Searching for the molecular mediators of motor learning in *Drosophila*

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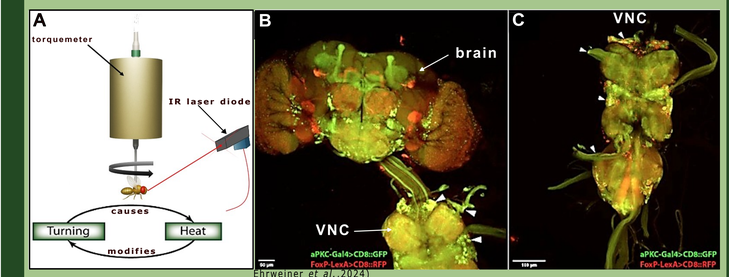
Background

In humans, language acquisition represents a form of operant motor learning. The operant process of speech learning shares key characteristics with the torque learning of *Drosophila melanogaster* in the torque meter: An initially variable behavior gets narrowed down by an operant feedback loop (Fig.1**A**). Here, the flies don't have any external cues about the experimental outcome but have to rely on their own behavior.

In both, humans and flies, this type of learning relies on a conserved molecular plasticity pathway that includes aPKC, an unspecific serine/threonine kinase, as well as the Forkhead transcription factor B. FoxP and aPKC coexpression only exist in the ventral nerve cord VNC) of the fly but not in its brain (Fig. 1**B**, Fig.1**C**). To date, other molecular components of this pathway and their role in operant learning remain elusive.

Here, we aim to identify aPKC/FoxP mediators in VNC motor neurons and to asses their role in operant learning by knocking out potential candidates via the CRISPR/Cas system. The motor learning performance of knock-out flies is analysed in the *Drosophila* torque meter.



Ehrweiner *et al.*,2024)

Figure 1: Motor learning relies on aPKC/FoxP expression in VNC motor neurons: Panel **A** depicts a scheme of the operant feedback loop. Flies learn to adjust their own behavior based on environmental feedback. This form of motor learning relies on a conserved molecular plasticity pathway that includes aPKC and FoxP. The coexpression of both genes only occurs in the VNC (Panels **B** and **C**) of the fly but not in its brain (Panel **B**) as indicated by the white arrows.

Generation of the aPKC-TurboID construct

1. Fusion of aPKC to the GS linker sequence

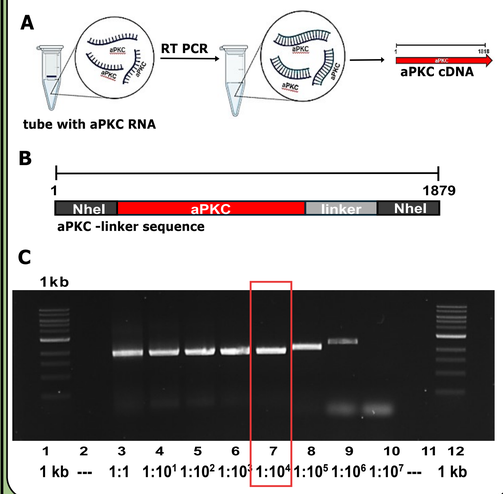


Figure 4: Fusion of GS linker sequence to aPKC. **A**: We fused aPKC cDNA to a glycine-serine (GS) linker by two sequential PCR steps, whereby the amplicon of the first PCR acted as a template for the second PCR step. **B**: Scheme of the final aPKC-GS linker fusion construct. **C**: Agarose gel following the second PCR reaction showing the aPKC-linker DNA band at the expected range of 2kb. Here, a dilution series of the amplicon of the first PCR reaction was generated. The red rectangle encloses the DNA band, which was cut out of the gel and used for TA cloning into the pGEMTeasy vector.

2. TA cloning of aPKC-linker construct

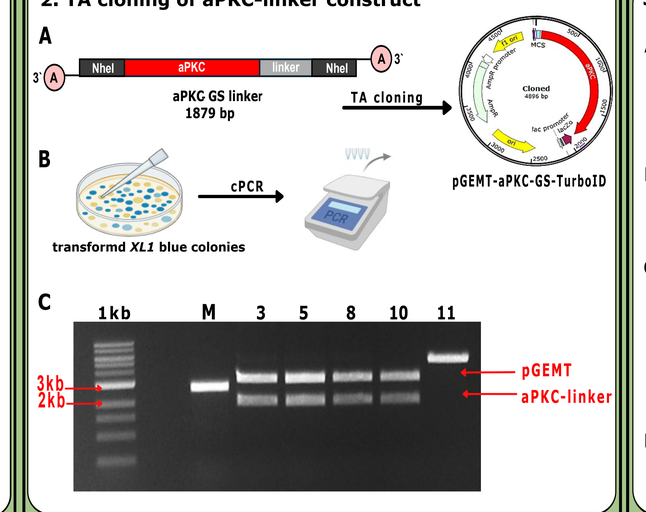


Figure 5: TA cloning of aPKC linker construct into pGEMTeasy vector. A: Scheme of the adenylated aPKC-linker construct. TA cloning and transformation of *XL1 blue* bacteria amplified our aPKC linker sequence. **B**: Schematic presentation of LBAMP plate with transformed blue and white *XL1 blue* colonies and subsequent colony PCR (cPCR). **C**: Agarose gel showing DNA bands of the aPKC-linker at 2kb and the pGEMT vector at 3kb after NheI digestion of single *XL1 blue* colonies. 3,5,8,10 and 11. We used the pGEMTeasy vector as a marker (M).

3. Cloning of aPKC-linker sequence into destination vector

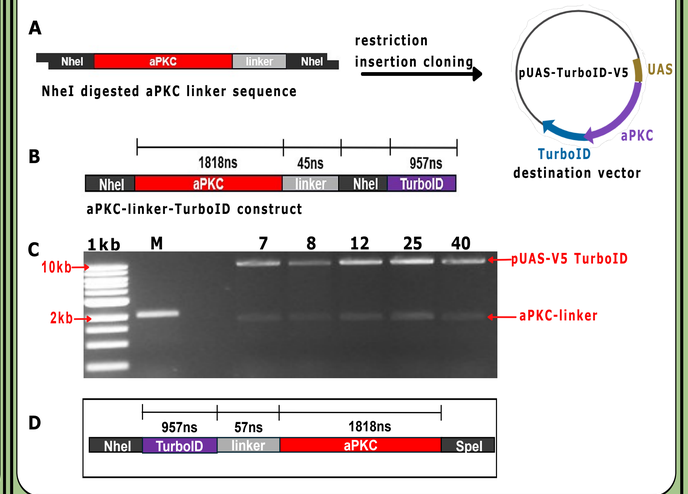


Figure 6: Generation of aPKC-linker-TurboID construct. A: Scheme of NheI-digested aPKC-linker sequence. Subsequent restriction ligation cloning of the aPKC-linker sequence into our destination vector that already carries the TurboID sequence generated the final fusion construct. **B:** Scheme of the dimensions of the aPKC-linker-TurboID sequence. **C**: DNA mini preparation of single transformed clones followed by NheI digestion confirmed the aPKC-linker sequence of the transformed clones 7,8,12,25 and 40. **D**: Additional version of the fusion construct. Here, TurboID is fused to the N-terminus of aPKC, while for the previous construct TurboID was fused to the C-terminus.

Experimental paradigms

Identification of aPKC/Foxp pathway intermediates

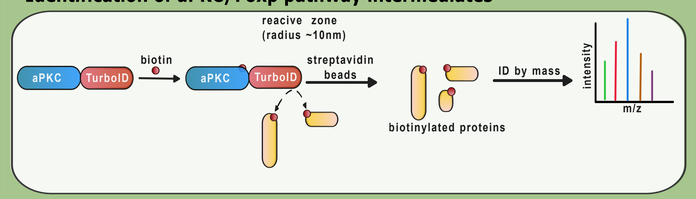


Figure 2: Identifying proteins biotinylated by our aPKC-TurboID construct. We generated transgenic flies that express a biotin ligase referred to as TurboID, which was genetically fused to aPKC. In response to biotin uptake of the fly, potential aPKC interaction partners will be biotinylated. These biotinylated proteins will be harvested using streptavidin- coated beads and identified by mass spectrometry.

Assessing the role of promising candidates in motor learning

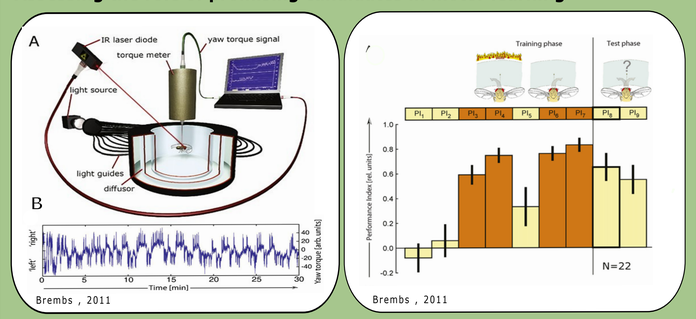


Figure 3: The torque meter set up used to train flies operantly. Knock-out flies for potential candidates will be tethered to the torque meter which measures the rotational force of the flies along the vertical body axis (yaw torque) during stationary flight. (Fig.3**A**). Figure 3**B** depicts the experimental sequence of the torque training paradigm. Here, yellow bars indicate test, orange bars training phases. The performance index (PI) score indicates the proportion of time spent on the unpunished side. Positive PI values indicate a preference for the unpunished, negative values for the punished side.

Generation of the aPKC-TurboID expressing fly lines

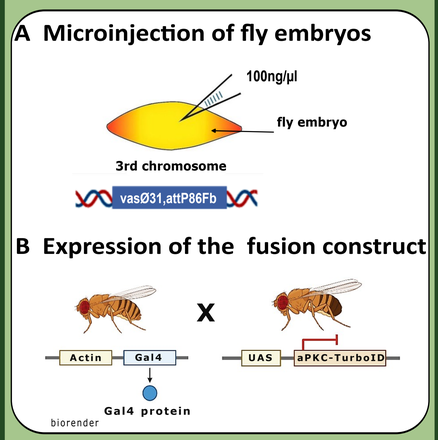


Figure 7: Establishing fly lines that express the aPKC -TurboID construct. A: For targeted gene insertion microinjection was performed in fly embryos that carry a phi C 31 integrase gene that inserts DNA at a specific landing site in the genome with high expression levels. **B:** Flies carrying our fusion construct are crossed with actin-Gal4 dirver lines to express the aPKC-TurboID sequence in all cells.

CadherinN knock out does not affect motor learning

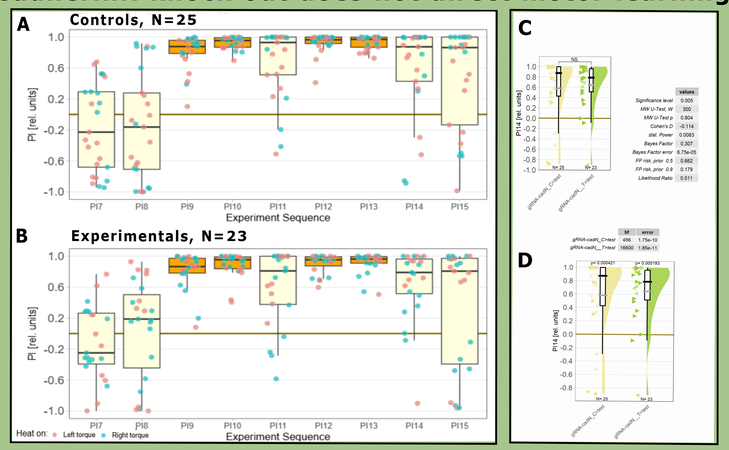


Figure 8: CRISPR/Cas mediated knock out of Cadherin N (CadN) does not affect motor learning. We temporally restricted the CadN knock-out to the adult stage by expressing a drug-inducible knockout system that only results in CadN knock out when flies are exposed to mifepristone (RU 486). Transgenic flies expressing the RU-inducible knockout system were subjected to a motor learning task in which one torque domain was coupled with punishing heat. Flies were either kept on instant food without RU at the adult stage for 2 days(**A**) or exposed to RU laced food for two days (**B**). **A** and **B**: Performance indices. Yellow bars indicate test phases, orange bars represent training phases. Black bars denote medians. Red and blue dots indicate the individual PIs and the punished torque direction. **C**: Multiple statistical tests revealed no difference between the control and the treatment group. Black bars denote medians, grey bars means. Triangles represent individual PIs and the punished torque direction **D**: One sample tests against zero reveal learning for both groups. Boxes indicate quartiles and whiskers non-ourlier range

Assessing the role of neuroligin2 for motor learning

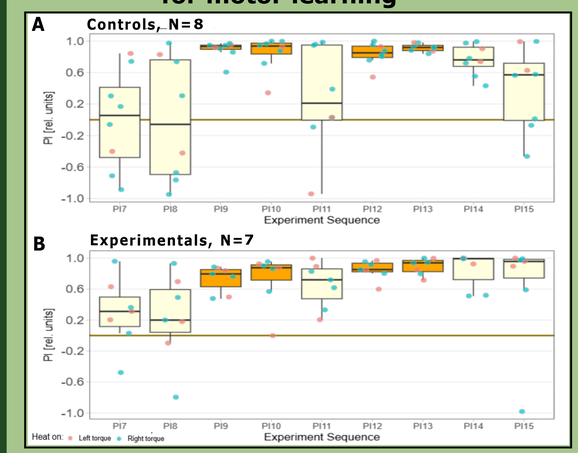


Figure 9: Motor learning indices of flies expressing the mifepristone inducible CRISPR/Cas knockout of neuroligin2 (Nlg2). Transgenic flies expressing the inducible Nlg2 knockout system were subjected to a motor learning task. Flies were either kept on instant food without RU at the adult stage for 2 days (**A**) or exposed to RU laced instant food for two days (**B**). **A** and **B**: Performance indices of flies in all experimental periods. Yellow bars indicate test phases, orange bars denote training phases. Red and blue dots indicate the individual PIs and the punished torque direction. Horizontal bars represent the median. Boxes indicate quartiles and whiskers non-ourlier range.

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Reference

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Wings of Change: aPKC/FoxP-dependent plasticity in steering motor neurons underlies operant self-learning in Drosophila.

Brembs, Björn (2011):Spontaneous decisions and operant conditioning in fruit flies.
In: Behavioural Processes 87 (1), S. 157–164. DOI: 10.1016/j.beproc.2011.02.005.
Abstract:

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