Evidence for motor neuron plasticity as a major contributor to motor learning in *Drosophila*

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Operant self-learning: a form of motor learning

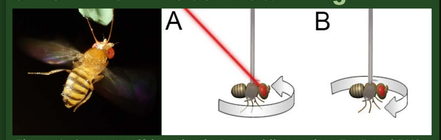

Figure 1: Operant self-learning in *Drosophila* requires FoxP and PKC
A fly is teathered by head and thorax flying stationarity in a featureless environment (photograph, left). The animal is tethered to a torque meter which measures the angular momentum around the fly's vertical body axis (yaw torque). The yaw torque range is divided into to torque domains of similar size, approximately corresponding to left and right turning attempts, respectively. In the example above, left turning attempts (A) are punished by an infra-red heat beam that is invisible to the fly, while right turning attempts (B) are not punished. Half of the flies are always punished for left, the other half for right turning attempts. Mutants and RNAi-mediated knock-downs of the gene FoxP are deficient in this task. Similarly, inhibiting all protein kinases C (PKC) also leads to impaired self-learning.

Inhibiting PKC in motor neurons impairs motor learning

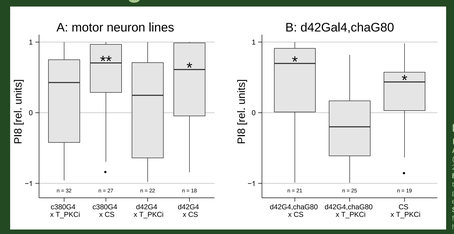

Figure 2: Inhibiting PKC in non-cholinergic motor neuron lines impairs self-learning.
A. Driving expression of the inhibitory peptide PKCi with either c380-Gal4 or D42-Gal4 (two nominally equivalent motor neuron driver lines) abolishes turning preference in a 2-min test period without heat directly after training.
B. Motor neurons in *Drosophila* are glutamatergic. To exclude cholinergic neurons potentially contained within the expression pattern of D42-Gal4, we used cha-Gal80 to prevent Gal4 expression in cholinergic neurons. Even with cholinergic neurons excluded, expression of PKCi using the D42 driver abolished operant self-learning.
Source: Colomb J, Brembs B. 2016. PKC in motorneurons underlies self-learning, a form of motor learning in *Drosophila*. PeerJ 4:e1971 https://doi.org/10.7717/peerj.1971

aPKC is required in FoxP neurons for motor learning

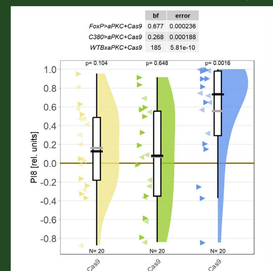

Figure 3: Using CRISPR/Cas9 to knock out the gene for the atypical PKC in motor neurons abolishes motor learning.
Plotted are performance indices for the first period after training (PI8). Knocking out aPKC in motor neurons or FoxP-neurons impairs operant self-learning. Expressing the CRISPR/Cas9 components 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, showed high PIs, a low p-value and a high Bayes Factor, indicating their self-learning was intact.

FoxP and aPKC are co-expressed in VNC MNs

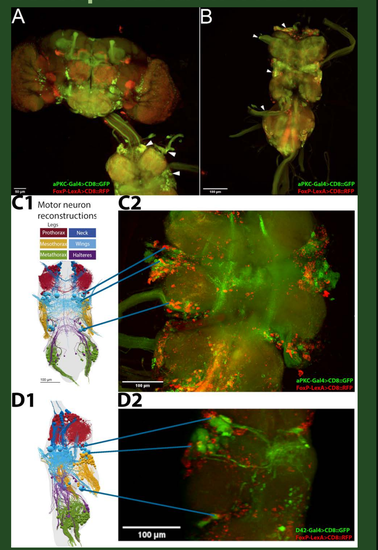

Figure 4: Co-expression analysis of FoxP and aPKC.
Confocal stacks of whole mount preparations of central nervous systems; **A-C**: green - aPKC-Gal4>CD8::GFP, red - FoxP-LexA>CD8::RFP; **D** green - D42-Gal4>CD8::GFP, red - FoxP-LexA>CD8::RFP. Confocal image stacks available at: 10.5281/zenodo.10047941.
A: Adult brain (top) with ventral nerve cord (VNC, bottom) attached. No co-expressing cells can be observed in the brain, whereas such neurons (yellow) are readily observable in all neuromers of the VNC (arrowheads).
B: VNC with aPKC/FoxP co-expression (yellow) both in cell bodies and fiber tracts in nerves (arrowheads).
C: **C1**- Dorsal view of motor neuron reconstruction (modified from (78). **C2**- Confocal image stack of dorsal view of the mesothoracic neuromer with putative wing MNs expressing both aPKC (green) and FoxP (red) marked.
D: **D1**- Lateral view of motor neuron reconstruction (modified from (78). **D2**- Confocal image stack of mesothoracic neuromer with all MNs (green) and FoxP neurons (red) marked. This lateral view supports the hypothesis that the ventral cluster of aPKC/FoxP neurons comprises wing MNs.

Motor neurons controlling turns co-express FoxP and aPKC

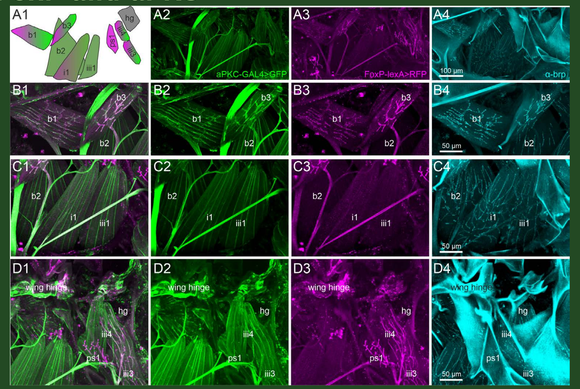

Figure 5: A subset of direct wing muscles is innervated by FoxP/aPKC co-expressing MNs. Representative projection view of MN terminals on the direct flight steering muscles in animals with GFP label in aPKC expressing cells (aPKC-Gal4>CD8::GFP, green), RFP expression in FoxP expressing cells (FoxP-LexA>CD8::RFP, magenta), and immunolabeling for the presynaptic active zone marker bruchpilot (brp, cyan) reveal which direct flight steering MNs express either aPKC, FoxP, or both, or none of them but only brp in presynaptic active zones. (**A1**) depicts the orientation, shape, and abbreviated names of direct flight steering muscles and summarizes which ones are innervated by aPKC expressing MNs (magenta), or by MNs without FoxP and aPKC expression (grey). (**A2**) Projection view of direct flight muscles and their innervation with GFP expression under the control of aPKC-GAL4 (green) at 20x magnification. (**A3**) Same preparation, image stack, and field of view but with RFP expression under the control of FoxP-lexA (magenta). (**A4**) Same preparation, image stack, and field of view with brp immunolabel (cyan) in presynaptic active zones of flight steering MNs. (**B1-B4**). Same preparation but with selective enlargement of the three basalare muscles (b1-b3), with all three labels in (**B1**). GFP label in aPKC-expressing cells (green, **B2**), RFP label in FoxP expressing cells (magenta, **B3**), and Brp label in presynaptic active zones (cyan, **B4**). Muscles b1 and b3 are innervated by steering MNs with aPKC and FoxP expression, but b2 is devoid of FoxP-expressing innervation. (**C1-C4**) Same preparation but with selective enlargement of second basalare (b2) and the adjacent prerale 1 (i1) and prerale II (ii3) muscles with all three labels (**C1**), GFP label in aPKC-expressing cells (green, **C2**), RFP label in FoxP-expressing cells (magenta, **C3**), and brp label in presynaptic active zones (cyan, **C4**). Only i1 is faintly labeled for terminals with aPKC FoxP expression. (**D1-D4**) Same preparation but with selective enlargement of the prerale II muscles iii3 and iii4, the adjacent pleurosternal muscle (ps1), and the posterior notal wing process muscles (hg) with all three labels (**D1**), GFP label in aPKC-expressing cells (green, **D2**), RFP label in FoxP-expressing cells (magenta, **D3**), and Brp label in presynaptic active zones (cyan, **D4**). The prerale II muscles iii3 and iii4 are innervated by terminals with aPKC and FoxP expression.

Motor learning breaks optomotor symmetry

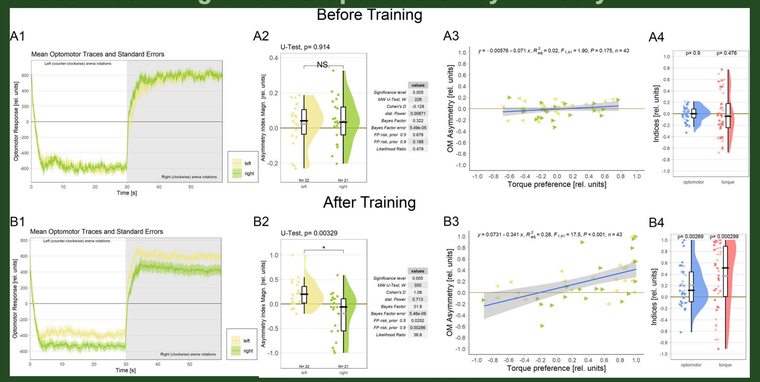

Figure 6: Associating one torque domain with heat changes optomotor behavior.
A: Measurements before training
A1 Averaged optomotor traces of flies punished either on the 'left'(yellow) or on the 'right' (green) torque domain. Both groups show similar response magnitudes in either direction of the optomotor stimulus. Errors are standard deviations.
A2 Optomotor asymmetry indices for flies punished either on the 'left'(yellow) or on the 'right' (green) torque domain. The values for both groups spread around zero. Positive values indicate a shift towards positive (right-turning) torque. Both frequentist and Bayesian analyses are displayed to the right of the plots and indicate no difference between the groups.
A3 Regression analysis between torque preference and optomotor asymmetry. Optomotor values were adjusted such that positive values indicate a shift towards the unpunished torque domain. No significant correlation was observed. Left-pointing arrowheads (yellow) denote flies punished on left-turning torque and right-pointing arrowheads (green) denote flies punished on right-turning torque.
A4 Comparison of optomotor asymmetry (left, blue) and torque preference(right, red) indices. Here again, optomotor values were adjusted such that positive values indicate a shift towards the unpunished torque domain. Both measures vary around the zero point and Wilcoxon tests against zero are not significant (p-values above each plot). Left- and right-pointing arrowheads denote punishment directions as before.
B: Measurements after training
B1 Averaged optomotor traces of flies punished either on the 'left' (yellow) or on the 'right' (green) torque domain. A reduction in the OMR magnitude can be observed on the punished, but not on the unpunished side. Errors are standard deviations.
B2 Optomotor asymmetry indices for flies punished either on the 'left' (yellow) or on the 'right' (green) torque domain. Positive values indicate a shift towards positive (right-turning) torque. The values for each group have now shifted towards the unpunished side compared to the values before training. Both frequentist and Bayesian analyses are displayed to the right of the plots and indicate a significant difference between groups.
B3 Regression analysis between torque preference and optomotor asymmetry. Optomotor values were adjusted such that positive values indicate a shift towards the unpunished torque domain. A significantly positive correlation was observed, such that higher torque preferences entailed larger optomotor asymmetry. Left-pointing arrowheads (yellow) denote flies punished on left-turning torque and right-pointing arrowheads (green) denote flies punished on right-turning torque.
B4 Comparison of optomotor asymmetry (left, blue) and torque preference (right, red) indices. Here again, optomotor values were adjusted such that positive values indicate a shift towards the unpunished torque domain. Both measures are shifted towards more positive values and Wilcoxon tests against zero are now significant for both variables (p-values above each plot). Left- and right-pointing arrowheads denote punishment directions as before.

aPKC is required for optomotor modulation after motor learning

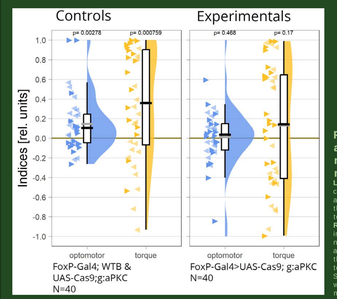

Figure 7: Knocking out aPKC abolishes optomotor asymmetry after motor learning.
Left: After motor learning, genetic control animals shows both optomotor asymmetry (blue) and a preference for the unpunished turning directions / torque domain (yellow).
Right: After motor learning, animals in which aPKC was knocked out in FoxP neurons showed neither optomotor asymmetry (blue), nor a preference for the unpunished turning direction / torque domain (yellow).
Shown are data from a 2-min period without heat immediately after eight minutes of training (PI8).