# Comm2 regulation of synaptic arborization at the neuromuscular junction and its impact on the locomotor system of *Drosophila Melanogaster*

Alixe Kirbihler
*Principal investigator*

Prof. McCabe
*Laboratory professor*

Marine Van Campenhoudt, Md Al Amin Khan and Rebecca Smith
*Supervisors*

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Ecole Polytechnique Fédérale de Lausanne (EPFL), Department of Life Sciences
Prof. McCabe Laboratory of Neural Genetics and Disease, Brain Mind Institute
Laidlaw Scholar Leadership and Research Program

## I. Introduction

The fundamental organization of motor synapses is key to understanding locomotion in health and disease.

*Commissureless 2* (*comm2*), is expressed in the Nervous System (NS) glia of Drosophila Melanogaster and is part of the 'comm' protein family (Sarro J et al., 2013) that bears a highly conserved 22 amino-acids sequence. In contrast to comm1 which has been revealed to play an important role in axon guidance (Tear G et al., 1996), its 'sister protein' comm2 has not been extensively studied. Therefore, its exact function in the organism remains ambiguous.

In adult flies, a motor neuron divides and connects with a specific muscle fiber (Soler et al., 2004; Baek and Mann et al., 2009) which is reminiscent of the human NMJ organization (Ross A. Jones et al., 2017). Studies conducted by Prof. McCabe's Lab has revealed that the loss of function (LOF) of *comm2* causes extensive branching at the larval NMJs (unpublished) leading to the hypothesis that the mutant flies would present an aberrant level of muscle activity and less efficient motor neuron circuitry.

To investigate our hypothesis, we recorded adult flies on a treadmill to analyze kinematics of the *Drosophila Melanogaster's* legs, representative of motor neuron activity. In this way, we were able to evaluate accurately the fine motor movements capacities of the mutant group in comparison with a control group (*w1118*).

Thus, the goal of this experiment is to characterize how synaptic arborization regulation by comm2 at the NMJ affects the locomotion of adult flies. It should enable us to gain a better understanding of locomotor systems, essential to our comprehension of motor neuron defects.

## II. Background knowledge and terminology

The *comm2* project began 5 years ago in Professor McCabe's laboratory with the study of 3rd instar larvaes that were *comm2* null mutants. They indeed were genetically modified by P elements mutant genesis so that they were no longer able to produce the functional protein associated with the *comm2* gene. It enabled us to observe the lack of this protein's impact on the organism and to determine its role at the NMJ.

Studying interactions at the NMJ is crucial to understanding the mechanism by which movement is transmitted, and what can prevent it from being initiated (particularly in the context of neurodegenerative motor diseases). The NMJ is where the synaptic buttons at the end of the outgrowth (also known as axons or branch) of motor neurons release neurotransmitters. On contact with them, the muscle fibre contracts, which, through a chain effect, induces movement.

The term "branch" is therefore used to refer to any arborisation of a motor neuron where there are two or more boutons.

The study set up used immunostaining techniques to observe the NMJ of the Ib muscle located in *Drosophila Melanogaster*'s legs.

An HRP marker (in pink) was used to target motor neurons. In addition, a second marker called Dlg was used to localize specifically the Ib muscle.

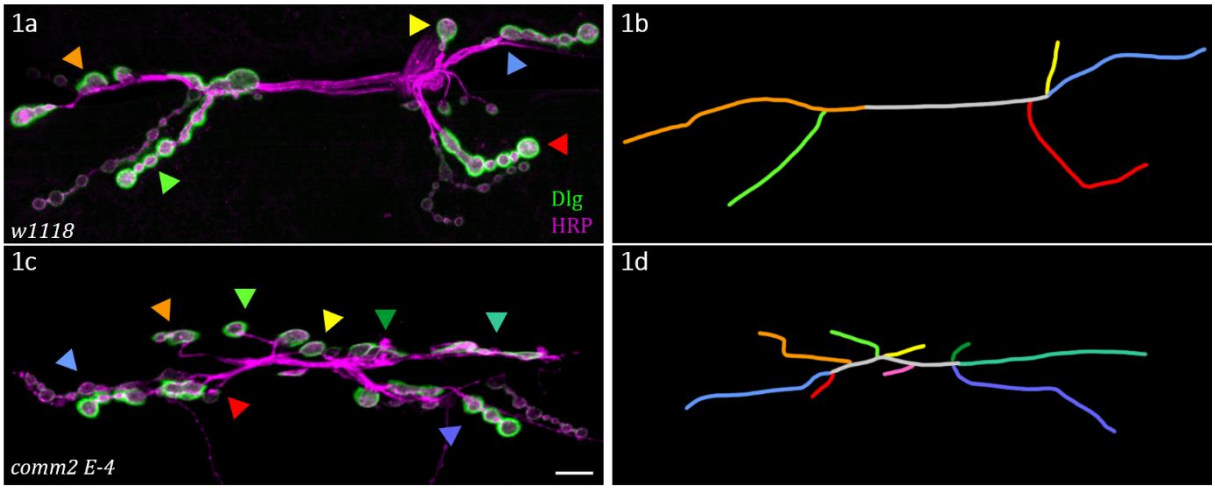


Fig. 1: **LOF of comm2 increases the number of branches and their length in Ib motor NMJs in Drosophila 3rd instar larvae**

(a): Ib branches (arrowheads) of *w1118* larvaes in m6/7 NMJ. (c): NMJ image of *comm2* null mutant larvae clearly shows that the number of branches is significantly increased compared to control NMJ. It was also observed that mutant Ib branches are shorter compared to controls (f). (b) & (d): schematic diagram depicting the branching pattern of aforementioned (a) & (c) NMJ image. *n*= biologically independent samples, Scale bar: 10µm.

These results were confirmed by statistical analysis, which reported extremely significant differences in branch number and size between the two genotype groups, as shown in Fig. 2.

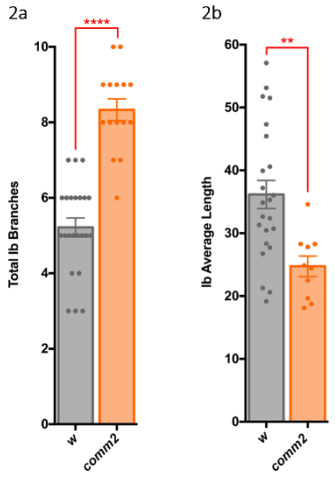


Fig. 2: **Quantification of the total number of branches and their length in Ib motor NMJs LOF *comm2* and *w1118* 3rd instar larvae**

(a): Number of total Ib branches in w1118 and comm2 quantification. t-test. (b): Quantification of branches' average length in *w1118* and comm2. t-test. *n*= biologically independent samples

However, these experiments were carried out only on L3 comm2 mutant larvae and not on adult flies, which is limiting the scope of our understanding. We therefore had to explore the evolution of the phenotype once the flies had grown to understand the impact of the LOF of this protein on a more complex motor system over the long term.

## III. Methods

This investigation has been designed around two experiments which will evaluate distinct behavioral features about the genotype groups in order to encapsulate a more precise characterization of the null mutant's motor movements capacities.
All of the flies went under the same experiments' conditions in an atmosphere of 29°C and 50% of humidity to ensure the validity of our results.

### A. Climbing

The subject of study is homozygous *comm2* males null mutant that are between one- and fourteen-days post eclosion (DPE). They will be compared with a control group of wild type *Drosophila Melanogaster* (*w1118*) of the same age.

For negative geotaxis climbing assays, male flies of appropriate genotypes were sorted upon eclosion and transferred to new food vials every two days throughout the course of experiments. Groups of 6-8 male flies were transferred to a fifteen cm long empty plastic vial containing no food. After a ten-minute acclimation period, the vial was tapped three times to activate their survival reflex and the ability to climb was video recorded (30fps) with a Rasperry Pi system. *FreeClimber* was then used to analyze the recorded videos and evaluate their vertical velocity in cm per second.

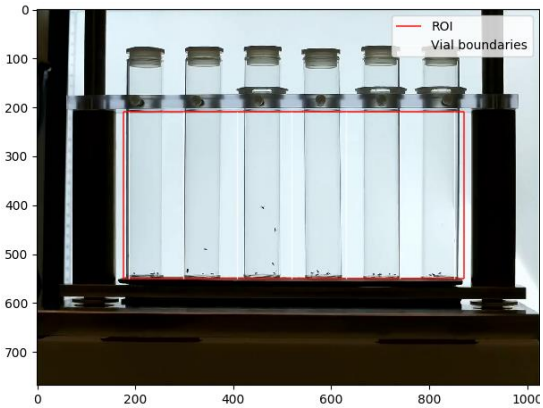


Fig.3: **Image of the climbing set up**

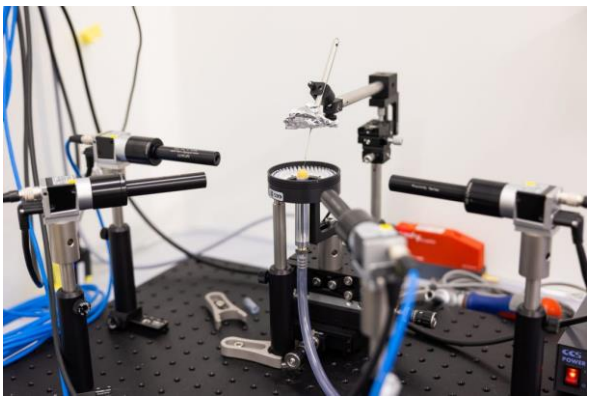

Fig. 4: **Image of the fly treadmill set up**

## B. Fly treadmill

The fly treadmill is an experiment that allows us to evaluate the flies' fine motor movements by recording how they walk.
The subject of study will be homozygous *comm2* null mutant males that are 10 and 20 days old. They will be compared with a control group of wild type *Drosophila Melanogaster* (*w1118*) of the same age.

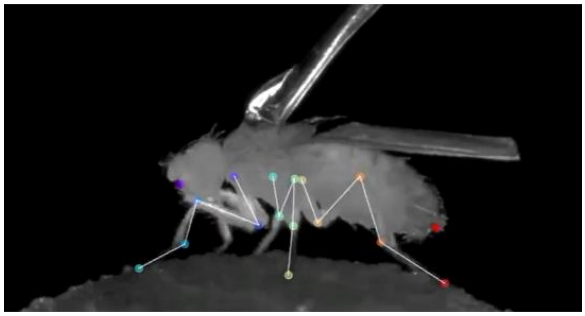

Fig. 5: **Video recording labelled by *DeepLabCut* of a fly on the treadmill**

After capturing their behavior and movements, we characterized the phenotype of the mutant by noting their differences and/or similarities with the control group. We especially focused on computing their average rest time on a one-minute walking session, as it is a very relevant behavioral feature to evaluate their motor movements capacities.

To do this, after capturing the movement of the groups of flies, we used *DeepLabCut* software, based on a neural network. *DeepLabCut* is then able to place the fly's body parts and provide us with their spatial coordinates over time in an Excel file.

A Python script had to be created from scratch to analyze the data. Derivation over time dx/dt was used to determine left legs' distal tip horizontal speed. The rest time intervals were defined as when the speed was close to zero (highlighted in yellow).

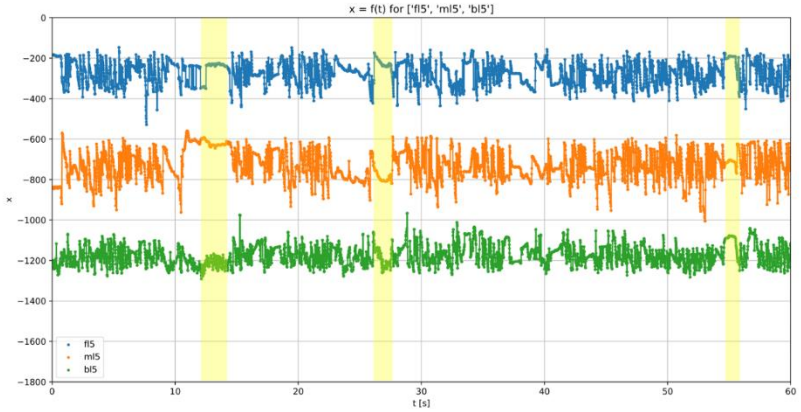


Fig. 6: **Position x of the left legs' distal tip over time**

The fly's legs were also rotated by the angle between the head-tail diagonal and the horizontal to ensure similar starting positions.

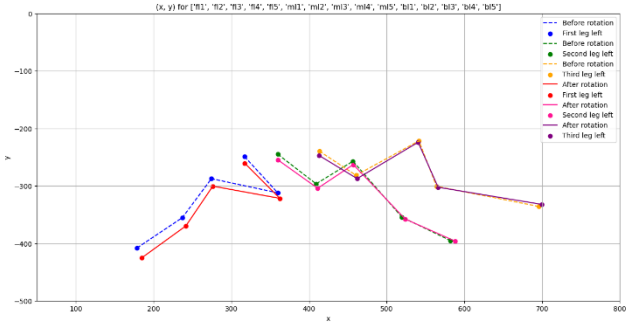

Fig. 7: **Comparison of the legs' position before and after the normalization**

At the end of this experiment, we were thus able to quantify the kinematics associated to extensive synaptic arborization by *comm2* LOF, hence its repercussions on flies' movement and behavior.

## IV. Results

Differences in vertical velocity between w1118 and comm2 LOF are not significant (ns). Two-Way ANOVA test : p= 0.0625 The data points were represented as Mean ± SEM.

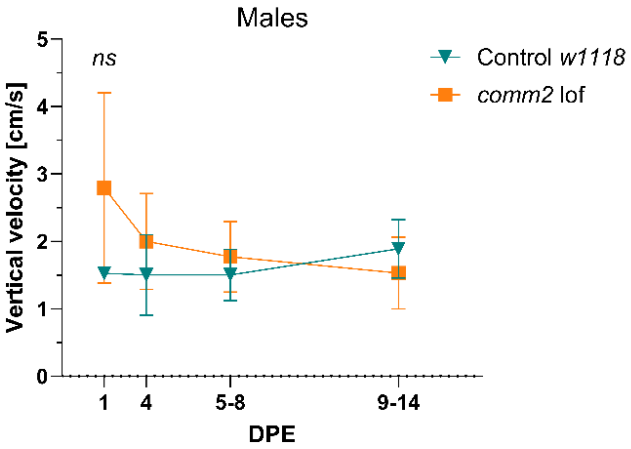


Fig. 8: **Climbing abilities comparison over age of adults *w1118* and *comm2* LOF**

Differences in vertical velocity between *w1118* and *comm2* LOF are not significant (ns). Two-Way ANOVA test: p= 0.0625. The data points were represented as Mean ± SEM

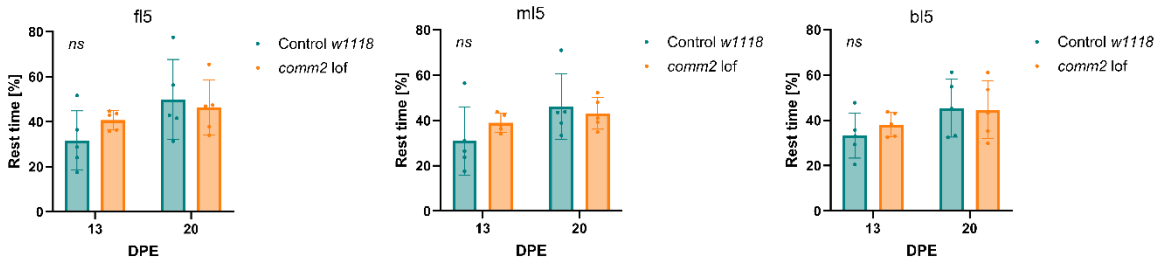


Fig. 9: **Proportion of resting time for each left leg comparison between *w1118* and *comm2* LOF groups at two different ages**

Not significant results in regards of the variables tested (genotype, age and body part). Two-Way ANOVA test (a) : p= 0.6456 (b) : p= 0.6327 (c) : p= 0.6741. The data points were represented as Mean ± SEM.

## V. Discussion

Considering the experiments carried out, we did not find significant differences between the LOF mutant group and the control group. Vertical velocity values during climbing and resting time in the 'fly treadmill' experiment did not show a strong correlation with genotype. This suggests that motor movement defects related to comm2 LOF at the NMJ may not be prominent.

However, due to time constraints, we could only test a limited population of flies (5 per age and genotype group, totaling 20). This limitation affects the robustness of our results, making them sensitive to outliers and less representative of the gene's impact. Thus, we are unable to confirm our hypothesis at this time.

However, neither can we fully refute it. Indeed, given the time constraints, it was not possible to test a large population of flies (5 per age and genotype group, i.e. 20 in all). To improve our findings, repeating these experiments with larger populations is advisable. Additionally, it's worth noting that the spatial coordinate data collected using *DeepLabCut* may lack precision, especially for flies with severe motor disorders. This imprecise labeling influenced our results.

Nevertheless, this investigation enhanced behavioral analysis of fine motor movements on a laboratory scale. We developed a Python script tailored to evaluate resting times during experimental sessions, and it holds potential for more advanced behavioral analyses. Future enhancements could include joint angle measurements to predict mutant phenotypes.

While our primary goal was to initiate characterization of the gene in Drosophila melanogaster, it succeeded in laying the groundwork for further research.

Notably, our findings align with studies showing the presence of a synaptic safety factor in neuromuscular organization (Wood SJ et al., 2001 and Soler C et al., 2004). This safety factor ensures neuromuscular junction functionality despite external stress or suboptimal

conditions. Therefore, the loss of the studied gene may be compensated for, making it challenging to observe a clear behavioral phenotype. Future electrophysiological experiments may shed more light on muscle activity in mutants.

## VI. Conclusion

The purpose of this investigation was to determine comm2 necessity in an organism. Even though its role has been linked to an overbranching phenotype in 3rd instar larvaes, the experiments undertaken did not reveal a subsequent motor movement defect in adult flies. However, it should be noted that the *comm2* LOF group showed a tendency towards longer periods of inactivity, as shown in the fly treadmill experiment.

Moreover, further research may be needed, in particular by studying a larger population of flies and focusing on a wider scope of behavioral characteristics. In fact, a comprehensive understanding of the mutant phenotype requires a more multifaced analytical approach.

It would also be interesting to extend the age parameters of the experiment to encompass older flies. This may allow us to better understand how the mutant group evolves with age.

To conclude, this investigation has paved the way for future researches into *comm2* specific function by first characterizing the gene.

## VII. Acknowledgements

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