How is *aPKC* activity related to operant self-learning performance in *Drosophila*?

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Abstract

The *atypical protein kinase C (aPKC*) is one of six protein kinase C genes in *Drosophila melanogaster*. In addition to cell polarity and asymmetric cell division, *aPKC* influences motor learning behavior. It is known from previous experiments that *aPKC* knockout leads to impaired operant self-learning, where flies learn from the consequences of their behavior without any cues from their surrounding. By missing the regulatory domain, *aPKCΔ* offers the possibility of upregulating *aPKC*. In this thesis, the effect of *aPKC* overexpression on operant self-learning was investigated in an open-loop learning experiment using a flight simulator. Confocal laser scanning microscopy was used to understand better which motor neurons in the ventral nerve cord *aPKC* and *FoxP* are expressed, whereby co-expression could be detected.
Overexpressing *aPKC* resulted in improvement of operant self-learning behavior.

Zusammenfassung

Die *atypische Proteinkinase C* (*aPKC*) ist eine von sechs Proteinkinase C Genen in *Drosophila melanogaster*. Neben Zellpolarität und asymmetrischer Zellteilung beeinflusst *aPKC* das motorische Lernverhalten. Aus früheren Experimenten ist bekannt, dass *aPKC*-Knockout zu einer Beeinträchtigung des operanten Selbstlernens führt, bei dem Fliegen aus den Folgen ihres eigenen Verhaltens lernen, ohne Hinweise aus ihrer Umgebung zu erhalten. Durch das Fehlen der regulatorischen Domäne bietet *aPKCΔ* die Möglichkeit, *aPKC* hochzuregulieren. In dieser Arbeit wurde die Wirkung von *aPKC* Überexpression auf operantes Selbstlernen in einem Open-Loop-Lernexperiment in einem Flugsimulator untersucht. Konfokalmikroskopie wurde verwendet, um besser zu verstehen, in welchen Motoneuronen im ventralen Nervenstrang *aPKC* und *FoxP* exprimiert wird, wobei Koexpression gezeigt werden konnte.

Die Überexpression von *aPKC* führte zu einer Verbesserung des operanten Selbstlernverhaltens.

1 Introduction

The psychologist Edward Thorndike observed that animals rewarded for responding to a set task responded more often than punished, noting in the Law of Effect that this link between behavioral response and the task was related to the degree of satisfaction or punishment. (Thorndike, 1911). This pattern of behavior was later defined as operant conditioning, which describes behavior that is not elicited by a stimulus and gets influenced in its frequency by a reinforcer exerted by the environment. Due to these consequences, the likelihood of the shown behavior may change in frequency (Skinner, 1953).

The flight simulator is ideal for simulating operant conditioning in the laboratory while working with *Drosophila melanogaster*. In this setup, the fly can independently determine the flight direction while being fixated in the arena. At the same time, the generated torque activates a sensory stimulus, which is applied whenever the fly flies to a predetermined penalized side. Flies tested in operant self-learning paradigm (yaw torque learning) should learn solely through the consequences of its behavior. There are no other influences from the environment. As an aversive stimulus, heat can be conducted, leading flies to remember and prefer the non-punished side (Wolf & Heisenberg, 1991).

Six protein kinase C (*PKC*) genes are divided into three subfamilies in *Drosophila melanogaster*. One of these *PKC* isoforms is *atypical PKC* (*aPKC*) (Shieh et al., 2002). *aPKCΔ* is an "N-terminally truncated form of aPKC … that lacks the Par-6-binding domain" (Betschinger et al., 2003, S. 329), which consequently offers the possibility to overexpress *aPKC*. Prior experiments proved that *PKC* is a crucial part of operant self-learning (Brembs & Plendl, 2008) and in particular *aPKC* knockout in motor neurons and *FoxP-iB* positive cells results in learning impairments, indicating that *aPKC* potentially modulates operant self-learning (Ehweiner & Brembs, 2021).

The *FoxP* gene family consists of transcription factors with a conserved DNA-binding domain through most species reaching from *Drosophila* to humans. *FoxP2*, responsible for speech acquisition in humans and vocal song learning in birds, shows the highest degree of similarities with *dFoxP* in *Drosophila* (Hannenhalli & Kaestner, 2009; Mendoza et al., 2014). Manipulation of *FoxP*, as well as *aPKC* expression, interferes with operant self-learning in *Drosophila* (Mendoza et al., 2014; Ehweiner & Brembs, 2021). The potential interaction of *FoxP* and *aPKC*, however, is arguable.

This thesis aims to reproduce an experiment in which *aPKC* overexpression improved operant self-learning using the flight simulator, to investigate how *aPKC* activity is related to operant self-learning performance in *Drosophila*. Another objective of this thesis is to map the anatomical relation between the expression pattern of *FoxP* and *aPKC* in the ventral nerve cord (VNC) of *Drosophila*.

2 Material and Methods

2.1. Fly stocks and maintenance

All flies were raised under a 12/12h light/dark cycle at 60% humidity and 25°C. Flies were flipped in fresh plastic vials daily for behavioral experiments to obtain constant fly density. The vials contained standard *Drosophila* cornmeal/molasses medium, fresh yeast and filter paper. Flies for dissections were raised in small glass vials containing standard *Drosophila* cornmeal/molasses medium and instant yeast. Flies were flipped every 3 to 4 days.

Stock	**Chromosome background**	**Source**
UAS-aPKCΔ	*w[*]; P{w[+mC]=UAS-aPKC.DeltaN}3*	Bloomington #51673
nSyb-GS	*nSyb-GS.B}attP2*	Bloomington #80699
aPKC-Gal4	*y[1] w[*]; Mi{Trojan-GAL4.un}aPKC[MI10848-TG4.un]/SM6a*	Bloomington #77814
$\frac{\textit{LexAop-mCD8::RFP,UAS-mCD8::GFP}}{\textit{CyO}}$; $\frac{\textit{FoxP-LexA}}{\textit{TM3}}$	*LexAop-mCD8::RFP,UAS-mCD8::GFP;TM3/TM6* *FoxP-LexA*	Andreas Ehweiner

Table 1: Complete list of fly lines.

2.2. *aPKCΔ* expression experiment

For this experiment, 20 female *UAS-aPKCΔ* virgins were crossed with eight *nSyb-GS* males. Freshly hatched offspring were kept under cold anesthesia, and only the female flies were selected and separated equally into two small fresh glass vials. One vial contained *Drosophila* food without yeast. The second vial was filled with instant *Drosophila* medium soaked in RU486 (200 μg/ml).

To gain temporal control of *aPKC* expression, the GeneSwitch/UAS expression system was used. The Gal4 Protein 'GeneSwitch' (GS) stays transcriptionally inactive without addition of

the steroid hormone RU486 (Mifepristone). By adding RU486 in food, GeneSwitch becomes transcriptionally active, expressing through UAS reporter line the *aPKCΔ* insert, leading to an *aPKC* overexpression (Osterwalder et al., 2001). To prepare the RU486 mixture, 20 mg Mifepristone, 1 ml 99% Ethanol, sugar and 99 ml $H_2O_{dest.}$ were mixed and used to dissolve instant food.

After two days, flies were transferred by another person into empty plastic vials, one labeled with '**A**' and one with '**B**.' One vial contained RU486-treated experimental flies and one an internal control group with non-treated flies. Female flies were kept under cold anesthesia to glue a thin copper wire triangle above their neck to immobilize the fly's head and thorax. Dentist glue was used and hardened with UV light for 10 s. Once hooked, flies were transferred into individual experimental chambers for another day with access to water and sugar. Then, on the experimental day, sugar was added (Brembs, 2008). Flies were hooked and tested blind (**Figure 1).** Treated and non-treated flies got hooked alternating. For one wheel (12 flies), first three '**A**' flies and then three '**B**' flies were hooked consecutively.

Once the experiment was completed, it was resolved that **group A** included flies treated with RU486 and **group B** formed the internal control group with non-treated flies.

As a control group, to verify the setup and laser adjustments, newly hatched *UAS-aPKCΔ x nSyb-GS* flies were transferred into a fresh plastic vial for one day. The next day, flies were hooked and tested as previously described non-treated flies.

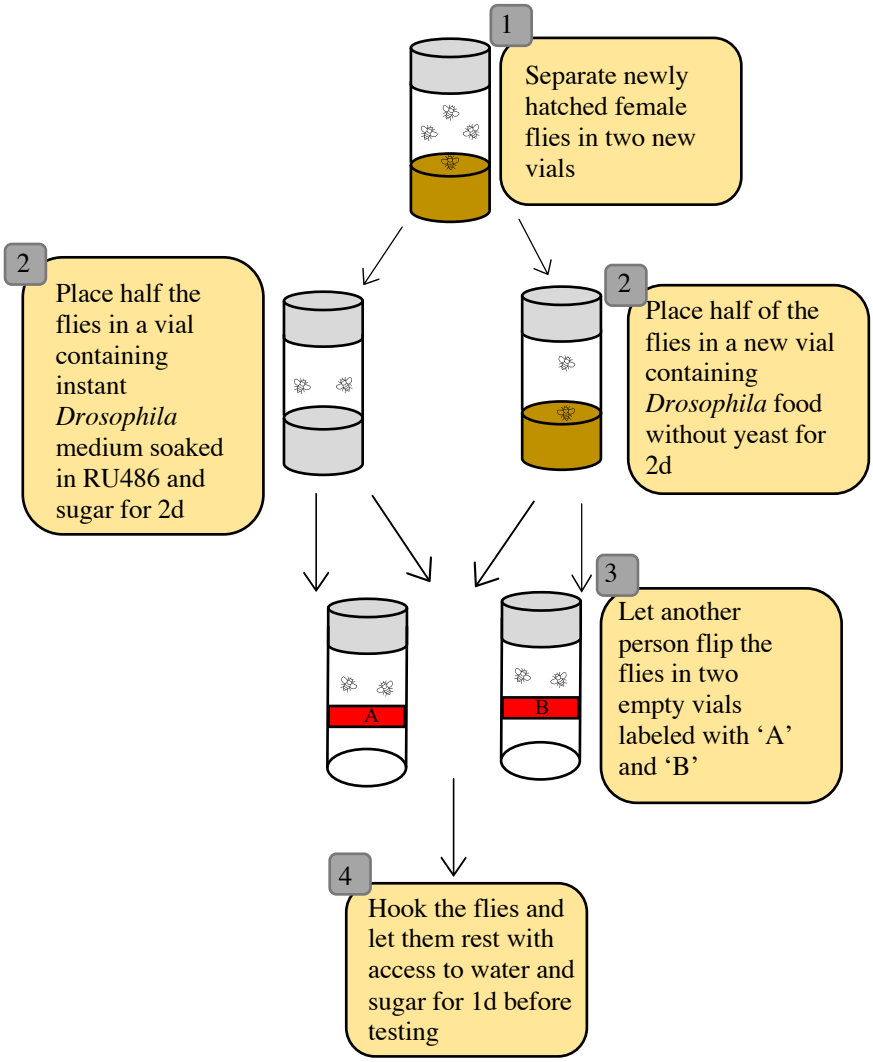

Figure 1: Fly maintenance set up for the *aPKCΔ* experiment: Newly hatched flies were sorted under cold anesthesia. Female flies were separated into two vials, one containing instant *Drosophila* medium soaked in RU486 and one with standard *Drosophila* food. After two days, flies got flipped by another person into empty vials labeled with 'A' and 'B'. Flies were then hooked and tested blind.

2.3. Torque meter setup

A newly built "Kopp" torque meter device was used, which in its basics combines elements from the "Shiming" (Tang & Juusola, 2010) and the "Götz" (Götz, 1964) device. Through the prior glued copper hook above the flies' neck, flies could get fixated in a metal clamp connected with the torque meter. Once attached to the device, flies were transferred to the center of the arena. Starting the experiment, the instrument measured and saved the angular momentum flies created while flying. The experiment was performed in darkness, so the only light source flies were exposed to came from a beamer (DLP Beamer, DELL) which evenly illuminated the arena. To measure the flying behavior, LabVIEW (3V19) was used. During yaw torque learning, no patterns were presented. Punishment was applied during training

whenever the fly's yaw torque reached the penalized side. (Brembs, 2008; Brembs & Heisenberg, 2000; Wolf & Heisenberg, 1991). Punishment was achieved through a laser (Streamline laser, Osela Inc.) pointing frontal onto the fly's head. The laser was at all times set at 3.5 V and 80% pulsing intensity (equals 160 ms width at approx. 4 Hz), which can be adjusted in LabView. The experiment could be followed using a digital microscope (USB-Digital microscope with a 40x – 1000x magnification, Bysameyee).

To establish the new device for reliable measurements, non-treated control flies were tested (**Table 2**).

2.4. Learning protocols

A standard learning experiment was set for 22 minutes (**Table 2;** Brembs & Plendl, 2008). Each experiment consisted of 4 optomotor periods, each 30 s, before and after training-test trials. The flies' optomotor response was set equal for the right and left torque. Each fly was only used once.

Period 5	Period 6	Period 7	Period 8	Period 9	Period 10	Period 11	Period 12	Period 13
Pretest	Pretest	Training	Training	Test	Training	Training	Test	Test
120 s	120 s	120 s	120 s	120 s	120 s	120 s	120 s	120 s
-	-	Punishment	Punishment	-	Punishment	Punishment	-	-

Table 2: Standard protocol for operant self-learning.

Right side and left side punishment were alternated to exclude side preference bias. The only exception were flies that exhibited an initial naïve preference for one side, as it should be ensured that these flies were exposed to the laser at least once. Right torque, created by flies turning right, was defined as the positive signal on the oscilloscope. Left torque, for left turns, was defined as the negative signal.

2.4.1. Adapted learning protocol

To test the effects of *aPKC* overexpression on operant self-learning, the protocol was adapted as follows (**Table 3;** Andreas Ehweiner, personal communication):

Period 5	Period 6	Period 7	Period 8	Period 9	Period 10	Period 11	Period 12	Period 13
Pretest	Pretest	Training	Training	Test	Training	Training	Test	Test
60 s	60 s	60 s	60 s	60 s	60 s	60 s	60 s	60 s
-	-	Punishment	Punishment	-	Punishment	Punishment	-	-

Table 3: Adapted protocol for operant self-learning.

2.5. Evaluations

To measure the learning success, a performance index (PI) was calculated:

$$PI = (a - b) / (a + b)$$

Where '*a*' stands for flying on the non-punished side and '*b*' for flying on the punished side. If flies avoided the punished side constantly, the PI equals *1*. Conversely, if flies always flew on the punished side, the PI equals *-1* (Dill et al., 1993). The statistical tests of single groups against zero are based on a Wilcoxon test (significance level set to p-value < 0.005). Results evaluations were performed using the *Drosophila* Time Series (DTS) Data Model, which can be downloaded from "https://github.com/brembslab/DTSevaluations."

2.6. Dissection of adult *Drosophila* ventral nerve cord

For dissections 10 female *aPKC-Gal4* virgins and eight $\frac{LexAop\text{-}mCD8::RFP,UAS\text{-}mCD8::GFP}{CyO}; \frac{FoxP\text{-}LexA}{TM3}$ males were crossed.

2.6.1. Dissection without antibody staining

Flies were put under CO_2 anesthesia and fixated in paraformaldehyde (PFA) 4% rotating at 4°C and 20 rpm for 2,5 h. Flies were washed 3 times for 15 minutes in phosphate-buffered saline (PBS). VNCs were dissected in PBS the same day. Once dissected, all PBS was removed and replaced with a drop of VECTASHIELD® *Antifade Mounting Medium*.

To scan and store the VNCs, two microscope cover glasses were glued to a microscope slide using clear nail polish, leaving a thin gutter in the middle. VNCs were transferred individually on the microscope slide. The VECTASHIELD® was removed and a third clover glass was glued to the microscope slide, covering the VNCs. A little gap was left on the top and bottom to refill the gutter with VECTASHIELD® using negative pressure. Once the gutter was filled, the openings were sealed using clear nail polish.

Confocal pictures were taken using a Leica SP8 confocal microscope (RRID: SCR_018169) with a 40x oil immersion objective. Images were edited in ImageJ-2.

2.6.2. Dissection with antibody staining

<u>Day 1</u>

The newly hatched flies were fixated in PFA 4% at room temperature for 1h. Once dissected, the VNCs were washed in phosphate-buffered saline with Triton-X (PBST) 0,1% 6 times for

10 minutes at room temperature, followed by blocking with 5% Normal Goat Serum (NGS) in PBST 0,1% for 2h at room temperature. Next, the blocking solution was replaced by primary antibody solution (**Table 4**) and incubated in darkness for 24h at 4°C.

0,5 μl	Anti-GFP (1:1000)
1 μl	Anti-RFP (1:500)
15 μl	NGS (3%)
483,5 μl	PBST 0,1%

Table 4: Primary Antibody solution.

Day 2

VNCs were washed in PBST 0,1% 6 times for 10 minutes at room temperature, followed by incubating in secondary antibody solution (**Table 5**) in darkness for 24h at 4°C.

2,5 μl	Goat-Anti-Rat; Alexa Fluor 555 (1:200)
2,5 μl	Goat-Anti-Chicken Alexa Fluor 488 (1:200)
15 μl	NGS (3%)
480 μl	PBST 0,1%

Table 5: Secondary Antibody solution.

Day 3

VNCs were washed in PBST 0,1% 6 times for 10 minutes at room temperature and mounted as described in section **2.6.1**. Confocal pictures were taken using a Leica SP8 confocal microscope (RRID: SCR_018169) with a 20x oil immersion objective and edited in Image-J-2.

3 Results

3.1. *aPKCΔ* expression experiment

3.1.1. Control experiment

As knockout of *aPKC* in all motor neurons leads to learning impairment (Ehweiner & Brembs, 2021) and overexpression of *aPKC* leads to learning improvement (Andreas Ehweiner, personal communication; **Attachment I**), the latter effect had to be confirmed by reproduction in "blind" experimenter manner.

Once it was reassured that non-treated control flies could learn in the described setup (*Material and Methods*; **2.3.**) using the standard learning protocol (**Table 2**; data not shown), control flies were tested for the adapted learning protocol (**Table 3**) to ensure the chosen laser setting resulted in no learning behavior to potentially be able to demonstrate a learning effect for treated flies. During training periods, flies showed efficient avoidance of the punished side, with PIs between 0,6 and 1 (**Figure 2A**, Periods 7, 8, 10 & 11). Yet, no learning behavior was observed during the test periods under omission of the punishing event (**Figure 2A**, Periods 12 & 13). Through the flight performance in **Figure 2A** and the non-significant memory expression in Period 12 (**Figure 2B**; $p = 0{,}804$; significance level set to p-value < 0.005), it could be proved that the chosen laser settings (*Material and Methods*; **2.3**.) resulted in flies showing no operant self-learning behavior.

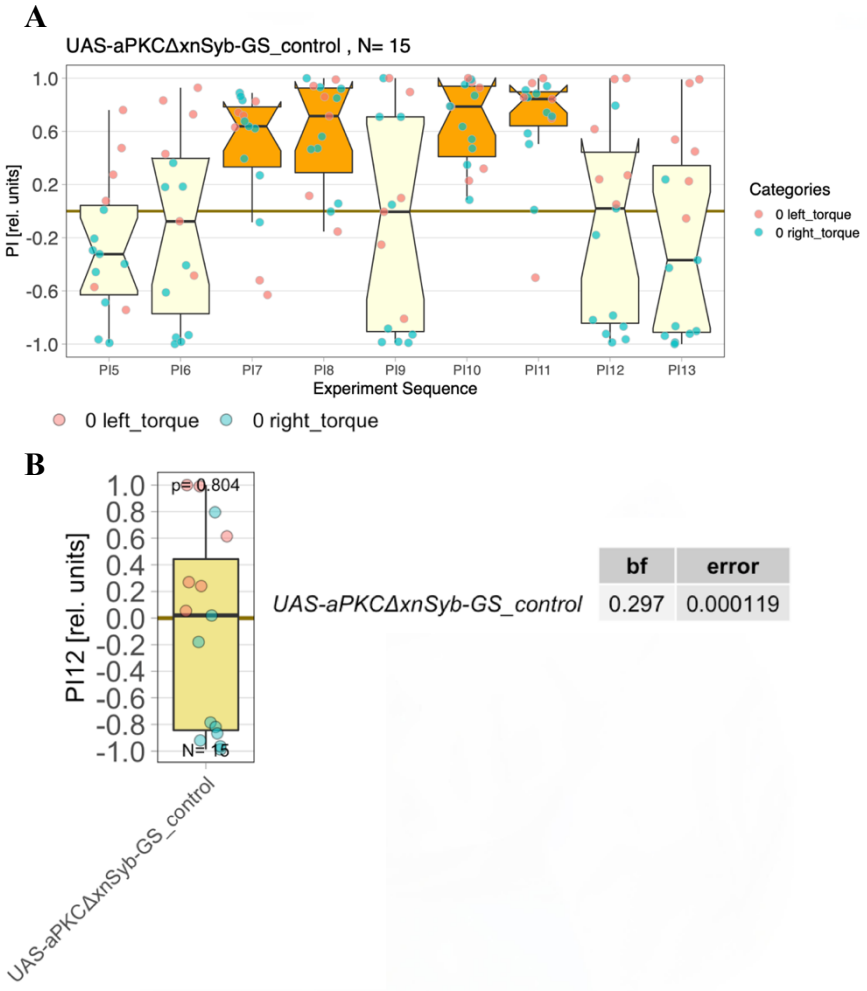

Figure 2: Graphic representation of flight behavior of 1 min. period control experiment; *UAS-aPKCΔ x nSyb-GS* flies; **A**: Performance Index box & dotplot without notches; Used learning protocol as described in **Table 2**; PI5 & 6: Pretest without punishment; PI7 & 8: Training periods with heat punishment; PI9: Test period without punishment; PI10 & 11: Training periods with heat punishment; PI12 & 13: Test periods without punishment; **B**: Statistical tests against zero (Wilcoxon-test).

3.1.2. *aPKCΔ* expression; Experimental and internal control group

To reproduce learning improvement, two groups of *UAS-aPKCΔ x nSyb-GS* flies were tested following the protocol in **Table 3**. One group (experimental group) was treated with RU486 to express *aPKCΔ* (*Material and Methods*, **2.2.**). The second one (internal control group) was not treated with the steroid hormone and therefore expressed an average level of *aPKC*. The internal control group showed an efficient punishment avoidance but no positive learning score during the test periods (**Figure 3B;** PI12 & 13), with non-significant PI (**Figure 3C**; p = 0,767; significance level set to p-value < 0.005). The experimental group also showed efficient punishment avoidance (**Figure 3A**) and flies showed an increased learning performance after

the first two training periods (**Figure 3A**, PI9).

Contemplating the two test periods at the end of the experiment, the experimental flies showed increased learning performance compared to the control flies (**Figure 3A and B**; PI12 & 13), even though the statistical analysis did not show a significant learning score (**Figure 3C**; p = 0,0149). Additionally, the experimental group's Bayes factor is roughly 14 times greater than the control group (**Figure 3C**; Experimental group bf = 3.09; Control group bf = 0.211).

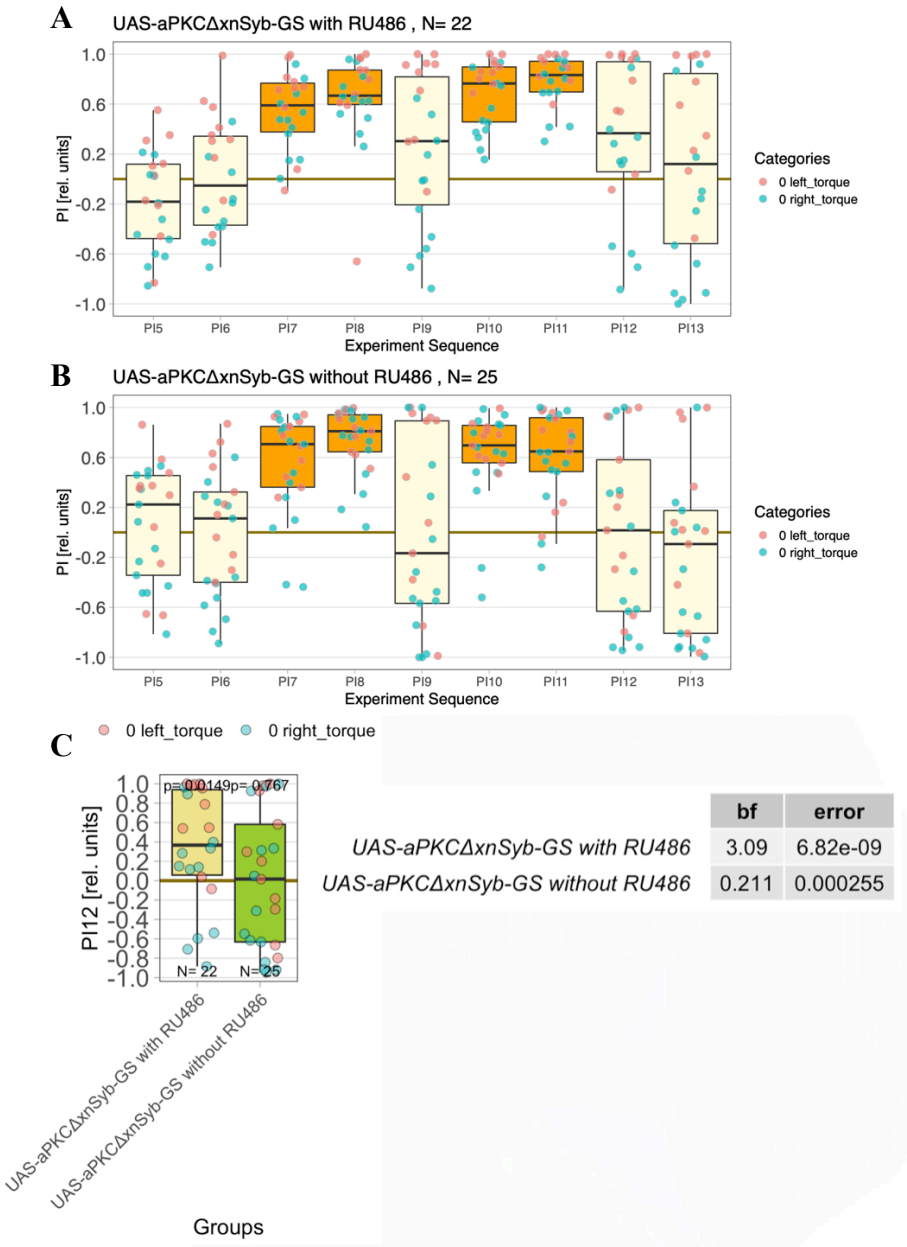

Figure 3: Graphic representation of flight behavior of the experimental group and the internal control group; *UAS-aPKCΔ x nSyb-GS* flies experimental group treated with RU486 and one control group without treatment; **A:** Performance Index box & dotplot without notches for RU486 treated group; **B:** Performance index box & dotplot without notches for non-treated group; Used learning protocol as described in **Table 3**; PI5 & 6: Pretest without punishment; PI7 & 8: Training periods with heat punishment; PI9: Test period without punishment; PI10 & 11: Training periods with heat punishment; PI12 & 13: Test periods without punishment; **C:** Statistical tests of single groups against zero (Wilcoxon-test).

3.2. *aPKC* and *FoxP* expression in adult *Drosophila* VNC

3.2.1. *aPKC* and *FoxP* expression without antibody staining

To further understand how *aPKC* and *FoxP* are responsible for motor learning, the expression pattern in the adult VNC was analyzed. As reference for the localization and connections of motor neurons, an illustrated ventral nerve cord video and figure from Maniates-Selvin et al. (2020) were used.

In **Figure 4A,** the expression pattern of *GFP*-expressing *aPKC* -positive neurons in the VNC is shown. '**a**' indicates projections leading to the neck of the fly, whereas '**b**' projects to the wings (Maniates-Selvin et al., 2020). In the ventral nerve cords' last segment, the abdominal neuromeres, a bright expression can be observed as in this area all abdominal neuromeres form a fusion (Court et al., 2020). **Figure 4B** shows the expression pattern of *RFP*-positive *FoxP*–expressing neurons, which is again strongly radiant in the abdominal neuromere. The remaining scanning revealed high noise-to-signal ratio with high level of background noise. However, *FoxP* expression could potentially be detected at neuropils projecting to the wings, labeled with '**b.**' To analyze the overlap of *aPKC* and *FoxP* expression, a composite image was created (**Figure 4C**).

Due to the overexposure of GFP and the high RFP background a clear overlap could not be identified with certainty, except in the abdominal neuromeres. Marked with '**b**' is a potential overlap of a motor neuron projecting to the wings (**Figure 4C**). To improve the results of the anatomical analysis, antibody staining was performed.

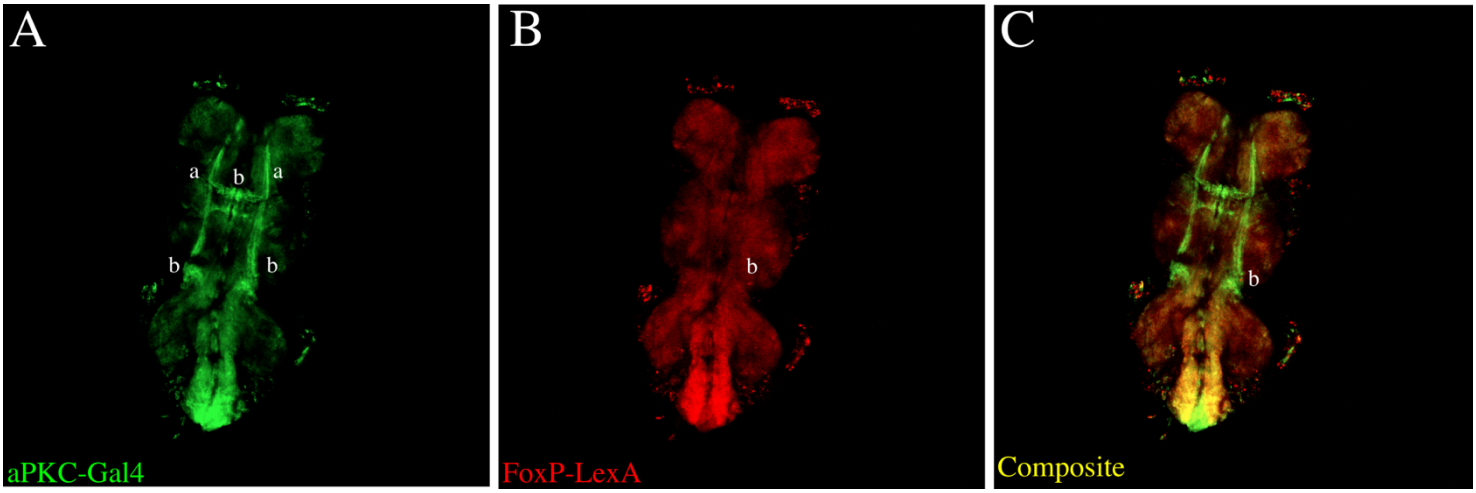

Figure 4: Confocal images of *Drosophila* VNC expressing aPKC and FoxP; A: Expression of *aPKC-Gal4* through GFP (green); **B:** Expression of *FoxP-LexA* through RFP (red); **C**: Composite; a: projection towards the neck; b: projection towards the wings.

3.2.2. *aPKC* and *FoxP* expression using antibody staining

Figures 5 and 6 A show the expression pattern of *GFP*-expressing *aPKC*-positive neurons in the adult VNC in dorsal (**Figure 5**) and ventral (**Figure 6**) views.

Antibody staining against GFP shows *GFP*-expressing *aPKC*-positive neurons in each segment of the VNC. In addition, *aPKC* expression could be detected in all nerve cords exiting the VNC and preserved by dissection. As reference figures from Maniates-Selvin et al. (2020) were used. In comparison, the expression of *RFP* in *FoxP*-positive neurons in stack was not as evident (**Figures 5 and 6B**). However, analyzing picture by picture, positive neurons can be seen in all segments but are not as dense as the *GFP*-expressing *aPKC*-positive neurons.

The composite primarily shows overlap in neurons projecting to the wings (**Figure 7**). In addition, overlaps in neurons projections to the neck and the meta- and mesothoracic leg could be detected (data not shown).

To better reveal the nerve tracts, confocal images were taken with overexposure. **Figure 8** potentially shows projecting to the metathoracic legs (**A**), wings (**B and C**) and prothoracic legs (**D**) throughout the stacks. Specifically, in **Figure 8D** both co-expression projecting to the prothoracic leg and neurons expressing *FoxP* and *aPKC* in parallel are visible.

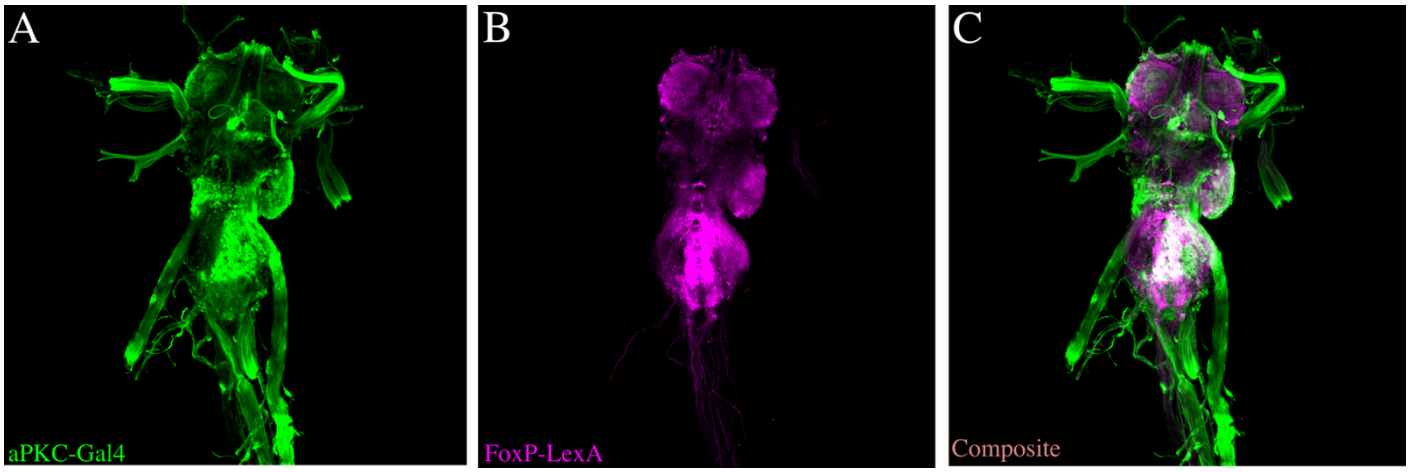

Figure 5: Confocal images with antibody staining of *Drosophila* VNC expressing *aPKC* and *FoxP* / dorsal view; A: Expression of *aPKC-Gal4* driving *GFP* (green); **B:** Expression of *FoxP- LexA* driving *RFP* (magenta); **C:** Composite.

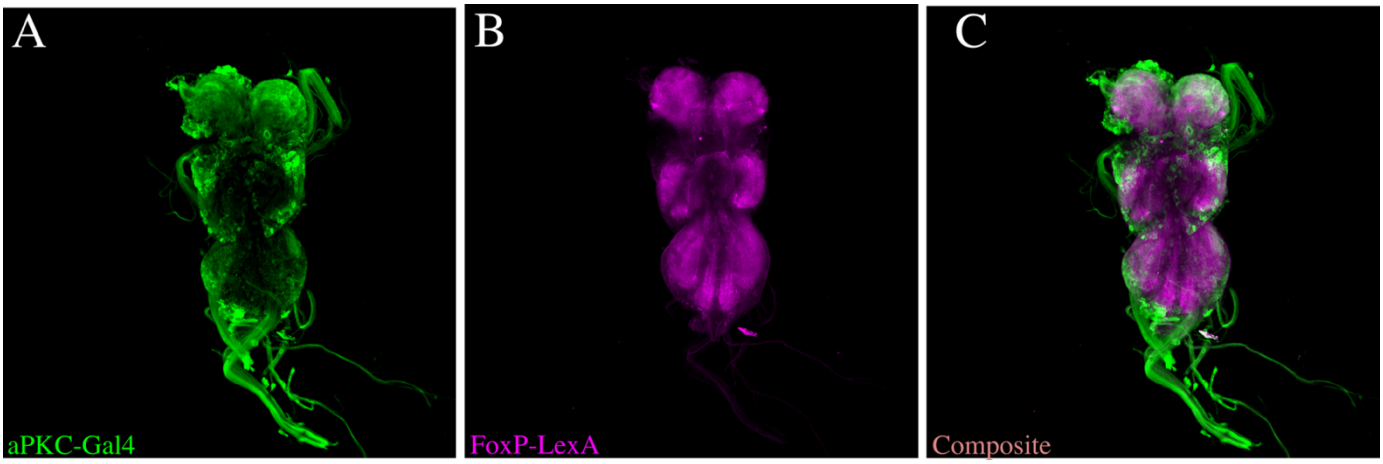

Figure 6: Confocal images with antibody staining of *Drosophila* VNC expressing *aPKC* and *FoxP* /ventral view; A: Expression of *aPKC-Gal4* driving *GFP* (green); **B:** Expression of *FoxP-LexA* driving *RFP* (magenta); **C:** Composite.

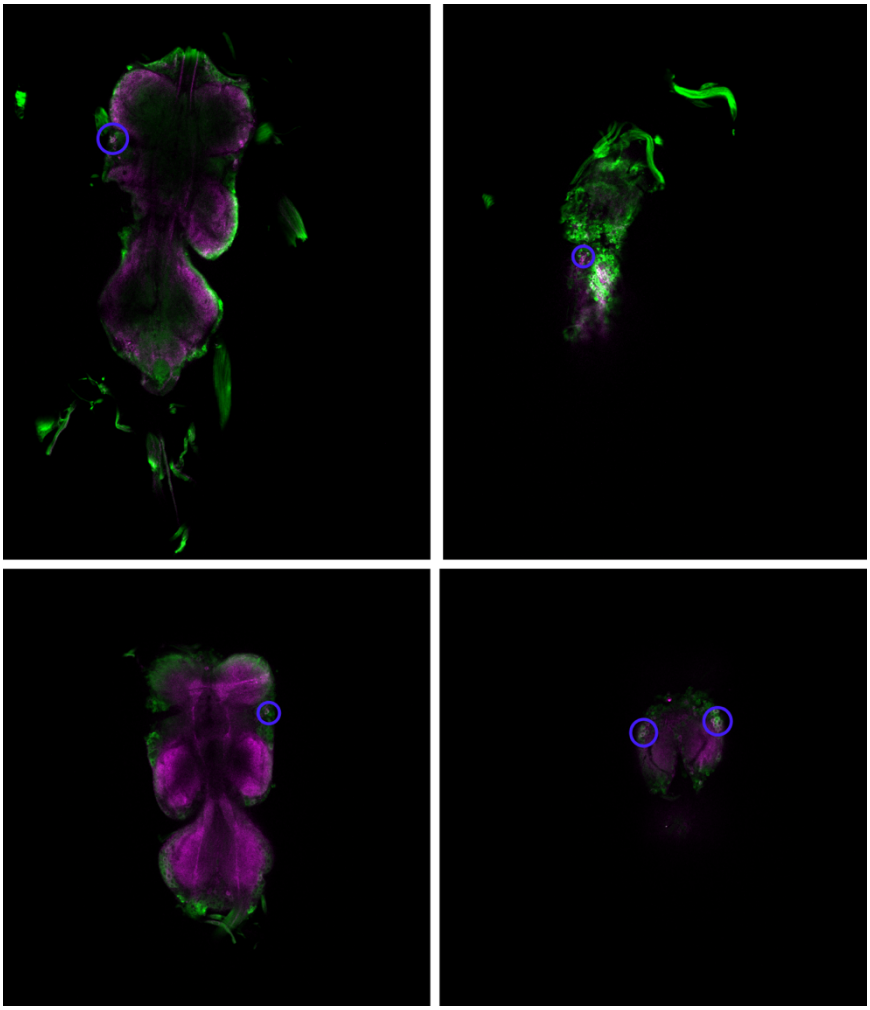

Figure 7: Composite confocal images expressing *aPKC* and *FoxP* showing overlap in neurons projecting to wings; Green: *GFP*-expression in *aPKC*-positive neurons; Magenta: *RFP*-expression in *FoxP*-positive neurons; White: Composite marked with blue circles.

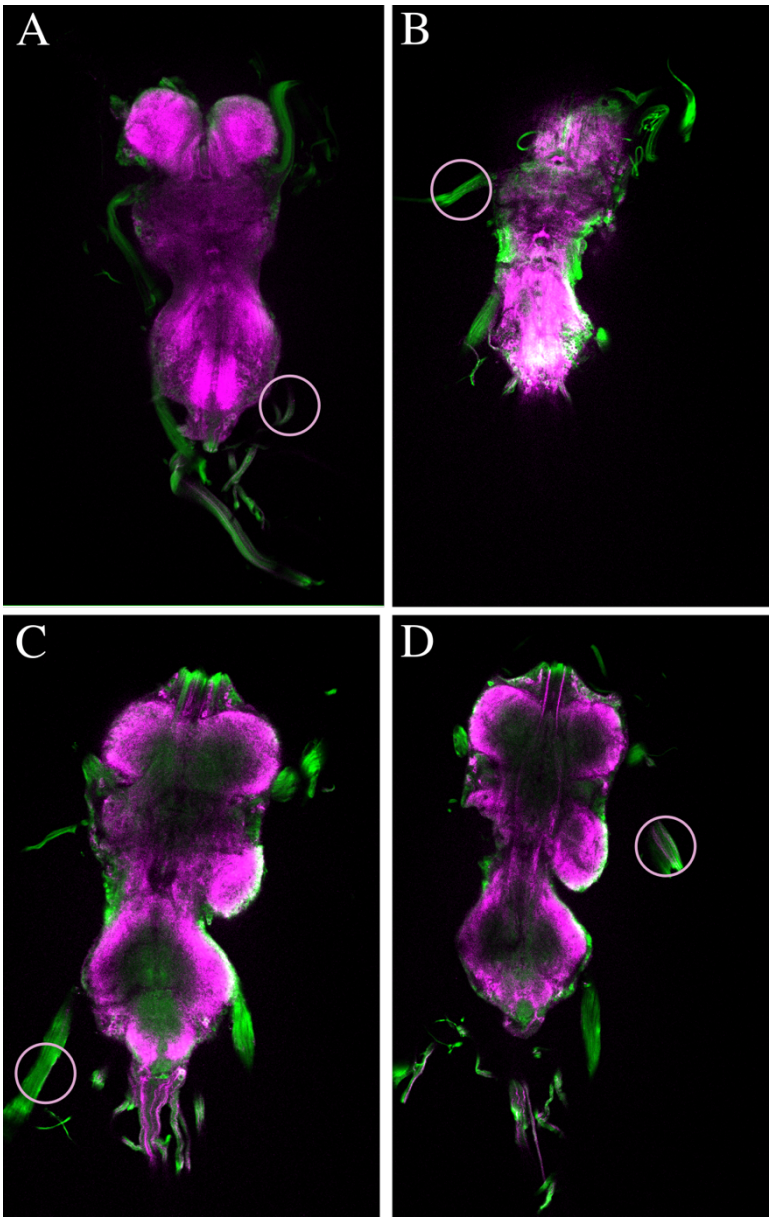

Figure 8: Composite confocal images expressing *aPKC* and *FoxP* showing overlap in nerve cords exiting the VNC; A: Overlap metathoracic leg; **B:** Overlap wings; **C:** Overlap wings; **D:** Overlap prothoracic leg.

4 Discussion

4.1. *aPKC Δ* expression experiment

In the presented work using the torque meter to investigate operant self-learning, it was possible to detect a behavioral change after overexpression of *aPKC* (**Figure 3**).

The control experiment with untreated flies and 1-minute periods was performed to find the right laser setting for the new setup (*Material and Methods*, **2.3**.). The laser has been set so low that non-treated control flies have lost their ability to learn (**Figure 2**). Thereby the control flies no longer showed any learning behavior so the subsequent effect of *aPKC* overexpression on the experimental flies could be compared.

To achieve *aPKC* overexpression, GeneSwitch activated through RU486 treatment was used (*Material and Methods,* **2.2.)**. The result of the internal control group's learning performance (**Figure 3B**) matched, as expected, the results of the 1-minute control experiment (**Figure 2A**), as no overexpression was performed. Even though the experimental flies did not show a significant learning score (**Figure 3C**), the learning behavior was increased compared to the internal control group's (**Figure 3 A and B**), indicating with multiple aspects that *aPKC* overexpression leads to improved operant self-learning.

To begin, looking at the PIs, the experimental flies showed a constant positive score during all three test periods, compared to the control flies' zero to negative PIs (**Figure 3 A and B**). Next, four tested experimental flies showed a strong preference for the punished side (**Figure 3A,** PI 12). A critical step in performing such conditioning experiment is eliminating the naïve side preference some flies tend to have. Besides the natural behavioral variability, such bias could impair the pooled results and tilt the statistical result to non-significant levels. It can be speculated that the negative PIs of those flies shifted the overall learning performance of the experimental group to less positive values and therefore lowered the learning scores' statistical significance. A greater sample size could potentially overcome the non-significant result. Lastly, additional statistical evaluations (**Attachment II**) defined tendencies of the experimental group to prefer the non-punished side. The control flies did not show such preference.

Considering that this experiment had similar results replicating initially conducted experiment by Andreas Ehweiner in 2021 (**Attachment I, Figure 9**), it is noteworthy to point out the differing parameters. The two experiments differ in laser intensity and pulsing rate, the torque meter device ('Götz' vs. 'Kopp' torque meter), the performing person and the laboratory environment (Andreas Ehweiner, personal communication). Despite slightly different

parameters, these comparable results underpin the reproducibility of this experiment. For further powerful evidence of reproducibility, the execution of the experiment by an independent laboratory would be conceivable (von Kortzfleisch et al., 2022).

Ehweiner and Brembs (2021) already described that *aPKC* is necessary for operant self-learning in *Drosophila*. This thesis showed that overexpression of *aPKC* improves learning performance compared to non-manipulated flies.

Having the enhancing effect of *aPKC* on operant self-learning, a potential dominance over other types of memories after *aPKC* overexpression could be investigated in the future, e.g., premature transition from goal-directed behavior to habitual responses.

4.2. *aPKC* and *FoxP* expression in adult *Drosophila* VNC

To detect the anatomical connections between the expression of *aPKC* and *FoxP* in the *Drosophila* VNC, confocal images were taken with endogenous expression (*Results,* **3.2.1.**) and antibody staining (*Results*, **3.2.2.**) to achieve different views of the expressing neurons through different working techniques. VNCs are shown in two different views because the second segment tore off during dissecting, yet many nerve tracts stayed attached (**Figure 5**). Therefore, the ventral view (**Figure 6**) was used while analyzing expression in the second segment. In addition, the antibody staining, particularly in **Figure 6**, shows no expression inside the VNC, which can be due to many reasons, e.g., the length of incubation with the antibody solution. Since many of the motor neurons are on the outer layers of the VNC (Maniates-Selvin et al., 2020) and therefore still visible, **Figures 5 and 6** complements each other in the analysis.

Because a connection between *FoxP* and *aPKC* is assumed and manipulation in both leads to impaired operant self-learning behavior using a flight simulator (Ehweiner & Brembs, 2021; Mendoza, 2014), co-expression in neurons projecting to wings was assumed. This could be demonstrated in **Figure 7** at several points.

Co-expression in nerve tracts from the VNC could be shown (**Figure 8**). However, considering that *aPKC* and *FoxP* are expressed throughout the VNC (**Figure 5 and 6**), co-expression should hypothetically be seen in more neural tracts. This difference may be due to the density and brightness of the *GFP*-expressing *aPKC*-positive neurons, which are more visible than *FoxP* even in overexposed confocal images.

Additionally, *GFP*-expressing *aPKC*-positive neurons can be seen in high density both in the VNC itself and in outgoing nerve tracts. Which is associated with the presented finding that *aPKC* overexpression improves operant self-learning behavior on the flight simulator and is essential for operant self-learning in general (Ehweiner & Brembs, 2021). Since

overexpression influences the flight behavior, potentially the dense expression in neurons projecting to the wings is essential. However, this requires further investigation into which muscles are innervated by the individual motor neurons and how these are used in operant self-learning tasks on the flight simulator.

4.3. Summary

The reproduced experiment on the flight simulator has shown that *aPKC* overexpression leads to improved operant self-learning performance in *Drosophila melanogaster*. Confocal images proved multiple co-expression of *aPKC*-positive and *FoxP*-positive neurons in adult *Drosophila's* VNC and outgoing nerve tracts.

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6 Attachment

I. *aPKCΔ* experiment by Andreas Ehweiner

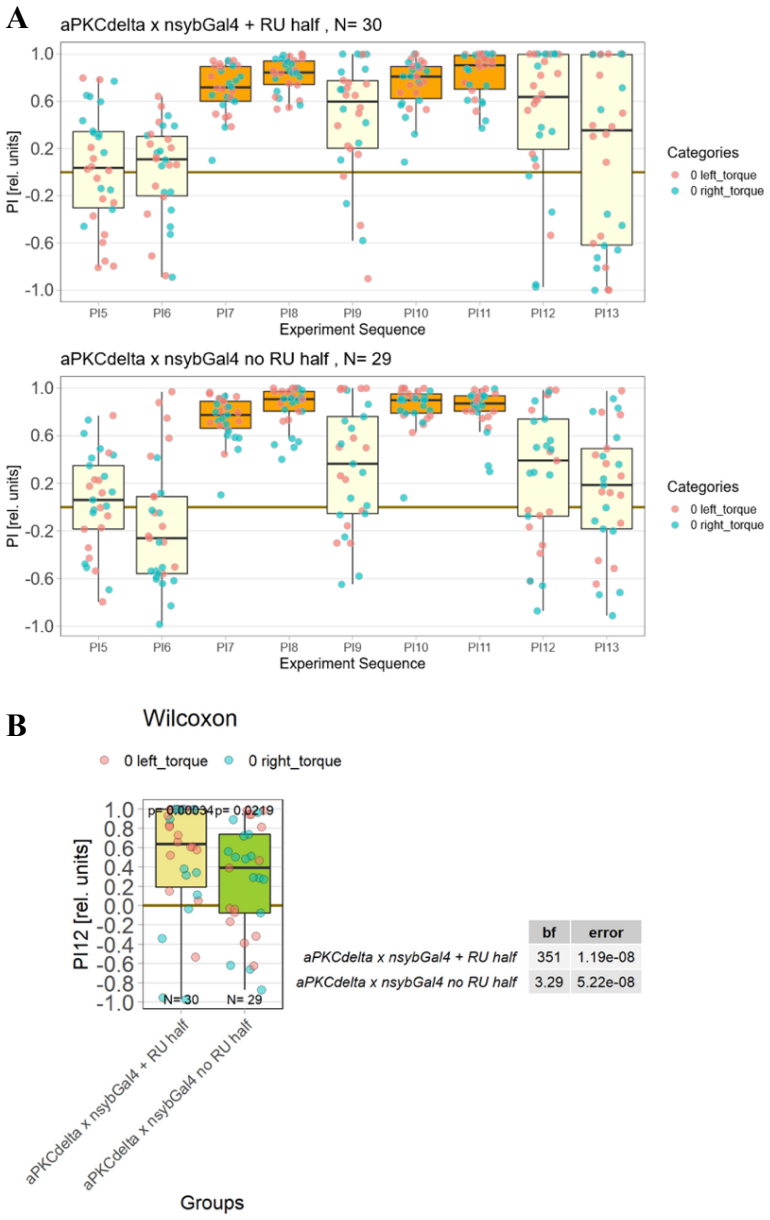

Figure 9: Graphic representation of flight behavior of the *aPKCΔ* experiment conducted by Andreas Ehweiner in 2021; A: Performance Index box & dotplot without notches; **B:** Statistical tests of single groups against zero (Wilcoxon-test).

II. Additional statistical evaluations of the *aPKCΔ* expression experiment

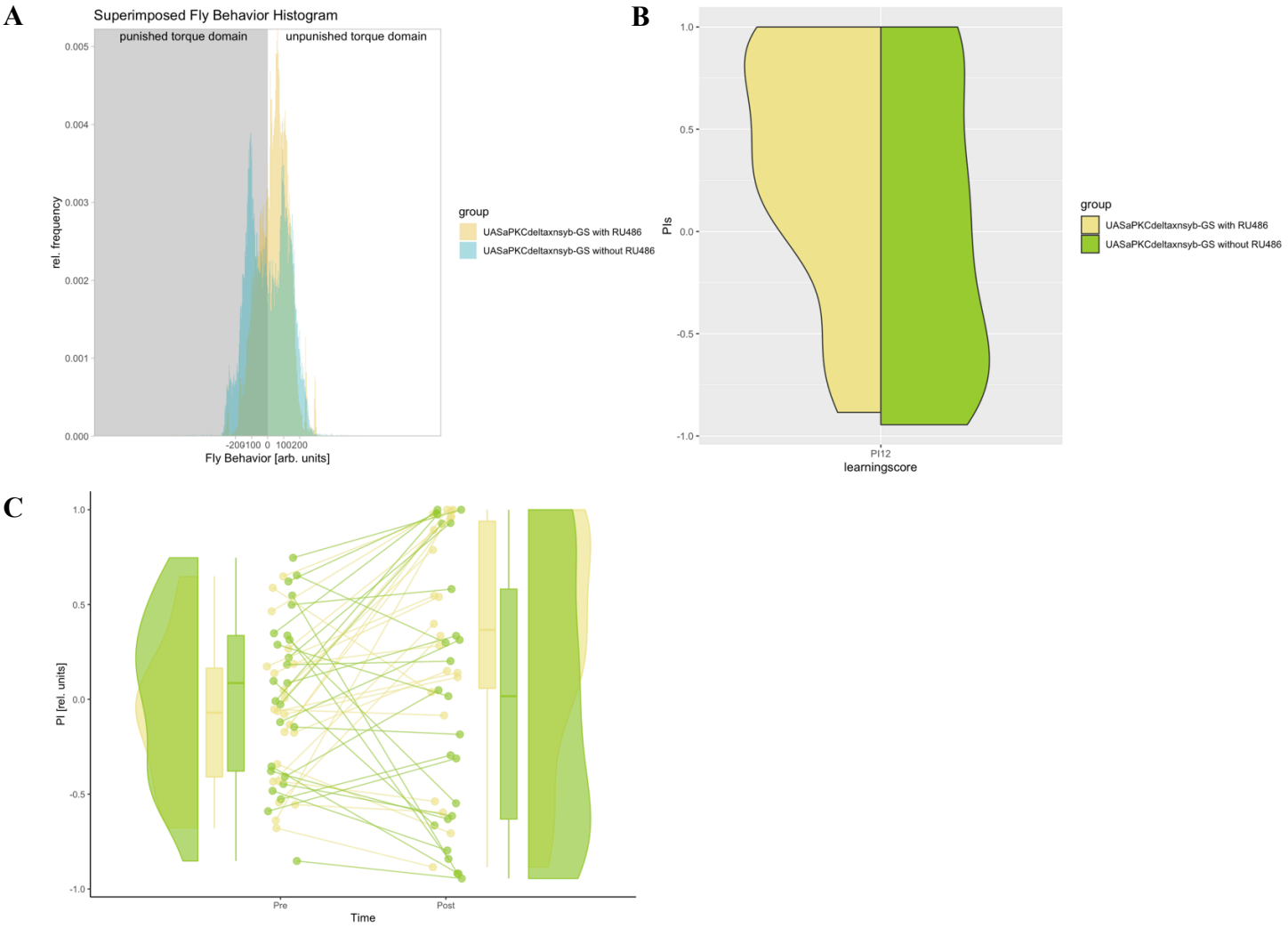

Figure 10: Additional statistical evaluations of the *aPKCΔ* experiment; A: Superimposed Fly Behavior Histogram; **B:** Raincloudplot; **C:** Split Violin Plot.

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8 Declaration of authorship

Die vorgelegten Druckexemplare und die vorgelegte elektronische Version der Arbeit sind identisch. Ich habe die Arbeit selbstständig verfasst, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und die Arbeit nicht bereits an einer anderen Hochschule zur Erlangung eines akademisches Grades eingereicht. Weiterhin bestätige ich, dass ich die von dem in § 27 Abs. 5 der geltenden Prüfungsordnung vorgesehenen Rechtsfolgen Kenntnis habe.

Datum	Unterschrift