Atypical PKC and persistent FoxP expression are key molecular components of operant self-learning, a form of motor learning, in *Drosophila* wing motor neurons

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Abstract

Motor learning is not only a key invention of the many new animals evolving new body plans during the Cambrian, it is also central to human existence such as in learning to speak or walk, rehabilitation after injury, or sports. Evidence suggests that all forms of motor learning may share an evolutionary conserved molecular plasticity pathway. Here we present novel insights into the molecular processes underlying a kind of motor learning in the fruit fly *Drosophila*, operant self-learning. We have discovered that the Forkhead Box gene *FoxP* is not required in the fly brain for this type of learning. Instead, atypical protein kinase C (aPKC) appears to be a central component in a plasticity process that takes place in *FoxP*-expressing motor neurons in the ventral nerve cord. Using CRISPR/Cas9 to knock out canonical aPKC interaction partners *bazooka* and the kidney and brain gene (KIBRA) in adult animals, we found that aPKC likely acts via non-canonical pathways in this form of learning. We also found that 14 but not 7 days after CRISPR/Cas9-mediated knockout of *FoxP* in adult animals, learning is impaired, suggesting that adult *FoxP* expression is required for operant self-learning.

1. Introduction

Motor learning is an essential component of human behavior and ubiquitous throughout the animal kingdom. The process of learning a motor skill can be influenced by a number of factors, such as the amount of training, the type of feedback or the presence/absence of environmental cues. Regaining lost motor functions after brain or spinal cord injury is considered a crucial component of rehabilitation. Human language is acquired by a form of motor learning and motor learning also appears to be a key invention that allowed the newly evolved animals in the Cambrian to become ambulatory (Keijzer et al., 2013; Arendt, 2021; Jékely, 2021). Despite the central role of motor learning for all animals including humans, compared to other forms of learning, relatively little is known about the molecular mechanisms of motor learning: how are which neurons modified to improve behavior?

Vocal learning such as learning to speak, is a form of motor learning that involves the Forkhead Box transcription factor family P (FoxP) in vertebrates (Lai et al., 2001; Zhang et al., 2002; Haesler et al., 2007; Fisher and Scharff, 2009; Takahashi et al., 2009; Bolhuis et al., 2010; Teramitsu et al., 2010; Scharff and Petri, 2011; French et al., 2012; Day et al., 2019;

Co et al., 2020). Other, non-vocal forms of motor learning also involve FoxP genes in vertebrates (Groszer et al., 2008; Schreiweis et al., 2014; French et al., 2019; Lozano et al., 2021) and invertebrates (Mendoza et al., 2014). Vocal learning also shares the involvement of protein kinase C (PKC) with other forms of motor learning (Sakaguchi and Yamaguchi, 1997; Metzger and Kapfhammer, 2003; Rochefort et al., 2011; Colomb and Brembs, 2016; Gallimore et al., 2018), raising the possibility of a conserved motor learning pathway extending beyond these two components.

Here we provide further evidence about the specific manner in which FoxP and PKC are involved in a form of motor learning in the fruit fly *Drosophila*, operant self-learning at the torque meter. In this experiment, motor learning doubly dissociates from other forms of learning such that genes involved in motor learning are not involved in other forms of learning and vice versa (Brembs and Plendl, 2008; Colomb and Brembs, 2010; Brembs, 2011). At the torque meter, a fly is tethered between head and thorax such that it can move all other appendages. When beating its wings, it generates forces that the torque meter measures. Specifically, it measures torque around the vertical body axis, yaw torque (Götz, 1964). Even in the absence of any guiding cues, flies can learn to associate one torque

domain (e.g., roughly corresponding to left or right, respectively turning maneuvers) with a punishing heat beam (Wolf and Heisenberg, 1991). This experiment not only conceptually mimics other motor learning paradigms in that feedback is made immediately contingent on specific motor actions, but also via its dependence on FoxP and PKC genes (Mendoza et al., 2014; Colomb and Brembs, 2016). This form of motor learning has been termed operant self-learning to distinguish it from other forms of operant learning and to denote that the subject is learning about its own behavior (Colomb and Brembs, 2010). Most other forms of operant learning include a guiding cue, be it a manipulandum or other stimulus signaling reinforcement. Adding a color cue predicting the heat beam concomitantly with the yaw torque behavior to the self-learning paradigm, it was discovered that in such circumstances, *FoxP* and PKC manipulations no longer disrupted learning. Instead, well-known genes involved in other forms of learning are required (Brembs and Plendl, 2008). Apparently, the animal preferentially learns the color cue when both are available, engaging a conserved world-learning mechanism (i.e., a mechanism that mediates learning about relationships in the world around the subject)(Brembs, 2009).

For operant self-learning, it is not known in which neurons the *FoxP* gene is required,

and which PKC gene is involved. It is also unknown which pathway is engaged by PKC and if FoxP expression is required also in adult flies for this form of motor learning. In this work, we address all three research questions.

2. Materials and Methods

2.1. Strains and fly rearing

If not stated otherwise, flies were raised on standard cornmeal/molasses medium (Guo et al., 1996) at 25°C and 60% humidity at a 12-hour light/dark cycle. For experiments requiring the expression of temperature sensitive Gal80, animals were raised at 18°C. To set up crosses for behavioral experiments, 20 females were placed together with five to eight males and were allowed to lay eggs for 24 hours. They were flipped daily into fresh vials, ensuring appropriate larval density. Flies were prepared the day before the experiment, allowing them time to recover. 24 to 48 hours old female flies were briefly immobilized using cold anesthesia. A thin triangular copper hook (0,05 mm diameter) was glued (3m Espe Sinfony, 3M Deutschland GmbH) between head and thorax, fixing both body parts to each other (Brembs, 2008). Each animal was kept individually in a small moist chamber with a few grains of sugar. For the *UAS-PKCi* experiments flies received a heat-shock at 35°C for four

hours before the experiment. For *tub-Gal80* expression, animals were raised at 18°C and placed at 30°C for two days. Experiments were always performed at room temperature. For experiments using the gene-switch system, newly hatched flies were placed on *Drosophila* instant medium (351.204, Schlüter Biologie, Eutin-Neudorf, Germany) containing the steroid hormone RU486 (200 µg/ml, CAS No.: 84371-65-3, Sigma-Aldrich, St. Louis, MO) for two days.

The UAS-g-KIBRA line was generated in the laboratory of Michael Krahn, Universität Münster. The cloned the following guides from KIBRA Intron-2: GTACTTACGACTGCTTCGAC and KIBRA Intron-4: GGGCACCGTG-CAGATCAGCA in pCDF6 and inserted in attP40.

Genotype	use	Bloomington	Flybase
;;nSyb-GS	driver line	80699	FBti0201287
;;GMR55G08-GAL4	driver line	50422	FBti0136906
;;GMR65A06-GAL4	driver line	39330	FBti0137511
;;GMR20H05-GAL4	driver line	47896	FBti0133817
;;ato-Gal4	driver line		
ELAV-Gal4;;	driver line		
ELAV-Gal4;Tub-Gal80ts;;	driver line		
;;FoxP-iB-Gal4/TM3	driver line		
;;UAS-g-aPKC	effector line	85862	FBti0210993
;;UAS-g-BAZ	effector line	84234	FBti0207133
;;UAS-g-PKC53e	effector line	76612	FBti0194968
;UAS-g-KIBRA	effector line		
;;UAS-Cas9;	effector line		
;;UAS-PKCi	effector line	4589	FBti0010565
;;UAS-t:gRNA(4xFoxP)	effector line		
;UAS-Cas9;;	effector line		
C380-Gal4;;	driver line	80580	FBti0016294
y[1] w[*]; Mi{Trojan GAL4.un} aPKC[MI10848-TG4.un]/SM6a	driver line	77814	FBti0196316
Wild type Berlin	wild type strain		

Table 1: Fly strains used in this work

2.2. Experimental setups

Two different torque meters and setups were used. Both torque meters were described

previously: The ‘Shiming’ meter (Tang and Juusola, 2010) and the ‘Götz’ meter (Götz, 1964)(RRID:SCR_017276), named after the authors. With flies attached to the torque meter via a clamp, both devices measure the rotational force (torque) around the animal’s vertical body axis. While the Götz meter is older than the Shiming meter, due to its rotational compensation it is technically more advanced and was included in a more modern version of the setup which was used in the later experiments, after the first data was collected using the Shiming setup. In both setups, the animal is surrounded by a cylindrical panorama (arena; diameter 58 mm), homogeneously illuminated from behind by either a projector (Götz: DLPLCR4500EVM, Texas Instruments) or a halogen lamp (Shiming: OSRAM 100W/12V), such that stationary flight in a controlled environment was achieved. An infrared laser (StockerYale Lasiris SNF series; 825 nm, 150 mW) was used as punishment in both setups. The laser was pointed from above onto the animal’s head, pulsed (approx. 200ms pulse width ~4 Hz) and the intensity adjusted empirically for maximal heat avoidance and learning. The experiment is fully computer controlled, using custom software (Shiming: LabView, National Instruments, RRID:SCR_014325. Götz: DOI: 10.5281/zenodo.7102195).

2.3. Experimental sequence

Before each self-learning experiment, the yaw torque range was adjusted using optomotor stimuli for each fly tethered to the torque meter. For the 'Shiming' setup, arena rotation for the optomotor stimulus was operated by hand. The direction of the rotation was reversed after the fly reached its peak optomotor torque. During optomotor presentations, the torque was adjusted to be zero-symmetric. Optomotor stimuli were presented automatically for flies in the 'Götz' setup. Because of this difference, experiments recorded with the Shiming device did not record optomotor periods such that their periods are numbered from 1 to 9 (Table 2), while optomotor periods for the Götz setup were included, such that the periods were numbered 1-17 (Table 3). Because we used four optomotor periods in the Götz setup, periods 5 to 13 are the periods in which self-learning was studied in this setup.

The main self-learning experiment then consisted of nine periods of two minutes duration. The laser was off for the first two periods, so that the fly could freely choose its direction of flight without punishment. In the following two training periods either the left or the right torque domain was associated with the punishing laser. The punished torque domain was alternated between experiments. The first two

training periods were followed by one test period without punishment. Afterwards, the fly was trained again with the same side punished as before for another two 2-min. periods. Finally, no heat was applied in the final two test periods, allowing the fly to express its spontaneous yaw torque preference. Figures always show the preference in the first test period after the last training period, i.e., period 8 in the Shiming setup and period 12 in the Götz setup (Tables 2, 3). Axis labels in the figures merely reflect this difference between the two setups, while the same first test period after the last training period was tested for learning in each case.

Period 1	**Period 2**	**Period 3**	**Period 4**	**Period 5**	**Period 6**	**Period 7**	**Period 8**	**Period 9**
Pretest	Pretest	Training	Training	Test	Training	Training	Test	Test
No heat	No heat	**Heat**	**Heat**	No heat	**Heat**	**Heat**	No heat	No heat

Table 2: Experimental sequence "Shiming Setup"

OM before				Experiment									OM after			
Period 1	Period 2	Period 3	Period 4	Period 5	Period 6	Period 7	Period 8	Period 9	Period 10	Period 11	Period 12	Period 13	Period 14	Period 15	Period 16	Period 17
OM	OM	OM	OM	Pretest	Pretest	Training	Training	Test	Training	Training	Test	Test	OM	OM	OM	OM
No heat	No heat	No heat	No heat	No heat	No heat	**Heat**	**Heat**	No heat	Heat	Heat	No heat	No heat	No heat	No heat	No heat	No heat

Table 3: Experimental sequence "Götz Setup". OM - optomotor

2.4. Data handling and statistical analysis

2.4.1. Data selection

To ensure proper punishment by the laser, each fly was exposed to the laser after the experiment, to ensure it was correctly adjusted. If the fly survived the laser for 15 seconds or more, the data were excluded from analysis. Data that did not show any or shifted optomotor responses, indicating either an unhealthy fly or an error with the measuring device, were also excluded. Data were also excluded if the fly did not experience the laser at least once during training. Lastly, flies with poor flight performance (constant stopping of flight) were also excluded from analysis. While these data were excluded from analysis, most of them were included in the published data folders, such that the inclusion criteria can be

independently tested for their effects on our results.

2.4.2. Data availability and analysis

The preference of a fly for right or left torque domain was quantified as the performance index $PI = (ta - tb)/(ta + tb)$. During training periods, tb indicates the time the fly is exposed to the heat and ta the time without heat. During tests, ta and tb refer to the times when the fly chose the formerly (or subsequently) unpunished or punished situation, respectively. Thus, a PI of 1 means the fly spent the entire period in the situation not associated with heat, whereas a PI of −1 indicates that the fly spent the entire period in the situation associated with heat. Accordingly, a PI of zero indicates that the fly distributed the time evenly between heated and non-heated situations and a PI of 0.5 indicates that 90 of the 120 s in that period were spent in the unpunished situation.

All data is analyzed using R (R Project for Statistical Computing) (RRID:SCR_001905). The collection of R-scripts evaluating the time series data can be found at 10.5281/zenodo.7101742. The data model pertaining to the XML raw data files and the YAML data set files can be found at 10.5281/zenodo.7101734. In brief, the XML data files contain both the meta-data for each single fly experiment as well as the time series data covering the entire

experiment. The single YAML file per dataset contains the experimental design, such as which data files belong to which experimental group, the kind of statistics to be performed, significance levels used, experimenter comments and data inclusion/exclusion. The main R-Script reads the YAML dataset files and performs the appropriate computations for quality control, analysis and statistics.

Quality control is performed on each single fly XML file and included in the published data sets. Each single fly experiment XML file is thus accompanied by a single fly HTML quality control report sheet containing both plots of the raw time series data as well as a number of evaluations necessary to assess the proper execution of the experiment and the quality of the resulting data.

Data analysis and statistics for each dataset are reported in an HTML dataset evaluation sheet. A complete dataset thus consists of one XML raw data file and one HTML quality control sheet for each fly, plus a single YAML dataset file and one HTML dataset evaluation sheet.

The datasets were published using a custom Python script (DOI: 10.5281/zenodo.7101741) that synchronizes the collected data on the local computer with the University of Regensburg publication server. Persistent

identifiers for each dataset are listed in the figure legends.

2.4.3. Statistics

PIs are statistically evaluated in two complementary ways, using both frequentist and Bayesian statistics. Following previous studies (Liu et al., 1999, 2006; Brembs and Hempel de Ibarra, 2006; Brembs and Wiener, 2006; Brembs and Plendl, 2008; Brembs, 2009), individual PIs of the first test after the last training period are tested against zero to evaluate the ability of the manipulated flies to show a preference towards the unpunished torque domain. We used a Wilcoxon test in the frequentist case and computed a Bayes Factor for the Bayesian version. We set the alpha value for the Wilcoxon test at 0.5% as suggested by (Benjamin et al., 2018), such that p-values below 0.005 and Bayes factors above 5 for the same group of flies would be considered compelling evidence that the flies were able to learn. Conversely, Bayes Factors below one together with p-values higher than 0.05 were considered evidence that the flies were not able to show self-learning. Finally, groups where the two statistics were in conflict or intermediate, were considered inconclusive. Thus, both the Bayesian and the frequentist criteria had to be met in order to claim that a genetic manipulation interfered with operant self-learning.

3. Results

3.1. Temporal genome editing: Self-learning impaired only after a 2-week *FoxP* knockout

FoxP was shown to be important for normal development (Castells-Nobau et al., 2019; Palazzo et al., 2020). Strong motor impairments were reported after a developmental *FoxP* knock-out, for instance rendering the animals unable to fly. Since the ability to fly is a basic requirement for torque learning, we used a CRISPR/Cas9-based approach to pan-neuronally knock out *FoxP* in adult flies. No effect was observed on self-learning two days after the start of the knock-out induction (Fig 1 A), but waiting for 14 days after the pan-neural knock-out yielded a significant self-learning impairment (Fig. 1B). As a test seven days after induction was also without effect (DOI: 10.5283/epub.52965), self-learning remains functional for at least 7-14 days after cessation of *FoxP* gene transcription in all neurons.

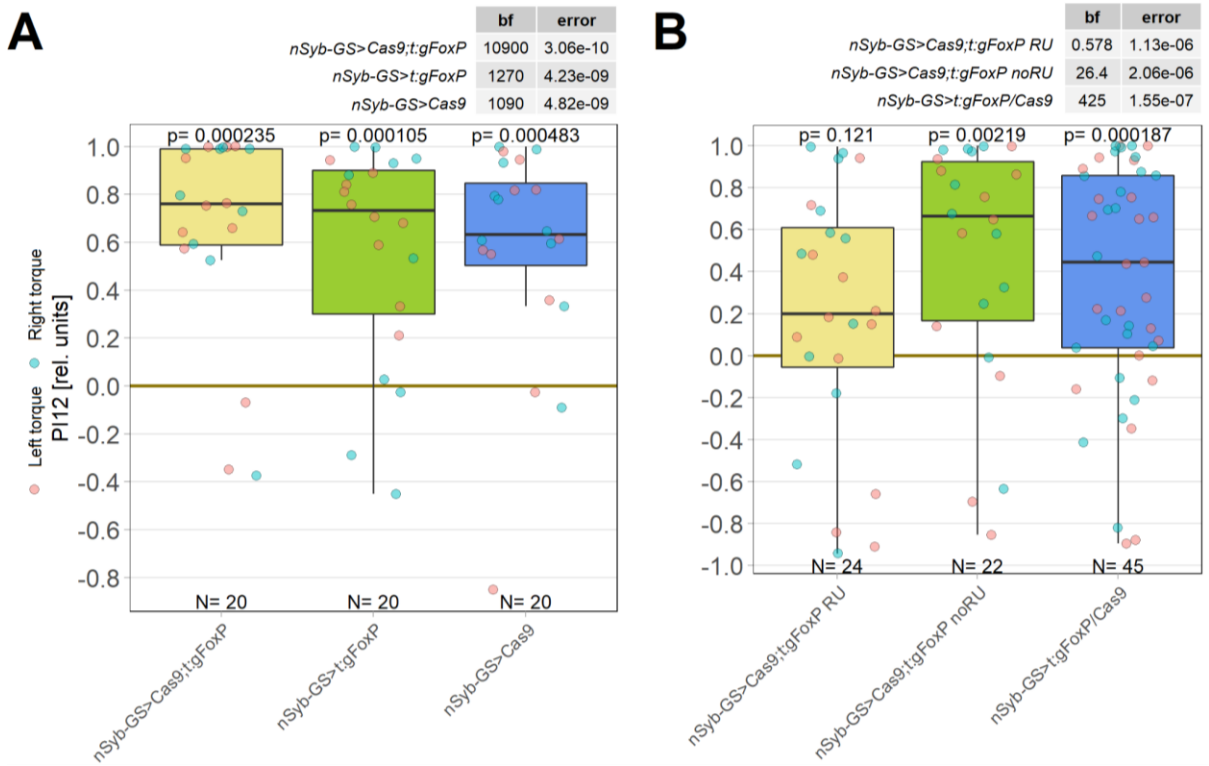


Figure 1: Knockout of *FoxP* in adult *Drosophila* shows learning impairments only after 14 days.

Plotted are performance indices for the first period after training (PI12). Each colored dot represents a single fly experiment. Red dots denote experiments where the fly was punished on its left turning torque domain, blue dots denote flies that were punished on their right turning domain. Box plots denote medians, quartiles and non-outlier range. Statistical analyses test for differences of PIs against zero.

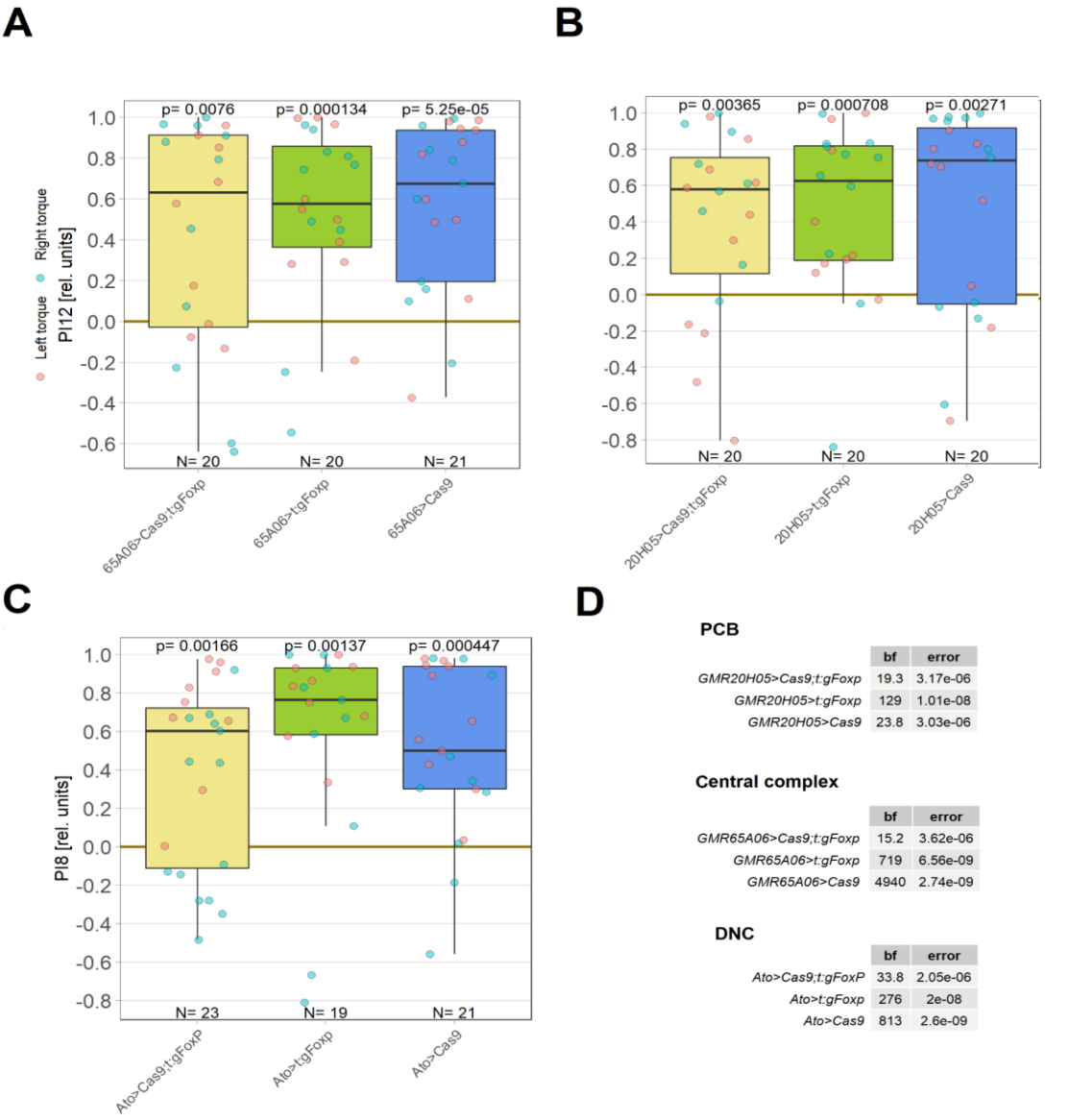
A. Self-learning two days after *FoxP* knock-out induction with RU486. Experimental animals (left, yellow) as well as gRNA (middle, green) and Cas9 (left, blue) control animals show all high PIs as well as large Bayes Factors above 1000 and p-values below 0.005, indicating that all groups showed unimpaired self-learning. Data: 10.5283/epub.52963

B. Self-learning 14 days after *FoxP* knock-out induction with RU486 and 12 days after cessation of RU486 administration. Experimental animals (left, yellow) show low PIs as well as a Bayes Factor of less than one together with a large p-value, whereas both the genetic control animals without RU486 treatment (middle, green) and the pooled RU486-treated gRNA and Cas9 controls (right, blue) show high PIs, large Bayes Factors and p-values smaller than 0.005, indicating an impairment in self-learning only in the experimental group. Data: 10.5283/epub.52964

3.2. Spatial genome editing: *FoxP* likely not required in the brain

After we established that self-learning remains intact up to 7-14 days without *FoxP* gene expression, we sought to identify brain

regions where FoxP gene expression was necessary for operant self-learning. As FoxP mutants show an impairment in self-learning, spatial CRISPR/Cas9-based genome editing was performed without restricting the manipulation to the adult stage. At the time these experiments were performed, no driver lines with overlapping expression in the Saddle and Vest regions were available. Therefore, we tested driver lines expressing in the protocerebral bridge (PCB, Fig. 1A), the PCB and adjacent central complex neuropils (see Materials and Methods for details, Fig. 2B) and the dorsal cluster neurons (Fig. 2C). No self-learning impairment was observed following *FoxP* knockout in these brain areas (Fig. 2), suggesting that FoxP expression is not necessary in these areas for operant self-learning.



PCB

	bf	error
GMR20H05>Cas9;t:gFoxp	19.3	3.17e-06
GMR20H05>t:gFoxp	129	1.01e-08
GMR20H05>Cas9	23.8	3.03e-06

Central complex

	bf	error
GMR65A06>Cas9;t:gFoxp	15.2	3.62e-06
GMR65A06>t:gFoxp	719	6.56e-09
GMR65A06>Cas9	4940	2.74e-09

DNC

	bf	error
Ato>Cas9;t:gFoxP	33.8	2.05e-06
Ato>t:gFoxp	276	2e-08
Ato>Cas9	813	2.6e-09

Figure 2: For operant self-learning, *FoxP is likely* not required in the brain.

Plotted are performance indices for the first period after training (PI8/12). Each colored dot represents a single fly experiment. Red dots denote experiments where the fly was punished on its left turning torque domain, blue dots denote flies that were punished on their right turning domain. Box plots denote medians, quartiles and non-outlier range. Statistical analyses test for differences of PIs against zero (above plots and D).

A. Flies with *FoxP* knocked out in the protocerebral bridge (left. yellow) as well as the gRNA (middle, green) and Cas9 (right, blue) control flies show high PIs, large Bayes factors (D) and small p-values, indicating their self-learning is intact. Data: 10.5283/epub.52956

B. Flies with *FoxP* knocked out in the protocerebral bridge and additional components of the central complex (left. yellow) as well as the gRNA (middle, green) and Cas9 (right, blue) control flies show high PIs, large Bayes factors (D) and small p-values, indicating their self-learning is intact. Data: http://doi.org/10.5283/epub.52951

C. Flies with *FoxP* knocked out in the dorsal cluster neurons (left. yellow) as well as the gRNA (middle, green) and Cas9 (right, blue) control flies show high PIs, large Bayes factors (D) and small p-values, indicating their self-learning is intact. Data: 10.5283/epub.52946

D. Bayesian statistics for the three datasets.

It was reported previously that protein kinase C (PKC)-dependent plasticity is required in motor neurons for operant self-learning (Brembs and Plendl, 2008; Colomb and Brembs, 2016). As *FoxP* is expressed in motor neurons, we knocked *FoxP* out in motor neurons using two different driver lines, C380 and D42. However, CRISPR/Cas9-mediated *FoxP* knock-out in motor neurons disrupted flight-performance of manipulated flies to an extent that precluded any torque learning experiments.

3.3. Self-learning requires a*PKC* in *FoxP* motor neurons

Colomb and Brembs (2016) discovered that blocking all *PKC* isoforms in neurons using the inhibitory peptide PKCi abolished operant self-learning. We replicated their results by expressing PKCi pan-neuronally both during development and restricted to adult flies. Expression during development did not impair self-learning, while adult expression abolished self-learning (Fig. 3A). Expressing PKCi only in cells expressing isoform B of the FoxP gene also abolished self-learning, suggesting that PKC activity is required in FoxP-positive neurons.

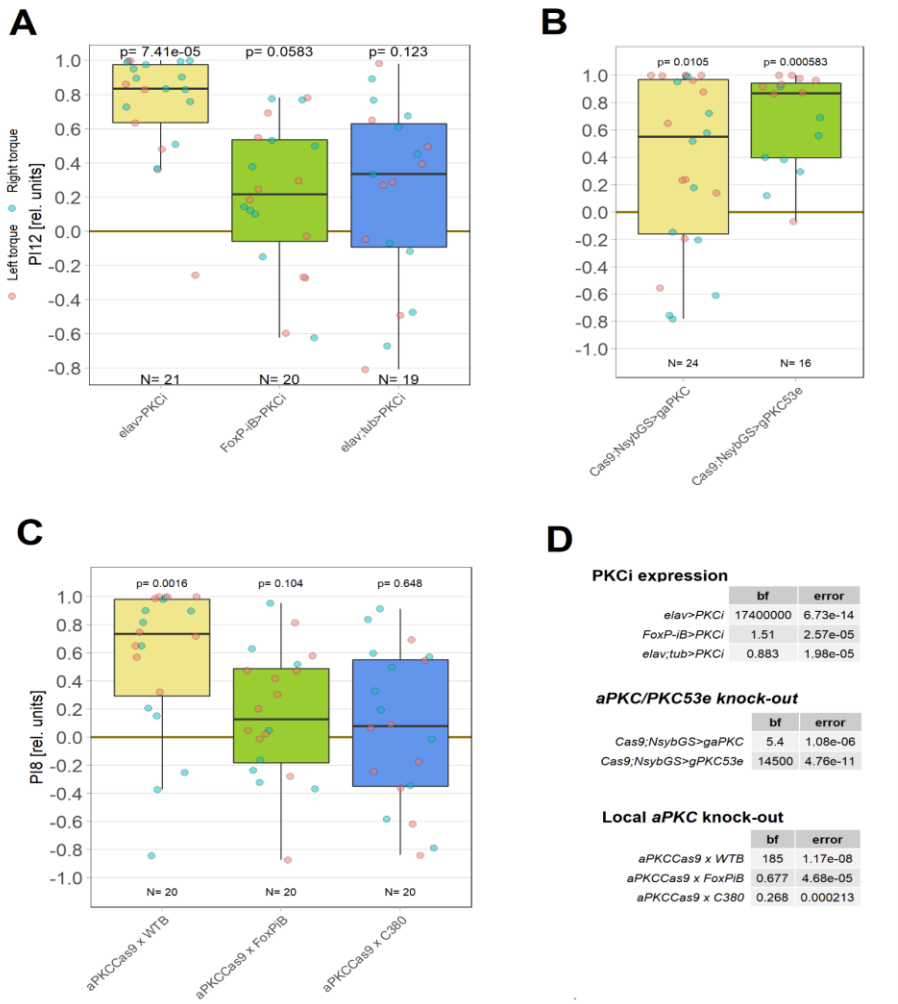


Figure 3: For operant self-learning, *aPKC* is required in motor neurons.

Plotted are performance indices for the first period after training (PI8/12). Each colored dot represents a single fly experiment. Red dots denote experiments where the fly was punished on its left turning torque domain, blue dots denote flies that were punished on their right turning domain. Box plots denote medians, quartiles and non-outlier range. Statistical analyses test for differences of PIs against zero.

A. Inhibiting all protein kinase C isoforms with the inhibitory peptide PKCi. Constitutive, pan-neuronal expression PKCi (left, yellow), leads to high PIs and a large Bayes Factor, indicating this manipulation left self-learning intact. Expressing PKCi either in FoxP-isoform B positive neurons (middle, green), or in all neurons but restricted to adulthood (right, blue) yields low PIs, high p-values and low Bayes factors, indicating self-learning is impaired. Data: 10.5283/epub.52958

B. Pan-neuronal knock-out of two different PKC genes with CRISPR/Cas9 in adulthood suggests aPKC is necessary for operant self-learning. Knocking out atypical PKC (yellow, left) yields moderate PIs, p-values and Bayes factors, indicating some effect on operant self-learning, while the high PIs, low p-values and high Bayes factor of the group where PKC53E was knocked out (right, green) indicate their self-learning is intact. Data: 10.5283/epub.52957

C. Knocking out aPKC in motor neurons or *FoxP*-neurons impairs operant self-learning. Expressing the CRISPR/Cas9 components either in *FoxP* isoform B-positive neurons (green, middle) or in motor neurons (blue, right) leads to low PIs, high p-values and low Bayes Factors, indicating their self-learning is strongly impaired. Control flies with only the CRISPR/Cas9 genetic elements but no driver, show high PIs, a low p-value and a high Bayes Factor, indicating their self-learning is intact. Data: 10.5283/epub.52944

D. Bayesian statistics for the three datasets.

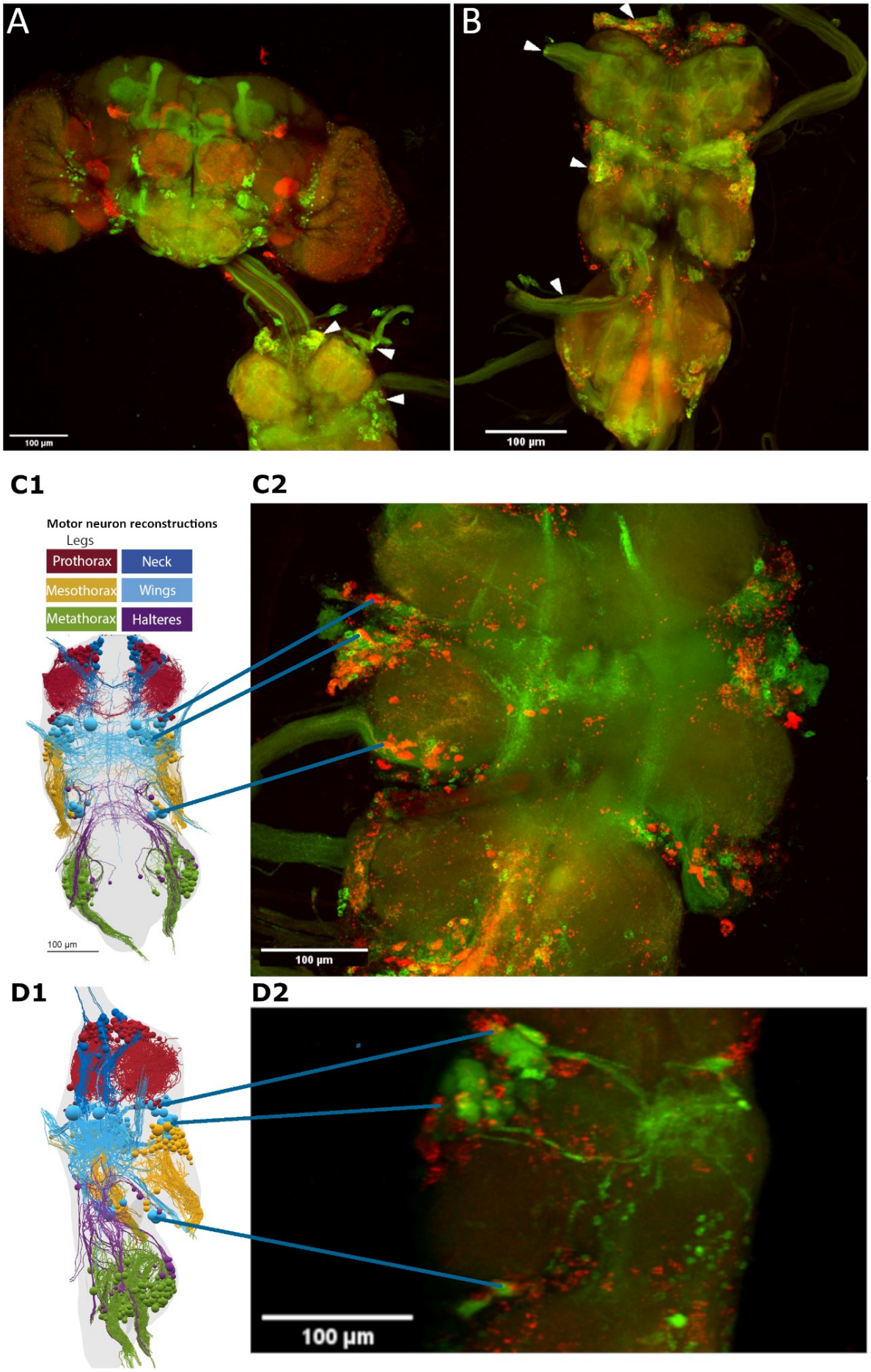
Screening RNA-Seq databases for PKC genes expressed in motor neurons and restricting any candidates to those where gRNA lines were available for CRISPR/Cas9-mediated gene knock-out, yielded only two genes: the atypical PKC (aPKC) and the diacylglycerol-activated PKC53E. Knocking out each gene panneuronally in adult flies and testing the manipulated animals for operant self-learning showed excellent learning performance in the PKC53E-manipulated flies (Fig. 3B), clearly ruling out PKC53E as the gene involved in operant self-learning. The performance of the aPKC-manipulated flies was somewhat lower, not reaching our criteria for significant learning, but at the same time too high to be confident in the result (Fig. 3B).

As PKC activity is required in motor neurons, we limited the aPKC knock-out to these neurons and this abolished self- learning (Fig. 3C). Testing the hypothesis articulated above that PKC activity is required in FoxP neurons, we also knocked out aPKC in FoxP neurons, which also abolished self-learning. (Fig. 3C).

3.4. *aPKC* and *FoxP* are co-expressed in wing motor neurons

The behavioral data presented above thus support the hypothesis that the plasticity mediating operant self-learning takes place in neurons that co-express both *FoxP* and *aPKC*.

Whole-mount confocal microscopy of fly central nervous systems expressing *aPKC*-Gal4 and *Foxp*-LexA suggest that neurons expressing both *aPKC* and *FoxP* exist only in the ventral nerve cord (Fig. 4A, B). We identified co-expressing neurons in all neuromers of the ventral nerve cord, with the ventral location of the mesothoracic *aPKC/FoxP* neurons suggesting potential wing motor neurons (Fig. 4C)(Phelps et al., 2021). To ascertain the identity of this ventral cluster of co-expressing neurons, we marked all motor neurons with the driver line D42-Gal4 and used FoxP-LexA to stain all *FoxP* neurons. With these labels, we identified a ventral sub-population of specific wing motor neurons expressing *FoxP*, which match the location of the *aPKC/FoxP* neurons identified before (Fig. 4D). In an initial analysis, we confirmed that at least some direct wing muscles, responsible for, e.g., wing amplitude modulations giving rise to some of the yaw turning forces, are innervated by motor neurons that co-express *FoxP* and aPKC (Fig. 5).


A
100 µm
B
100 µm
C1
Motor neuron reconstructions
Legs
Prothorax
Neck
Mesothorax
Wings
Metathorax
Halteres
100 µm
C2
100 µm
D1
D2
100 µm

535

Figure 4: FoxP and aPKC coexpression in motor neurons. Confocal stacks of whole mount preparations of central nervous systems; A-C: green - aPKC-Gal4>CD8::GFP, red - FoxP-LexA>CD8::RFP; D: green - D42-Gal4>CD8::GFP, red - FoxP-LexA>CD8::RFP. Confocal image stacks available at:

A. Adult brain (top) with ventral nerve cord (VNC, bottom) attached. No coexpressing cells can be observed in the brain, while such neurons(yellow) are readily observable in all neuromers of the VNC (arrowheads).

B. VNC with *aPKC/Foxp* coexpression (yellow) both in cell bodies and fiber tracts in nerves (arrowheads).

C. C1: Dorsal view of motor neuron reconstruction (modified from (Phelps et al., 2021). C2: Confocal image stack of dorsal view of the mesothoracic neuromer with putative wing motor neurons expressing both *aPKC* (green) and *FoxP* (red) marked.

D. D1: Lateral view of motor neuron reconstruction (modified from (Phelps et al., 2021). D2: Confocal image stack of mesothoracic neuromer with all motor neurons (green) and FoxP neurons (red) marked. This lateral view supports the hypothesis that the ventral cluster of *aPKC/FoxP* neurons indeed comprises wing motor neurons.

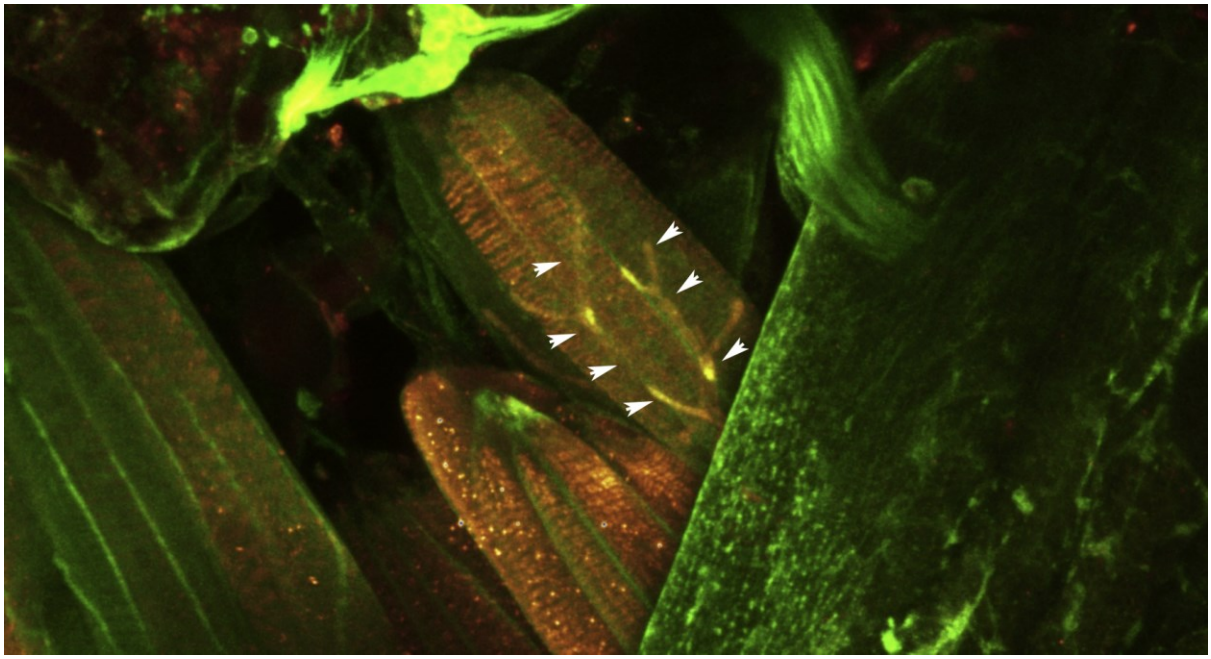


Figure 5. Direct wing muscles innervated by FoxP/aPKC co-expressing motor neurons. Preparation of a direct wing muscle from transgenic flies expressing in green aPKC-Gal4>CD8::GFP and in red FoxP-LexA>CD8::RFP. Yellow terminals on the muscle (arrowheads) indicate co-expressing neurons innervating the muscle. Image: Carsten Duch, Universität Mainz.

3.5. Canonical *aPKC* pathways not involved in self-learning

Having established that *aPKC* is required in *FoxP*-positive motor neurons, we next sought to identify further components of the *aPKC*-dependent plasticity underlying operant self-learning in *Drosophila*. It is not uncommon for plasticity mechanisms in the adult animal to recruit genes with a function during neuronal development (Greenspan, 1995; Mayford and Kandel, 1999; Leslie and Nedivi, 2011). A prominent interaction partner of *aPKC* during *Drosophila* nervous system development is *bazooka* (*baz*), a crucial component of the highly conserved PAR complex (Pichaud, 2018; Thompson, 2022). However, knocking out *baz* in all adult neurons did not disrupt operant self-learning (Fig. 6A), suggesting the Par complex signaling pathway is not involved in operant self-learning. A second prominent aPKC interaction partner is the kidney and brain protein (KIBRA) acting in the conserved Hippo pathway (Yoshihama et al., 2011, 2012) and also proposed to be involved in learning/memory (Sacktor, 2012; Heitz et al., 2016; Zlomuzica et al., 2018; Ferguson et al., 2019; Ji et al., 2019). Knocking out KIBRA in all adult neurons also did not disrupt operant self-learning (Fig. 6B).

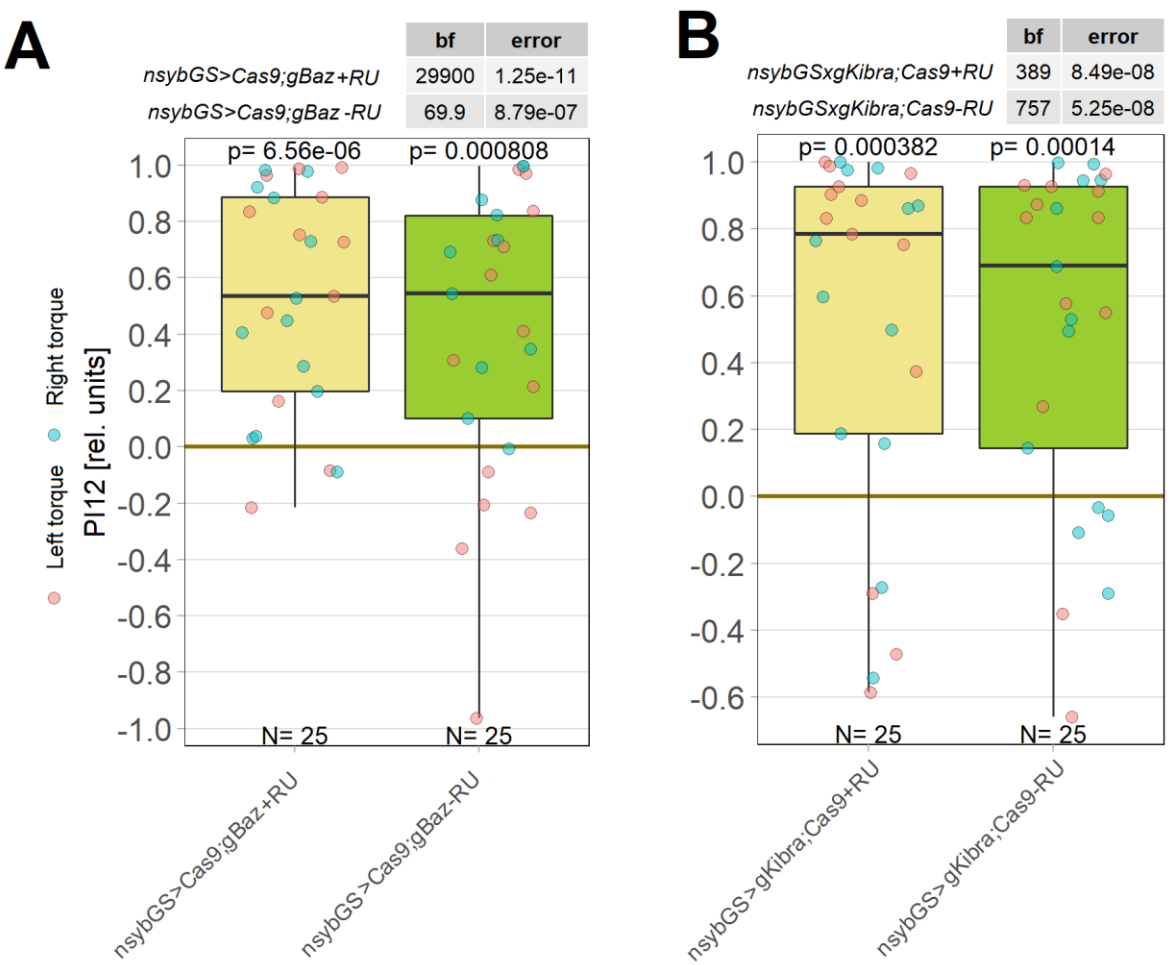


Fig. 6: Neither *bazooka* nor KIBRA are involved in operant self-learning.

Plotted are performance indices for the first period after training (PI12). Each colored dot represents a single fly experiment. Red dots denote experiments where the fly was punished on its left turning torque domain, blue dots denote flies that were punished on their right turning domain. Box plots denote medians, quartiles and non-outlier range. Statistical analyses test for differences of PIs against zero.

A. Knocking out *bazooka* using CRISPR/Cas9-mediated genome editing in adult animals has no effect on operant self-learning. Both flies that were fed the steroid hormone RU486 (left, yellow) and the genetically identical flies without the hormone (right, green), show high PIs, low p-values and high Bayes Factors, indicating a preference for the unpunished torque domain. Data:

B. Knocking out KIBRA using CRISPR/Cas9-mediated genome editing in adult animals has no effect on operant self-learning. Both flies that were fed the steroid hormone RU486 (left, yellow) and the genetically identical flies without the hormone (right, green), show high PIs, low p-values and high Bayes Factors, indicating a preference for the unpunished torque domain. Data:

4. Discussion

Operant self-learning in *Drosophila* is a form of motor learning that appears to be conserved among bilaterians. The transcription factor *FoxP* is involved in various forms of motor learning in chordates (Lai et al., 2001; Haesler et al., 2007; Groszer et al., 2008; Fisher and Scharff, 2009; Bolhuis et al., 2010; French et al., 2012, 2019; Schreiweis et al., 2014; Day et al., 2019; Co et al., 2020), as is PKC (Sakaguchi and Yamaguchi, 1997; Watanabe et al., 2002, 2006; Metzger and Kapfhammer, 2003; Yoshida et al., 2003; Sakaguchi, 2004; Gallimore et al., 2018). PKC is also involved in motor learning in the feeding behavior of the lophotrochozoan *Aplysia (Lorenzetti et al., 2008)* and both are involved in motor learning in the ecdysozoan *Drosophila* (this work and (Brembs and Plendl, 2008; Mendoza et al., 2014; Colomb and Brembs, 2016). This wealth of evidence supports a 'deep homology' for motor learning in bilaterians (Scharff and Petri, 2011).

In this work, we present three new insights into the neurobiological mechanisms underlying this form of motor learning.

4.1. Self-learning requires *aPKC* in *FoxP* wing motor neurons

While previous evidence suggested that some PKC activity was required in motor neurons for operant self-learning (Colomb and

Brembs, 2016), it was not clear which PKC gene was involved and in which motor neurons. Improving upon the previous attempts to identify the responsible PKC gene, here we used the CRISPR/Cas9 technique to first rule out that *PKC53e* is involved and then show that the atypical PKC (aPKC) is necessary for operant self-learning (Fig. 3). Restricting transgenic manipulations to *FoxP* isoform-B positive neurons and using anatomical experiments to confirm behavioral results suggesting that *FoxP* is not necessary in the brain for operant self-learning (Figs. 3, 4), we now hypothesize that *aPKC* activity is required in *FoxP*-positive wing motor neurons in the ventral nerve cord (VNC). An initial analysis of direct wing muscles, responsible for, e.g. wing beat amplitude (a major contributing factor to yaw torque), revealed *aPKC/FoxP* co-expressing neurons innervating these muscles (Fig. 5), corroborating our hypothesis. A second interesting, but so far unstudied population of *aPKC/FoxP* co-expressing neurons resides in the abdominal neuromer of the VNC. As flies use their abdomen analogously to a rudder during turns in flight (Götz et al., 1979; Zanker, 1988), involvement of these neurons seems plausible in addition to wing motor neurons.

While few studies in insects have shown motor neuron plasticity, the *Aplysia* sensorimotor synapse is a classical model for research on

plasticity mechanisms. There is a rich literature on motor neuron plasticity in this preparation, some of which reports PKC-dependent mechanisms (Cai et al., 2008; Fulton et al., 2008; Glanzman, 2008; Villareal et al., 2009; Reissner et al., 2010; Jin et al., 2011; Bougie et al., 2012; Choi et al., 2014; Hu et al., 2015, 2017a; Ferguson et al., 2019; Alexandrescu and Carew, 2020). Also in mammals (including humans) motor neuron plasticity in the spinal cord is a readily observable phenomenon in non-clinical and clinical settings (Wolpaw and Lee, 1989; Wolpaw et al., 1989; Carp and Wolpaw, 1994; Carp et al., 2001; Wang et al., 2006; Wolpaw, 2012; Eftekhar et al., 2018; Kaneko et al., 2022; Rochat et al., 2022; Simonyan et al., 2022). The discovery of aPKC-dependent plasticity in *Drosophila* motor neurons expands this body of literature to a genetically tractable organism and inasmuch as clinical practice relies on motor neuron plasticity, may even help instruct the development of clinical applications.

So far, in flies, PKC activity has only been shown to be important for memory consolidation/maintenance in world-learning experiments, but not for learning/acquisition (Kane et al., 1997; Drier et al., 2002). Similarly, in different forms of world-learning in *Aplysia*, aPKC is thought to be involved in memory consolidation or maintenance, but leaving learning intact (Michel et al., 2011; Bougie et al., 2012; Hu

et al., 2017a, 2017b; Lyons et al., 2017; Sacktor and Hell, 2017). Also in vertebrates, most reports cite the role of PKC in memory, rather than learning (Sacktor, 2012; Sacktor and Hell, 2017; Gao et al., 2018). While in some of these processes the type of PKC is not known, in others atypical PKCs are involved and in again others classical PKCs. In the literature, there does not appear to be a clear picture of a single form of PKC-mediated plasticity, even though several strands of similarity can be discerned between processes. Notably, the one other preparation where PKC activity has been linked to learning, is also conceptually most closely resembling the one we used here, operant reward learning in *Aplysia* feeding. However, in this preparation, the Calcium-dependent Apl-I PKC not the atypical Apl-III PKC appears to be mediating the plasticity (Lorenzetti et al., 2008). Given the degeneracy between the different PKC genes and the fact that they not only can compensate for long-term PKCi-mediated inhibition (this work and (Colomb and Brembs, 2016), but also for each other (Tsokas et al., 2016), more research is needed to elucidate how these different mechanisms of plasticity evolved and are related to each other.

4.2. Self-learning is mediated by a non-canonical *aPKC/FoxP* pathway

Neither *bazooka* (*baz*) nor the kidney and brain gene (KIBRA), two prominent interaction partners of aPKC (Wieschaus and Noell, 1986; Yoshihama et al., 2011, 2012; Sacktor, 2012; Heitz et al., 2016; Soriano et al., 2016; Pichaud, 2018; Zlomuzica et al., 2018; Ferguson et al., 2019; Thompson, 2022), showed an effect on operant self-learning when they were knocked out in the adult (Fig. 6), potentially raising doubts about the effectiveness of the CRISPR/Cas9 method in these cases. However, we have ensured that both manipulations successfully knocked out the genes. First, we found that the *baz* knock-out, rather than impairing self-learning, increases learning performance (manuscript in preparation), suggesting the PAR complex may be sequestering *aPKC* and thereby limiting its availability for self-learning. Our KIBRA knock-out had a severe effect on flight performance when elicited during development, suggesting that also this manipulation was, in principle, effective. Interestingly, at least during development, *baz* and KIBRA have been reported to have opposite effects on the function of *aPKC*, with the PAR complex (*baz*) and the Hippo pathway (KIBRA) mutually inhibiting each other (Zhang and Wei, 2022). While *baz* is thought to mediate *aPKC* ac-

tivity, KIBRA is thought to exert negative regulatory activity on *aPKC*. Thus, knocking out each one of them should have revealed a decrement in self-learning in at least one of them, if the processes during development were recapitulated during self-learning. On the other hand, experiments in which KIBRA has been shown to be involved in learning/memory, have suggested a positive rather than a negative regulatory role (Hu et al., 2017b; Ferguson et al., 2019), albeit with an emphasis on long-term memory rather than learning (Zha and Sossin, 2022). Whichever way *aPKC* may be interacting with components of these canonical pathways, the literature predicts that at least one of our manipulations should have revealed a decrement in operant self-learning. The fact that this prediction was falsified strongly suggests that *aPKC* exerts its function in a non-canonical manner in operant self-learning plasticity. We are currently pursuing research on the possibility that *bazooka* may constitute a negative regulator of aPKC activity in operant self-learning (manuscript in preparation).

4.3. Persistent *FoxP* effect on operant self-learning in adults

As *FoxP* mutants are impaired in operant self-learning (Mendoza et al., 2014), two hypotheses about the role of the prominent transcription factor arise. First, *FoxP* may be directly

involved in the learning process via some unknown, cytosolic function. A transcription factor function appears unlikely, due to the short duration of our experiments. Second, *FoxP* may exert its effects as a developmental regulator, being crucial for the development of the circuits mediating operant self-learning. As the developmental role of FoxP genes is well documented (Takahashi et al., 2009; Bowers et al., 2014; Rocca et al., 2017; Castells-Nobau et al., 2019; Co et al., 2020; Palazzo et al., 2020) and there are few domains in the gene that would lend themselves for a hypothetical cytosolic function, the latter hypothesis appeared more plausible. The result that adult knockout of *FoxP* had no immediate effect in operant self-learning (Fig. 1A) seemed to corroborate this hypothesis. In contrast, supporting a continued role of FoxP genes in motor learning also after development are data from songbirds where FoxP2 gene expression is not only regulated by singing (Teramitsu and White, 2006; Teramitsu et al., 2010; Chen et al., 2013; Thompson et al., 2013), but where normal *FoxP2* expression is necessary in adults to maintain learned song (Day et al., 2019; Xiao et al., 2021). To also test the second hypothesis, we aged the flies after the *FoxP* knockout and tested them 7 and 14-days later. Flies still showed operant self-learning after seven days without *FoxP* transcription (DOI:

10.5283/epub.52965), but were impaired after 14 days (Fig. 1B). These results suggest a role of adult *FoxP* expression in maintaining operant self-learning capabilities after development, analogous to the role of FoxP2 in songbird vocal learning (Day et al., 2019; Xiao et al., 2021).

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