where neither we nor [20] were able to detect FoxP expression) had no effect in Buridan’s paradigm (figure 9), despite these neurons being deposited at doi:10.6084/m9.figshare.12607862). We also corroborated previous results [20] that FoxP is expressed in a large variety of neuronal cell types (figure 3). Our genomic manipulations created several new alleles of the FoxP gene which had a number of behavioural consequences that mimicked other, previously published alleles [20]. Specifically, we found that constitutive knock-out of either FoxP-IB alone or of all FoxP isoforms affects several parameters of locomotor behaviour, such as walking speed, the straightness of walking trajectories or landmark fixation (figures 6 and 7). We discovered that mutating the FoxP gene only in particular neurons can have different effects. For instance, knocking FoxP out in neurons of the dorsal cluster (where FoxP is expressed) or in MB Kenyon cells (where neither we nor [20] were able to detect FoxP expression) had no effect in Buridan’s paradigm (figure 9), despite these neurons being...

![Figure 9](image_url)
Figure 10. (Caption overleaf.)
required for normal locomotion in Buridan’s paradigm [41,54,56]. By contrast, without FoxP in the protocerebral bridge or motor neurons, flies show similar locomotor impairments as flies with constitutive knock-outs (figure 10). These impairments appear to be due to developmental action of the FoxP gene during larval development, as no such effects can be found if the gene is knocked out in all cells in the early pupal or adult stages (figure 11).

### 5.1. Foxp expression

#### 5.1.1. Neuronal expression of FoxP is widespread but not in mushroom bodies

The exact expression pattern of FoxP has been under debate for quite some time now. Initial work combined traditional reporter gene expression with immunohistochemistry [21] (table 4).
Lawton and colleagues [21] created a FoxP-Gal4 line where a 1.5 kb fragment of genomic DNA upstream of the FoxP coding region was used to drive Gal4 expression. These authors validated the resulting expression pattern with the staining of a commercial polyclonal antibody against FoxP. We used the same antibody in this work and observed perfect co-expression with our reporter (figure 1c). The Lawton et al. description of the FoxP expression pattern as a small number of neurons distributed in various areas of the brain, particularly in the protocerebral bridge, matches our results and those of [20].

Subsequent reports on FoxP expression patterns also used putative FoxP promoter fragments to direct the expression of Gal4 [35,62]. DasGupta and colleagues [35] used a 1.4 kb sequence upstream of the FoxP transcription start site, while Schatton & Scharff [62] used 1.9 kb. Their larger fragment contained the sequences of the two previously used fragments.

![Figure 12. FoxP expression pattern in the adult Drosophila brain. (a) Rostral and caudal sections with neuropil areas marked for FoxP-iB expression (above) or for other FoxP isoforms excluding FoxP-iB (below). (b) Volume rendering of adult neuropil with marked approximate FoxP-positive cell body locations in the cortex. Scale bars, 50 µm.](image)

**Table 4.** The four previous reports and the present work describing FoxP expression patterns.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Method</th>
<th>Expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lawton et al. [21]</td>
<td>Gal4 (1.5 kb fragment, pGabTB)</td>
<td>small number of neurons in various areas of the brain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>not mushroom bodies</td>
</tr>
<tr>
<td>DasGupta et al. [35]</td>
<td>Gal4 (1.4 kb fragment, pBGUw)</td>
<td>only mushroom bodies</td>
</tr>
<tr>
<td>Schatton &amp; Scharff [62]</td>
<td>Gal4 (1.9 kb fragment, pBGUw)</td>
<td>mushroom bodies and other areas</td>
</tr>
<tr>
<td>Castells-Nobau et al. [20]</td>
<td>GFP-tag (fosmid)</td>
<td>abundant number of neurons in various areas of the brain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>not mushroom bodies</td>
</tr>
<tr>
<td>Present work</td>
<td>Genomic Gal4-tag (CRISPR/Cas9 HDR, pTGEM(0))</td>
<td>abundant number of neurons in various areas of the brain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>not mushroom bodies</td>
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</tbody>
</table>
In contrast to [20,21] and our work here, both studies reported expression in overlapping sets of MB Kenyon cells. While DasGupta and colleagues implied that there was no expression outside of the MBs, Schatton and Scharff were not explicit. However, Schatton et al. independently reported that they had observed strong expression also outside of the MBs ([63] and A Schatton, C Scharff 2018-2020, personal communication).

The fourth and latest study reporting on FoxP expression in Drosophila [20] avoided the problematic promoter fragment method and instead tagged FoxP within a genomic segment contained in a fosmid [64], intended to ensure expression of GFP-tagged FoxP under the control of its own, endogenous regulatory elements. This study was the first to circumvent the potential for artefacts created either by selection of the wrong promoter fragment or by choosing an inappropriate basal promoter with the fragment (see below). However, since they also used insertion of a transgene, their expression pattern, analogous to that of a promoter fragment Ga4 line, may potentially be subject to local effects where the fosmid with the tagged FoxP was inserted. Castells-Nobau and colleagues [20] also used the antibody generated by Lawton et al. [21] to validate their transgenic expression patterns. The results reported with this technique are similar to those of Lawton et al. [21] and our work (summarized in figure 12b).

In an attempt to eliminate, the last source of error for determining the expression pattern of FoxP in Drosophila, we used CRISPR/Cas9 with homology-directed repair to tag FoxP in situ, avoiding both the potential local insertion effects of the previous approaches and without disrupting the complex regulation that may occur from more distant parts in the genome. For instance, in human cells, there are at least 18 different genomic regions that are in physical contact with the FOXP2 promoter, some of which act as enhancers [65]. The effects of these regions may be disrupted even if the entire genomic FoxP locus were inserted in a different genomic region as in [20]. Interestingly, the first promoter fragment approach [21] and the fosmid approach [20] agree both with our most artefact-avoiding genome editing approach and the immunohistochemistry with an antibody validated by at least three different FoxP-KO approaches. This converging evidence from four different methods used in three different laboratories suggests that FoxP is expressed in about 1800 neurons in the fly nervous system, of which about 500 are located in the ventral nerve cord. Expression in the brain is widespread with both localized clusters and individual neurons (figure 12b) across a variety of neuronal cell types. Notably, the four methods also agree that there is no detectable FoxP expression in the adult or larval MBs. By contrast, in honey bees, there is converging evidence of FoxP expression in the MBs [62].

5.2. Understanding false-positive FoxP detection in mushroom bodies

This comparison of our data with the literature prompts the question why two different promoter fragment approaches [35,62] suggested FoxP expression in the MBs (confirmed by a ribosome-based approach, see below) when there is no FoxP protein detectable there.

A first observation is that Lawton et al. used the classic hsp70-based pGaTB vector [66] to create their Ga4 line, while both DasGupta et al. as well as Schatton and Scharff used the more modern Drosophila synthetic core promoter (DSCP)-based pBGUw vector [67]. The two vectors differ with regard to their effects on gene expression. In addition to carrying two different basal promoters, the modern pBGUw sports a 3’UTR that is designed to increase the longevity and stability of the mRNA over the pGaTB vector, which can result in twofold higher Ga4 levels [68].

This observation is complemented by single-cell transcriptome data [69]. FoxP RNA can be detected in more than 4100 brain cells, likely overcounting the actual FoxP expression more than threefold. For instance, FoxP RNA is detected in over 1000 glial cells where none of the published studies has ever detected any FoxP expression (see also figure 2).

Taking these two observations together, it becomes plausible that there may be transient, low-level FoxP transcription in some MB neurons (and likely thousands of other cells as well), which in wild-type animals rarely leads to any physiologically relevant FoxP protein levels in these cells. Only when gene expression is enhanced by combining some arbitrary promoter fragments with genetically engineered constructs designed to maximize Ga4 yield such as the pBGUw vector, such transient, low-abundance mRNAs may be amplified to a detectable level.

These considerations may also help explain why the ribosome-based method of [34] was able to detect FoxP RNA in MB Kenyon cells: the transcript they detected may have been present and occupied by ribosomes, but ribosomal occupancy does not automatically entail translation [70]. It remains unexplained, however, how DasGupta et al. failed to detect all those much more strongly expressing and numerous neurons outside of the MBs. All of the above is consistent with other insect species showing FoxP expression on the protein level in their MBs [62], as only limited genetic alterations would be needed for such minor changes in gene expression.

The stochasticity of gene expression is a well-known fact [71–79] and known to arise from the transcription machinery [80]. Post-transcriptional gene regulation is similarly well-known [81–86]. It is thus not surprising if we observe that many cells often express transcripts that rarely, if ever, are translated into proteins. The final arbiter of gene expression must therefore remain the protein level, which is why we validated our expression analysis with the appropriate antibody. On this decisive level, FoxP has not been detected in the MBs at this point.

5.3. Different FoxP isoforms are expressed in different neurons

Our genome editing approach allowed us to distinguish differences in the expression patterns of different FoxP isoforms. The isoform specifically involved in operant self-learning, FoxP-iB, is only expressed in about 65% of all FoxP-positive neurons. The remainder express either FoxP-iA or FoxP-iIR or both. Neurons expressing only non-iB isoforms are localized in the antennal lobes, the fan-shaped body, the lobula and a glomerulus of the posterior ventrolateral protocerebrum (figure 5). Combined with all three isoforms differing in their DNA-binding FH box, the different expression patterns for the different isoforms adds to the emerging picture that the different isoforms may serve very different functions.
5.4. FoxP and locomotor behaviour

5.4.1. FoxP is involved in both spatial and temporal parameters of walking

Alterations of FoxP family genes universally result in various motor deficits on a broad scale in humans [4,6,7,87] and mice [88–90] for both learned and innate behaviours. Also in flies, manipulations of the FoxP locus by mutation or RNA interference have revealed that FoxP is involved in flight performance and other, presumably born, locomotor behaviours [18,20–22] as well as in motor learning tasks [18].

The locomotor phenotypes described so far largely concerned the temporal aspects of locomotion, such as initiation, speed or duration of locomotor behaviours. Here, concerned the temporal aspects of locomotion, such as phenotype becomes impossible to dissect.

that the specific contribution of a gene to a behavioural most instances, as so many different behaviours are affected which renders coarse neurogenetic approaches useless in our results further exemplify the old insight that coarse assaults on gene function such as constitutive knock-outs of entire genes or isoforms very rarely yield useful, specific phenotypes [91]. Rather, it is often the most delicate of manipulations that reveal the involvement of a particular gene in a specific behaviour. This fact is likely most often due to the pleiotropy of genes, often paired with differential dominance which renders coarse neurogenetic approaches useless in most instances, as so many different behaviours are affected that the specific contribution of a gene to a behavioural phenotype becomes impossible to dissect.

In the case of FoxP, it was already known, for instance, that the different isoforms affect flight performance to differing degrees [18] and that a variety of different FoxP manipulations affected general locomotor activity [20–22]. Here we show that a complete knock-out of either FoxP-iB or all isoforms affected both spatial and temporal parameters of locomotion, but the insertion mutation FoxP

5.4.2. Locomotion does not require FoxP expression in all FoxP neurons

Taken together, the results available to-date reveal FoxP to be a highly pleiotropic gene with phenotypes that span both temporal and spatial domains of locomotion in several behavioural modalities, lifespan, motor learning, social behaviour and habituation. It is straightforward to conclude that only precise, cell-type-specific FoxP manipulations of specific isoforms will be capable of elucidating the function this gene serves in each phenotype. With RNAi generally yielding varying levels of knock-down and, specifically, with currently available FoxP RNAi lines showing only little, if any, detectable knock-down with RT-qPCR (data deposited at doi:10.6084/m9.figshare.12607667 and Annette Schenck, personal communication), CRISPR/Cas9-mediated genome editing lends itself as the method of choice for this task. Practical considerations when designing multi-target gRNAs for FoxP prompted us to begin testing the CRISPR/Cas9 system as an alternative to RNAi with an isoform-unspecific approach first, keeping the isoform-specific approach for a time when we have collected more experience in this technique. In a first proof-of-principle, we used CRISPR/Cas9 to remove FoxP from MB Kenyon cells, DCNs, motor neurons and the protocerebral bridge.

MBs have been shown to affect both spatial and temporal aspects of locomotion [e.g. 51–60] and Castells-Nobau et al. [20] reported a subtle structural phenotype in a subset of MB Kenyon cells that did not express FoxP. As detailed above, two groups have reported FoxP expression in the MBs and it appears that some transcript can be found in MB Kenyon cells. With a substantial walking defect both in FoxP1955 mutant flies (which primarily affects FoxP-iB expression [18]) and in flies without any FoxP (figure 7), together with the MBs being critical for normal walking behaviour, the MBs were a straightforward candidate for a cell-type-specific FoxP-KO. However, flies without FoxP in the MBs walk perfectly normally (figure 9c). There are two possible reasons for this lack of an effect of our manipulation: either FoxP protein is not present in MBs or it is not important in MBs for walking. While at this point we are not able to decide between these two options, our expression data concurring with those from previous studies [20,21] suggest the former explanation may be the more likely one (see also above). Remarkably, a publication that did report FoxP expression in the MBs [35] did not detect the walking deficits in FoxP1955 mutant flies despite testing for such effects. Motor aberrations as those described here and in other FoxP manipulations [20–22,36] constitute a potential alternative to the decision-making impairments ascribed to these flies in DasGupta et al. [35].

DCNs were recently shown to be involved in the spatial component (landmark fixation) of walking in Buridan’s paradigm [41], but removing FoxP from DCNs showed no effect (figure 9b), despite abundant FoxP expression in DCNs (figure 9a). It is possible that a potential effect in stripe fixation may have been masked by already somewhat low fixation in both control strains. On the other hand, even at such control fixation levels, significant increases in stripe deviation can be obtained (e.g. Figure 10a). Before this is resolved, one explanation is that FoxP is not required in these neurons for landmark fixation in Buridan’s paradigm, while the neurons themselves are required.

Motor neurons are involved in all aspects of behaviour and have been shown to be important for operant self-learning [61]. With abundant expression of FoxP in motor neurons (figure 3a), we considered these neurons a prime candidate for a clear FoxP-KO phenotype. Indeed, removing FoxP specifically from motor neurons only, mimicked the effects of removing the gene constitutively from all cells (figure 10a,b). It is noteworthy that this manipulation alone was sufficient to affect both temporal and spatial parameters, albeit only one of the two driver lines showed clear-cut results (D42). One would not necessarily expect motor neurons to affect purportedly ‘higher-order’ functions such as landmark fixation. It is possible that the higher tortuosity in the trajectories of the flies where D42 was used to drive our UAS-gRNA construct is largely responsible for the greater angular deviation from the landmarks in these flies and that this tortuosity, in turn is caused by the missing
FoxP in motor neurons. Alternatively, D42 is also driving in non-motor neurons where FoxP is responsible for landmark fixation. The driver line C380 showed similar trends, albeit not quite statistically significant at our alpha value of 0.5%, suggesting that potentially the increased meander parameter may be caused by motor neurons lacking FoxP.

The protocerebral bridge is not only the arguably most conspicuous FoxP-positive neuropil, it has also been reported to be involved in temporal aspects of walking [93–95]. Moreover, the protocerebral bridge provides input to other components of the central complex involved in angular orientation [22,96–98]. Similar to the results in motor neurons, removing FoxP from a small group of brain neurons innervating the protocerebral bridge, phenocopies constitutive FoxP mutants.

Taken together, the motor neuron and protocerebral bridge results suggest that both sets of neurons serve their locomotor function in sequence. At this point, it is unclear which set of neurons precedes the other in this sequence.

5.4.3. FoxP is only required during larval development to ensure normal locomotion

There is ample evidence that the FoxP family of transcription factors acts during development in a variety of tissues [9,99,100]. What is less well known is if adult FoxP expression serves any specific function. A recent study in transgenic mice in operant conditioning and motor learning tasks showed postnatal knock-out of FOXP2 in cerebellar and striatal neurons affected leverpressing and cerebellar knock-out also affected motor-learning [90]. At least for these tasks in mammals, a FoxP family member does serve a postnatal function that is independent of brain development (brain morphology was unaltered in these experiments). Also in birds, evidence has been accumulating that adult FoxP expression serves a song plasticity function [101–106]. Our temporally controlled experiments (figures 8 and 11) suggest that at least locomotion in Buridan’s paradigm can function normally in the absence of FoxP expression in the adult, as long as FoxP expression remains unaltered during larval development. Future research on the role of FoxP in locomotion and landmark fixation hence needs to focus on the larval development before pupation.

Data accessibility. All original raw data are deposited with figshare (http://figshare.com), references in the text. All raw data are publicly accessible with an Attribution 4.0 International (CC BY 4.0) license. Data sources for specific figures are referenced in the text. A compressed file with all the data is also available [33].


Competing interests. We declare we have no competing interests.

Funding. This project was funded by the DFG (Deutsche Forschungsgemeinschaft, grant no. BR 1892/17-1).

Acknowledgements. Technical assistance was provided by Klara Krmpotic (Buridan experiments with FoxP1-IB-Gal4) and Marcela Loza. We are indebted to David Deitcher for sharing his FoxP antibody with us. We are grateful to three anonymous reviewers for their insightful suggestions, which significantly improved the quality of the manuscript.


