OptoPAD: dissecting the neuronal control of *Drosophila* eating habits by merging the flyPAD with optogenetics

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Abstract

The goal of this project is to develop technology to manipulate genetically identified neurons and investigate the circuit basis of behaviour in *Drosophila melanogaster* using optogenetics. We use a combination of the FlyPAD (Fly Proboscis and Activity Detector – a behavioural system, that detects the physical interaction of flies with food by capacitive-based measurements) and *Bonsai* (a visual programming framework for the acquisition and online processing of data streams), in order to build a new behavioural paradigm where flies choose between two sources of food with different "neuronal consequences" depending on their actions. We built a system (OptoPAD) capable of simultaneous recording of feeding behaviour and manipulation of neuronal activity in 32 individual flies expressing different opsins.

The new system makes use of a 32-arena FlyPAD system where a high-power RGBA LED illuminates each individual arena. Using *Bonsai*, each capacitance signal is processed in real time and when the fly behaviour matches predefined parameters, an activation message is sent to an Arduino board leading to the activation of the LED and hence triggering the desired optogenetic manipulation.

Using this system we demonstrate that activation of *Gr5a*- positive neurons triggers appetitive behaviour and activation of *Gr66a*-positive neurons drives aversion from food. This system allows the dissection of the circuit basis of feeding homeostasis and the closed-loop manipulation of circuits to study aversive, appetitive or anticipatory behaviours in *Drosophila*.

Keywords: FlyPAD, Bonsai, optogenetics, channelrodopsin, closed-loop

1. Introduction

Animals need to eat in order to survive, making feeding an essential component of animal behaviour. In fact, feeding is one of the few behaviours that can give us an insight into an animal's physiological status, since they tend to choose their diet according to their needs. Fruit flies (*Drosophila melanogaster*) have emerged as a powerful model to study the neuronal and molecular mechanisms underlying feeding behaviour as they can be easily tracked and genetically manipulated[1]; however, their small size makes it difficult to quantify their food intake.

In order to overcome this disadvantage, we have recently developed an automated, high resolution behavioural monitoring system called FlyPAD (fly Proboscis and Activity Detector), which uses capacitive-based measurements to detect the physical interaction of individual flies with food, generating metrics related to total food intake, hunger and satiation[2].

In recent years, neuroscientists have developed different approaches in order to understand the relationship between neuronal activity and behaviour. One approach is to directly manipulate neuronal activity and correlate it with changes in behaviour. This methodology can be performed both in open- or closed-loop experiments. Open-loop means that neuronal activity is manipulated independently of the animal's behaviour, in contrast during closed-loop experiments, the behaviour of the animal determines the way in which neuronal activity is manipulated, allowing higher temporal precision of the manipulation (which animals can learn as it comes closer to the physiological situation where an action triggers a response) and fully autonomous neuronal manipulation.

The goal of this project is to build a behavioural setup capable of simultaneously recording feeding behaviour and manipulating the electrical activity of neurons in *Drosophila* in a closed-loop manner.

2. State of the Art

We can travel more than a century into the past and find authors interested in how the body maintains its stability in the face of a variable external environment. For example, Bernard in 1878 in his book "Leçons sur les phénomènes de la vie, communs aux animaux et aux végétaux" stated that "the conditions which must be maintained constant in the fluid matrix of the body in order to favour freedom from external limitations are water, oxygen, temperature and nutriment (including salt, fat and sugar)"[3,4]. Indeed, since the first definition of homeostasis, nutrition has always formed a core component of it. It is well known that animals, in order to survive and create healthy offspring (species maintenance), must eat. Moreover, they must eat according to their internal needs.

Understanding the way the brain is able to modulate eating behaviour according to its body needs is a way

of getting closer to food related diseases, such as obesity, and possibly to finding some answers as to whether it has a neuronal explanation[5].

2.1. Measuring Feeding Behaviour

Fruit flies (*Drosophila melanogaster*) have been a key model to study evolutionarily conserved biological processes such as ageing, nutrient sensing and feeding because of their short life cycle and their array of genetic tools to rapidly unravel the complexity of biological systems. Although this model has been widely used to study feeding behaviour, precise quantification of food intake has been very difficult to achieve, due to the small quantities flies eat and also due to their small size (hard to measure reliable weight differences)[6,7]. During recent decades several methods have been developed to study different aspects of food intake and feeding behaviour in Drosophila[1].

Information is getting more and more computerized. Thus, the automated and high-throughput gathering of data is becoming possible and scientists are developing methods that will allow them to get more digital data in less time.

The FlyPAD (fly Proboscis and Activity Detector) is an automated, high-resolution behavioural system, which uses capacitive-based measurements to detect the physical interaction of individual flies with food. When an animal, standing on one electrode that surrounds the food source, touches the food, which is placed on a second electrode, with its proboscis or leg, changes in the dielectric constant between the two electrodes occur, therefby creating a deviation in the capacitive signal. This sensor has a resolution of 100Hz with a sensitivity of 1fF. One setup can record up to 64 channels simultaneously and independently.

This method makes use of an off-line (after the experiment) algorithm for detection of activity bouts (changes in the capacitive signal that represent the contact of the fly with the food, interacting either with its proboscis, leg or body). Flies tend to eat in feeding burst within the activity bouts. Furthermore, due to the great sensitivity and time resolution this system allows, we were able to show that flies eat from solid sources of food in a very stereotypical way. In fact, flies may use a central pattern generator in order to control a feeding motor programme. Flies rhythmically extend and retract their proboscis on the food (5Hz). This behaviour was reflected in a highly rhythmic, square wave-like pattern in the capacitance signal. The signal was processed using a "sip-detection" algorithm to study feeding behaviour of flies dynamically across time[2].

This method is presently one of the most sensitive method to measure feeding behaviour of flies as it is sensitive to individual sips. Moreover it allows for high-throughput experiments as the data analysis takes much less time than with the "traditional" methods, which rely on manual scoring.

2.2. Gustatory System in *Drosophila*

In order to survive, animals must distinguish between edible and contaminated food.

Unlike vertebrates, which possess only one gustatory organ in the body, insects have their taste organs distributed over different parts of their body. Neurons expressing gustatory receptors (gustatory receptor neurons – GRNs) can be found in sensory bristles on the proboscis, internal mouthpart organs, legs and wings[8].

By 2001, Clyne, Dunipace, Scott and co-workers analysed the *Drosophila*'s genome using a bioinformatics algorithm and identified all 68 GR genes (each one coding for a seven-transmembrane domain protein)[9–11].

The fruit fly, like other insects and mammals, tends to search for sweet food and to avoid bitter compounds as they are usually toxic[12]. In *Drosophila*, this distinction has been reported to be mediated by many different GRNs, particularly by the activity of two different GRN populations that lack anatomical overlap[10,13,14]. These cells are named *Gr5a*- and *Gr66a*-positive neurons. Studies have shown that flies lacking *Gr66a* expression fail to avoid bitter substances and that those lacking *Gr5a* are not attracted to sugar[15]. Furthermore, the segregated projection of these two types of neurons reveal that there is a spatial map of taste quality in the fly's brain[13]. Ultimately, we can say that activation of *Gr5a* cells mediates attractive/appetitive behaviours while the activation of *Gr66a* cells mediates aversive responses[14].

2.3. What is Optogenetics?

Optogenetics refers "to the integration of optics and genetics to achieve gain- or loss-of-function of well-defined events within specific cells of living tissue"[16].

In 1971 and 1973 Oesterhelt and Stoeckenius[17,18], identified the first bacteriorhodopsin, a microbial light-activated ion pump. In the subsequent decades, the microbial opsin family that comprises this pump was later enlarged by hyperpolarizing light-driven chloride importers – the halorhodpsins[19] and by depolarizing light-gated cation channels – the channelrhodop-sins[20,21].

Each opsin requires a vitamin A-related cofactor (retinal) to form a functional molecule, the rhodopsin[16]. Upon absorption of a photon, retinal isomerises and triggers a sequence of conformational changes within the opsin partner, therefore allowing ions to cross through the cell's membrane[22].

In 2005, Boyden and Deisseroth demonstrated that the introduction of microbial opsin genes (in particular

channelrhodopsin-2)[21] to mammalian neurons could reliably generate defined sequences of spikes or synaptic events in response to light with millisecond-timescale temporal resolution[23].

Unlike mammals, flies and worms do not possess sufficient levels of endogenous retinal for opsins to function in all cell types. Therefore, supplementary retinal in the food is needed to enable the use of optogenetic tools in these animals[24]. In order to use opsin genes in *Drosophila*, one needs to generate transgenic animals, by injecting a construct into the fly which, when incorporated, will allow expression of opsin genes under the control of UAS, either using a P-element insertion[25] or site specific integration using Phage ΦC31 integrase system[26].

Nowadays there are over twenty single-component optogenetic tools[16,22]. I will give a more detailed insight on two particular tools: ReaChR and Chrimson channelrhodopsins.

Although flies are an incredible model to dissect neuronal and molecular mechanisms underlying behaviour or circuit function, the use of optogenetics in adult flies has been difficult to implement due to two main reasons. The majority of channelrhodopsins available are blue light-sensitive channels. Flies can see blue light[27], so stimulation might induce visually driven behavioural artefacts. On the other hand, blue light is strongly absorbed by the cuticle of the fly, which may prevent activation of the channels if the targeted neurons are not close to the surface[28].

In 2014, two new red-shifted channelrhodopsins were described and from then on, have been quickly adopted by *Drosophila* researchers.

One of them is called ReaChR (Red activatable Channelrhodopsin), which has improved membrane trafficking, greater photocurrents and fast kinetics when compared to a previously described red spectral shifted channelrhodopsin (C1V1(E122T)). Its maximum response is given at ~590nm. This opsin was proposed to be a robust means to stimulate neurons with red-orange to red light[29]. In fact, this tool was used successfully to dissect the neuronal control of Drosophila‘s male courtship, using 1.1 mW/mm^2 [28].

The other red-shifted channelrhodopsin was described by Klapoetke and colleagues at MIT, who discovered multiple channelrhodopsin homologs from different species, via de novo sequencing. The new red-light drivable channelrhodopsin, discovered from the species *Chlamydomonas noctigama*, was nicknamed Chrimson and, with a spectral peak at 590nm, was described as “45nm more red-shifted than any other previously known channelrhodopsin”. This tool was effectively tested in *Drosophila* by measuring the percentage of flies expressing Chrimson in *Gr64f* neurons (sweet taste receptors) that elicited PER (Proboscis Extension Reflex), when stimulated (with 0.015 mW/mm^2) with amber light (617nm). Chrimson proved to be a reliable and fast means to optogenetically stimulate flies[30].

2.4. Whats is Closed-loop?

“Closed-loop” refers to the control systems that uses the output of a system to control or modify the input. These systems measure the error between the desired and the actual state of the plant and uses it to alter the signal of the input[31].

Closed-loop experiments in *Drosophila* have manly been used to study optomotor responses, where various visual stimuli lead to different motor behaviours, for example, following the movement of a vertical bar. These motor responses are usually studied in the context of walking or flying. Researchers can study locomotion either in freely moving or tethered flies.

Studying freely moving animals (walking or flying) require video tracking of the animal, where its position serves as input for modifications either in the visual stimulus (projection or display panels)[32,33], in the direction or strength of wind in a wind tunnel system[34] or even in the target of one laser in order to heat parts of fly’s body[35].

In setups where the fly is tethered, closed-loop systems have received more specialisation and development.

When studying walking in Drosophila, flies are left to walk on an air-suspended ball. The rotation of the ball is recorded and the path the fly takes is integrated in a virtual map of the movement of the fly. The fly is able to navigate in a virtual world as its movement is being mapped onto the projected visual stimulus, thus closing the loop[36–39].

The same virtual world navigation occurs in studies using flight simulators, where the fly is suspended and either measurements of the yaw torque[40,41] or of wing beat amplitude and frequency[42–47], serve as input for the navigation in the virtual reality that is being presented to the fly.

Furthermore, closed-loop systems in Drosophila play a key role in studying memory and learning in flies. Examples of conditioning in flies, are the flight simultator developed by Wolf and Heisenberg in 1990[48], where the fly’s yaw torque is input for modifications in the visual stimulus, and the heat box by Wustmann in 1996[49], where the position of the fly inside a box serves as input to control a heater that functions as an aversive stimulus.

3. Hardware and Software

We built a new setup based on the previous behavioural measurement system, the FlyPAD. One computer is able to receive the capacitive data of the FlyPAD from the USB port using *Bonsai*, a “modular, high-performance, open-source visual programming

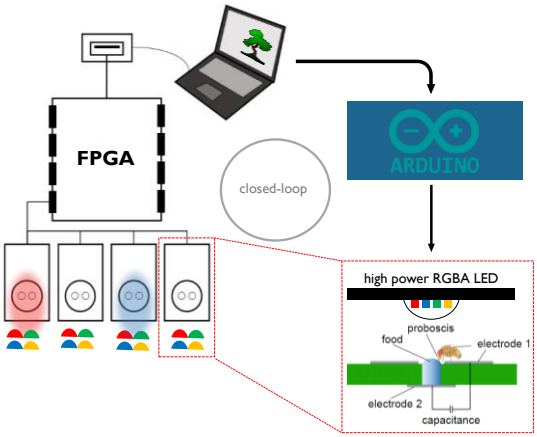

Fig. 1 Schematic of the OptoPAD *From the FlyPAD on the left to the clockwise direction we find the PC running Bonsai and receiving the capacitive data stream; Bonsai is able to send activation messages to different pins of the Arduino (the actuator), which will activate the different colours of the LEDs on top of the FlyPAD behavioural arenas where the animals are interacting with food.*

framework for the acquisition and online processing of data streams" conceived and developed at the Champalimaud Foundation[50]. To save the data from the FlyPAD, *Bonsai* allows an interface to record the capacitance signal at 100Hz.

Figure 1 illustrates the concept of the new behaviour recording and manipulation setup, from now on called OptoPAD.

In order to build this system we had to design two types of boards (using Eagle 6.2.0): the OptoPAD LED Board, which hosts the High Luminous Efficacy 10W RGBA LED from *LedEngin, Inc.* (ref. no. LZ4-00MA10) (four different wavelengths: 460 nm – blue, 523 nm – green, 590 nm – amber and 625 – red) and four N-Ch 100V 1.1A Infineon MOSFET transistors that serve as switches for each of the four colours; the OptoPAD Main Board, which hosts the Arduino board and distributes the electricity from the Corsair CS750M ATX Power Supply to power all the 64 OptoPAD LED Boards of the whole system (Fig.2a).

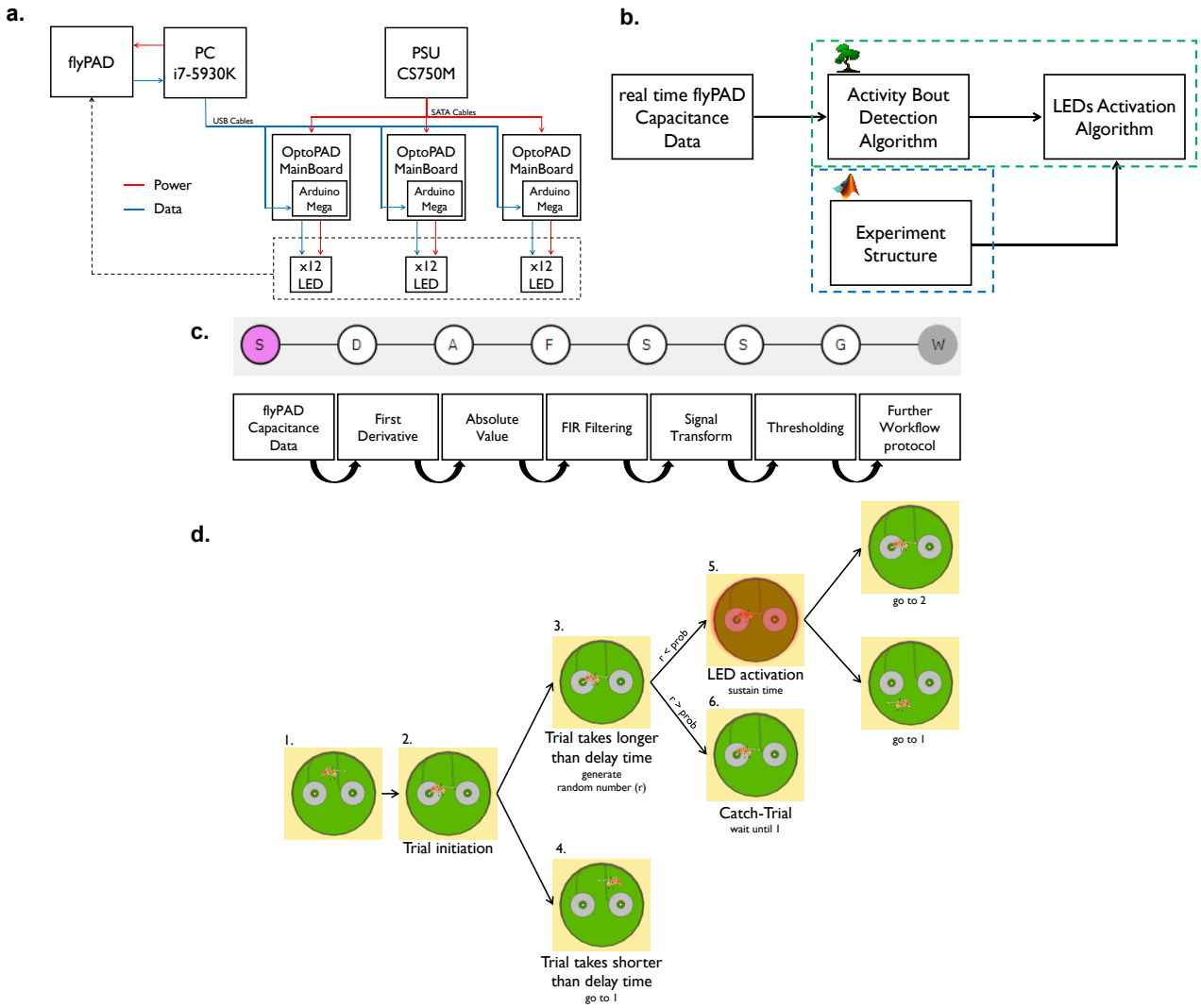

Fig. 2 Hardware and Software a. *Complete hardware system schematics;* ***b.*** *Data flow in the complete OptoPAD system;* ***c.*** *Bonsai: Activity Bout Detection Algorithm;* ***d.*** *Experiment structure: 1. Fly walks in the arena; 2. Fly finds a food source; 3. Fly keeps interacting with food for longer than the "delay" time; 4. Fly does not interact with food for longer than the "delay" time; 5. LED is active for "sustain" time; 6. Catch-Trial: system waits until the activity bout is over*

The real time capacitance data from the FlyPAD enters the computer via Serial communication; the capacitance signal from each of the 64 channels is processed independently in *Bonsai*, where the activity bout detection algorithm detects if the fly is interacting with the food (Fig. 2c); subsequently, *Bonsai* imports the "experiment structure" file, created in MATLAB and finally runs the LED activation algorithm according to the experiment structure (Fig. 2d), which is defined by 8 parameters: colourCh1, colourCh2, delayCh1, delayCh2, sustainCh1, sustainCh2, probabilityCh1, probabilityCh2. (Overview of the software in Fig. 2b)

4. Results

Firstly, we had to optimise our activity bout detection algorithm. We optimized our protocol by minimising the difference between the number of identified activity bouts by the offline data processing algorithm and our new *Bonsai* workflow, by coding it into MATLAB. This way we could manipulate the parameters (in particular, the FIR filter window length and the value of the threshold) with the same data set in order to discover which pair of values would be optimal.

A minimum difference was found with FIR Window Size equal to 50 samples (500ms) and Threshold value equal to 120 units. Using these values we could reliably identify activity bouts (Fig. 3).

We next set onto study the latency of our system.

Calculating the difference between these samples and the expected samples when the LED was supposed to turn on, resulted on a mean latency of 86.5ms (minimum – 5 samples; maximum – 12 samples).

Although this latency seems long for a behavioural setup, assuming that the fly's stereotypical eating pattern is performed at 5Hz, with this latency we are activating a neuronal consequence with a precision of

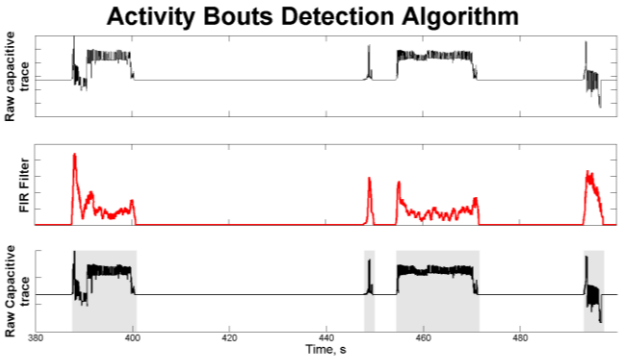

Fig. 3 Activity Bouts Detection Algorithm *The first plot shows a detail of the raw capacitance signal of a fly interacting with food; the second is the same signal depicted previously already processed by the algorithm until the FIR filtering (derivative, absolute value and FIR filter); the final plot represents the same capacitance signal with the grey shadows identifying activity bouts.*

±1 sip. Furthermore, the FlyPAD system streams data to the Serial Port in batches of six samples, giving rise to the variability observed in the calculated latency. Improving the communication protocol between the FlyPAD and the computer would lead to a decrease of the latency of at least 60ms.

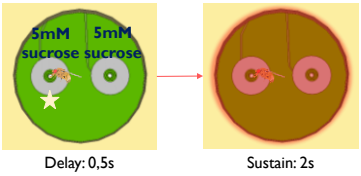

Fig. 4 Appetitive Behaviour Experiment Schematics

4.1. Appetitive Behaviour

In this experiment our hypothesis was that pairing food ingestion with artificial activation of *Gr5a*-positive neurons would elicit appetitive behaviour. In order to test whether this was the case, we allowed flies to choose from two different sources of food. Even

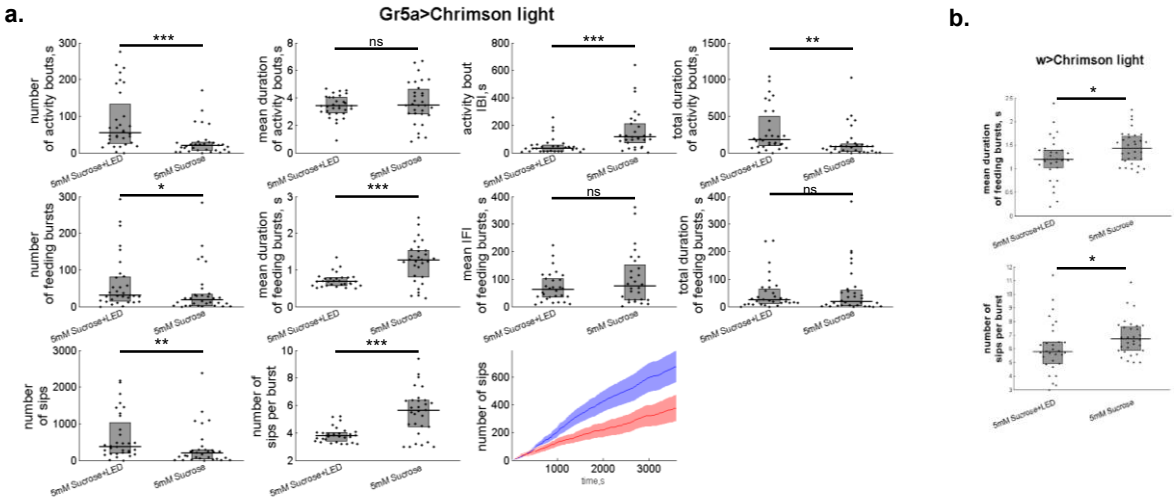

Fig. 5 Appetitive Behaviour Experiment Results a. *Feeding microstructure of Gr5a>Chrimson flies;* ***b.*** *Mean duration of feeding bursts and number of sips per burst of Chrimson control flies. Box plots display the median and interquartile range. ns – not significant (P>0.05), * – P≤0.05, ** – P≤0.01, *** – P≤0.001, significance was tested by Wilcoxon Rank-Sum test with Bonferroni correction, (Gr5a>Chrimson light, n=31; w>Chrimson light, n=32).*

though both sources had the same composition (5mM sucrose solution), only one of them was paired with red light stimulation for 2s, half a second after fly would started interacting with it (Fig. 4)

We found that the experimental flies prefer to eat from the source of food that was paired with the optogenetic stimulation, they had more sips in this source compared to the source that was not paired with light. This effect was only present in flies expressing Chrimson in the *Gr5a*-positive neurons. In all controls no difference was observed between the two food sources in terms of number of sips.

Several parameters in the microstructure of feeding strengthen the conclusion that pairing food exploration with activation of *Gr5a* neurons is appetitive (Fig. 5a). The number of activity bouts, number of feeding bursts and number of sips in the light-paired channel are greater than in the channel with no light activation. Furthermore, the activity bout Inter-Bout Interval is smaller in the channel that triggers the light stimulation when compared to the other source of food, meaning that the probability of returning to the food paired with LED activation is higher. However, some metrics support the opposite conclusion as the average time flies spend eating (mean duration of feeding burst) and also the number of times flies sip within the feeding burst is smaller in the optogenetic stimulation channel. In addition, flies harbouring only *UAS-Chrimson* without the *Gal4* also decrease the average time of feeding burst and the number of sips per burst (Fig. 5b), which can be explained by an unspecific expression of Chrimson in other neurons that might trigger a startle response. Furthermore, the shortening of the feeding burst may also be explained by the PER triggered by the activation of the *Gr5a* neurons, which could interrupt the ongoing feeding burst [28].

We can therefore conclude that it is possible to induce appetitive behaviour in flies using this setup and pairing the exploration of a food source with *Gr5a* neuron activation increases the attractiveness of that food source, as shown in the increased number of sips and a higher frequency of visits.

4.2. Aversive Behaviour

In the "Aversive Behaviour" experiment our hypothesis was that pairing food ingestion with artificial activation of *Gr66a*-positive neurons, would elicit aversion towards a source of food. We tested this hypothesis by allowing flies to choose from two different sources of food. Even though both sources had the same composition (10% yeast solution), one of them would lead to activation of the red LED for 2s after one second of interaction with that food (Fig. 6).

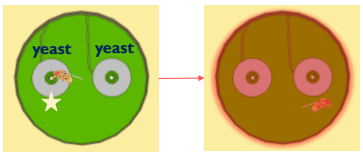

Fig. 6 Aversive Behaviour Experiment Schematics

One second after the initiation of an activity bout (when the light is turned on), experimental flies (expressing ReaChR in *Gr66a*-positive neurons) interrupt the stereotypical pattern of eating in the channel coupled with light (Fig.7a). Control flies, however, show no signs of disturbance (i.e. they continue eating) when the red light is shining (Fig.7b).

Consistent with triggering aversive behaviour, all feeding microstructure parameters are altered when comparing both sources of food (Fig. 7c). These results provide evidence that we are able to elicit

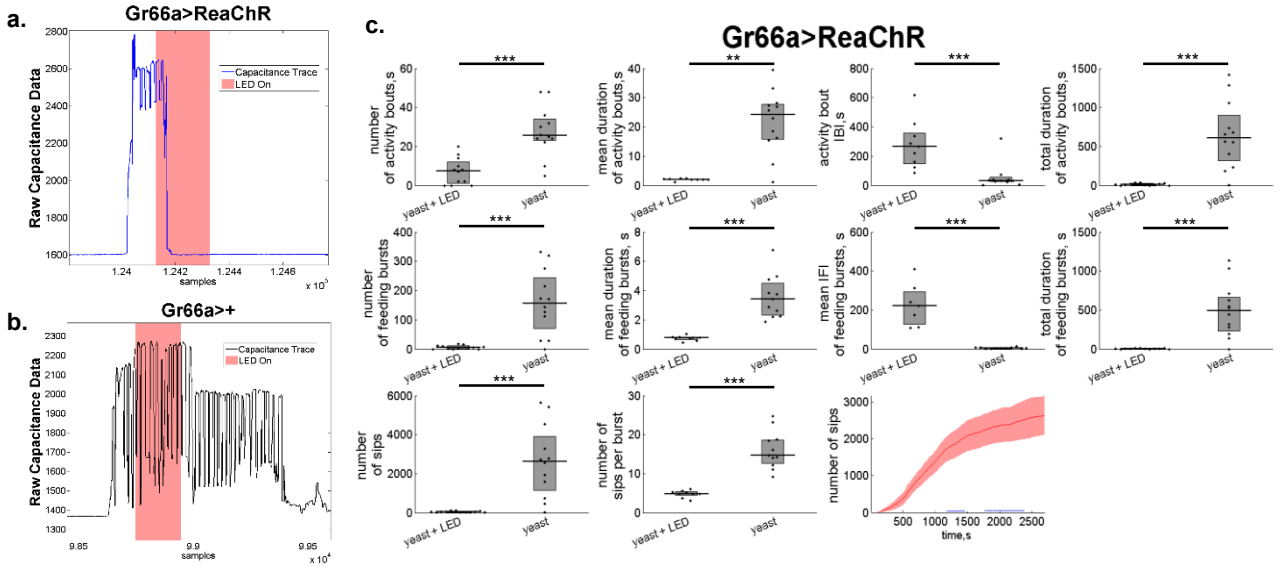

Fig. 7 Aversive Behaviour Experiment Results a. *Activity bout trace of a fly expressing ReaChR in Gr66a-positive neurons;* ***b.*** *Activity bout trace of a Gal4 control fly; (a. and b. red shade depicts when the LED was switched on)* ***c.*** *Feeding microstructure of flies expressing ReaChR in Gr66a-positive neurons. Box plots display the median and interquartile range. ** – P≤0.01, *** – P≤0.001, significance was tested by Wilcoxon Rank-Sum test with Bonferroni correction, n=12.*

aversive behaviour with this setup: flies decrease the number of activity bouts, feeding burst and sips upon light stimulation. Also, flies decrease the average time they spend interacting with food (mean duration of activity bout) or eating (mean duration of feeding burst), increase the Inter-Bout intervals and Inter-Feeding intervals (i.e. decreased probability of returning to the food) when optogenetic stimulation occurs.

In the control experiment we performed, no statistically significant changes were found, which again strongly suggests that flies in our setup do not have any bias towards or against red light. Therefore, with this setup we are able to manipulate behaviour and impose aversiveness to a source of food.

4.3. Preference Switch

Optogenetic activation of *Gr66a*-positive neurons elicits an aversive response towards the source of food paired with the stimulation. Our hypothesis for this experiment was that with periodic change of the contingency (which food source triggers light stimulation), flies would be able to change their preferred food source, showing evidence that we could dynamically manipulate preference between food sources.

In this experiment light was paired only with a source of food (channel 1) for five minutes, where in the subsequent five minutes only channel 2 would be associated with optogenetic stimulation, returning to the channel 1-activation protocol afterwards. Controls never change the way they eat, however, experimental flies stop eating from the source of food that triggers the light (Fig. 8).

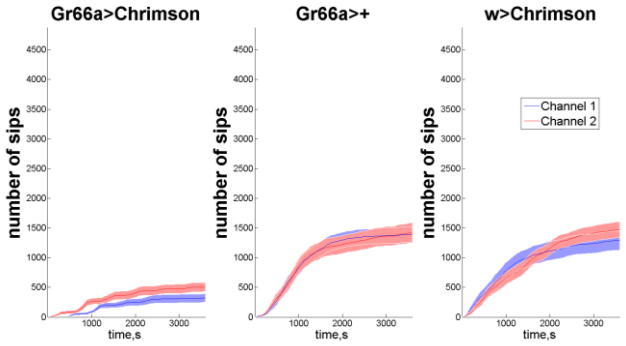

Fig. 6 Cumulative number of sips across the experiment condition (Gr66a>Chrimson) and the different controls
The blue trace represents the cumulative number of sips in channel 1 and red is the same parameter in channel 2. Line represents the mean and the shading the standard error of the mean (Gr66a>Chrimson, n=42; Gr66a>+, n=43; w>Chrimson, n=38)

Flies expressing Chrimson in *Gr66a*-positive neurons tend to eat less (close to 1000 sips in total), compared to the control flies of this experiment (close to 3000 sips in total). In fact, the plateau of the cumulative number of sips, which is usually correlated to satiation, in the control flies, occurs at a lower number of sips counting in the experimental flies. This result shows evidence that, even though we thought the plateau was associated with satiation, these flies are not able to find the best feeding strategy in order reach satiety, due to the unpredictable contingency of the experiment.

With this result we were able to demonstrate that we could dynamically manipulate *Drosophila* preference between food sources, through optogenetic activation of *Gr66a*-positive neurons.

5. Discussion

We developed a new behavioural setup that allows recording of feeding and closed-loop optogenetic manipulation of neurons in *Drosophila*. With this system, we were able to induce appetitive behaviour towards a source of food by stimulating *Gr5a*-positive neurons and to induce aversive behaviour through activation of *Gr66a*-positive neurons.

Despite most studies that use flies and manipulation of GRNs being also directed to the study of learning and memory [51,52], all the setups used so far work in an open-loop manner, where flies cannot associate their own actions to a reward or punishment. The developed setup is a behavioural system that can be used to study memory and learning in a more naturalistic environment in comparison to previous setups, in which flies are not tethered and are freer to interact with food.

In this context, the OptoPAD may allow researchers to explore many different questions regarding whether the activation of a particular neuronal population will trigger different feeding behaviours.

5.1. Hardware and Software

The developed setup is capable of recording and manipulating the activity of 32 flies simultaneously. However, and knowing that this system makes use of 4 USB Ports (1 for the FlyPAD and 3 for the Arduinos), one can scale-up the amount of flies studied according to the number of available USB Ports in the computer. The throughput is enough to have a significant number of experiments performed in a working day.

With the OptoPAD, one can use nearly any available channelrhodopsin, as the system is equipped with 4 colour LEDs in all the behaviour arenas. One limitation is that with the hardware and software developed so far, the LED can only be off or at maximum intensity: it is therefore not possible to vary light intensity. This caveat could be overcome by adding potentiometers that control the current which passes through the LEDs (hardware solution) or by implementing a power width modulation that would control the LEDs' duty cycles (software solution). Once being able to activate more than one colour at the same time and change the intensity of each of the colours, it would be possible to emit at any visible wavelength.

Regarding the results described in this work, the resolution of stimulation is around 86.5ms (average latency of 8.65 samples). Although this value might seem too long for behaviour experiments, we know that fly's stereotypical movement of sips happen at about 5Hz, meaning that flies extend and retract their proboscis in periods of 200ms. Therefore, we are stimulating flies with the precision of ±1 sip. Furthermore, the FlyPAD system streams data in batches of six samples, thus giving rise to the variability of latency (minimum – 5 samples; maximum – 12 samples). The system's latency can be reduced by improvement of the protocol that streams data from the FlyPAD to the Serial Port. By re-writing this algorithm, OptoPAD's latency could be dramatically reduced, by at least 60ms (6 samples).

We performed an optimisation of Bonsai parameter to better detect the activity bouts. This optimisation resulted in a FIR filter window size of 50 samples and a threshold of 120 units. When executing the optimisation, we chose to test only FIR windows sizes up to 50 samples, since by using larger windows we would postpone the offset of the identified activity bout. There is one example that demonstrates the problem of larger window sizes. For example, if we set the delay (activity bout minimum duration in order to trigger stimulation) to 1s and the fly only interacts with food for 500ms, as the FIR filter is set to a window of 50 samples (500ms), the fly will get light stimulation as the filter is summing across 500ms. At the limit, the last sample of the interaction will be enough for the sum of the following samples to be above the threshold (0.5s interaction + 0.5s filtering = 1s activity bout). Nonetheless, this problem does not exist for activity bouts that last longer than the delay time.

Further optimisations could be implemented. The output of the FIR filter could be divided by the number of samples. This way we would have a normalized output value and the threshold would not need to be so high in order to detect activity bouts. Furthermore, instead of having a lot of computations such as the derivative, absolute value and the FIR filter, we could use a band pass filter or a pattern recognition filter that would detect groups of square wave-like signals at approximately 5 Hz (sips).

5.2. Experiments

Our results are in line with the current knowledge, as activation of *Gr5a*-positive neurons cause flies to stay in the location that was paired with activation, whereas the activation of *Gr66a* neurons led to the opposite effect[14]. However in this study there are a lot of possible reasons for the modulation of preference. These flies could have stayed in the preferred substrate eating, grooming, sleeping or even laying eggs. In order to disentangle these possible explanations, we decided to run experiments that would allow us to understand what are the consequences of the activation of those neurons on feeding behaviour.

We were able to create a "virtual reality" for the fly where one source of food was more appetitive than other, even though they were the same solution (5mM sucrose). Almost all the results support this conclusion. In fact, the number of activity bouts, feeding bursts and sips was augmented in the source of food that would trigger the optogenetic stimulation (activation of *Gr5a*-positive neurons) and the Inter-Bout interval was diminished in the same source of food, meaning that flies had higher probability of returning to the "sweeter" food than going back to the food that did not trigger light. Nevertheless, the duration of feeding bursts and number of sips per burst were reduced in the "light/sweet" source of food. This result stands in apparent disagreement with the conclusion of inducing appetitive behaviour. However, flies that only harbour *UAS-Chrimson* show the same phenotype reducing their feeding burst durations on the source of food with light stimulation. This result seems to show that these flies have unspecific expression of the channelrhodopsin, which by triggering other neurons can explain the interruption of feeding. In previous studies, continuous light stimulation of *Gr5a*-positive neurons expressing ReaChR induces PER in the beginning of the stimulation [28], which may lead to the interruption of the ongoing feeding burst. In addition to that, one might speculate that while the light is active, flies do not feel the need to perform the motor program of feeding as their "sweet" gustatory neurons are already active, even with no more food being ingested.

In relation to the last two experiments they can be discussed together, since, even though the targeted neurons express different channelrhodopsins (ReaChR and Chrimson), we can draw the same conclusion from their results: by activating *Gr66a*-positive neurons, flies show aversive behaviour towards the food paired with the stimulation.

We demonstrated that the feeding behaviour was interrupted as soon as these neurons were activated. All the features of the microstructure of feeding were significantly different from the behaviour on the source of food that would not trigger the stimulation. The number of activity bouts, feeding bursts and sips were drastically diminished in the "light/bitter" source of food. Flies have a lower probability of returning to the spot of food that will trigger light and also have a lower probability of repeating a feeding burst there (longer Inter-Bout interval and Inter-Feeding interval). Also, the amount of time they spend eating from that food source (that triggers light) is minimal when compared to the other source.

Furthermore, we show that by reversing the contingency of the experiment, flies rapidly change their preferred food source. When analysing the "Preference Switch" experiment after the "Aversive

Behaviour", we were expecting to observe the same overall results over both parts. However, the stagnation of the number of sips displayed by the experimental flies was an unexpected result. In fact, through all experiments we performed, flies show a plateau on the cumulative number of sips that can be explained by satiation[2]. In this case, we observe that the control flies perform close to 3000 sips before showing satiation (Fig. 8). Nonetheless, the observed "satiation plateau" of the experimental flies occurred close to 1000 sips. This also stands in contrast to the experiment in which the aversive channel was not switched (Fig. 6c). Therefore the "Preference Shift" design leads to a premature cessation of feeding, suggesting that this plateau is not related to satiation. A possible explanation could be a learned helplessness effect[53], as flies might not being able to find the strategy to deal with the unpredictable contingency of the experiment.

6. Future Work

OptoPAD requires some improvements, particularly regarding its software. Modifying the activity bout detection protocol by designing a filter that would be specific to the stereotypical feeding behaviour of flies would be the first step of improvement. This would not only enable the OptoPAD to be a feeding recording and manipulation setup, but would help us to understand if flies are able to change strategies of feeding in a "sip" scale. However, this does not mean that the existing software is not efficient *per se*.

The next step towards studying learning and memory in Drosophila with this system is to find a new behavioural paradigm in which flies can learn the contingency of the experiment. From preliminary data collected using the OptoPAD, we could find features of behaviour that suggest that flies can learn to change their feeding motor pattern, conditioned by an aversive optogenetic stimulation. Furthermore, the OptoPAD allows the creation of a "virtual reality" environment where one source of food is more available, if it has a low probability of triggering an optogenetic punishment; or less available, if it has a high probability of activating a punishment. We can create predictable or unpredictable environments for flies by changing the contingency of availability with time or probability of stimulation.

The OptoPAD could be used to study operant conditioning in flies. Genetic modifications of the memory centres of *Drosophila* (e.g. the Mushroom Body) will allow studies on how and where flies store particular memories, how they can remember and, hopefully, serve as a model for the study of neurodegenerative diseases, such as Alzheimer's Disease.

7. Materials and Methods

Drosophila stocks were maintained on standard medium (containing, per litre, 80g cane molasses, 22g sugar beet syrup, 8g agar, 80g corn flour, 10g soya flour, 18g yeast extract, 8ml propionic acid and 12ml nipagin (15% in ethanol)) at 18ºC with 12:12 light/dark cycle. The following lines were used:

1. *Gr66a-Gal4/Cyo*
2. *Gr5a-Gal4 (II); Gr5a-Gal4 (III)*
3. *w-;;UAS-ReaChR/TM6 Sb Tb*
4. "virginized" stock *+/yhs-hid;;pUAS-Chrimson-mVenus/TM3, Sb*

Appetitive Behaviour Experiment Design

Gr5a-Gal4 (II);Gr5a-Gal4 (III) males were crossed to *+/+;+/+;pUAS-Chrimson-mVenus/TM3, Sb* virgins and always kept in the dark. Flies were left to mate at 25ºC for three days. Parents were discarded and on the 14th day after crossing progeny were sorted against balancers and males were selected.

Gr5a-Gal4 (II)/+;Gr5a-Gal4 (III)/pUAS-Chrimson-mVenus (*Gr5a*>Chrimson) males were starved for 24h in a vial with paper towel soaked with a 5mL aqueous solution of 400µM all-trans retinal.

After these 24h, flies were tested in the OptoPAD with both sources of food containing a solution of low sucrose concentration (5mM sucrose solution). We used a low sucrose solution so that the food would not be very appetitive per se but would be appetitive enough to elicit rare feeding events. Our hypothesis was that we could manipulate the fly's feeding behaviour to eat more from a source of food that would trigger sweet sensors via optogenetic stimulation than from a similar source without the stimulation.

Aversive Behaviour Experiment Design

Gr66a-Gal4/Cyo;+/+ males were crossed to *w-;+/+;UAS-ReaChR/TM6 Sb Tb* virgins and always kept in the dark. Flies were left to mate at 25ºC for three days. Parents were discarded and on the 14th day after crossing progeny were sorted against balancers and females were selected.

Gr66a-Gal4/+;UAS-ReaChR/+ (*Gr66a*>ReaChR) females were deprived from protein for 3 days in a vial with paper towel soaked with a 5mL aqueous solution of 100mM sucrose and 400µM all-trans retinal. To these female flies, 3 males were added to the vials to make sure the females were mated.

After 3 days, flies were tested in the OptoPAD setup with both sources of food containing a 10% yeast solution (a highly appetitive food for female flies).

Preference Switch Experiment Design

Gr66a-Gal4/Cyo;+/+ males were crossed to *+/+;;pUAS-Chrimson-mVenus/TM3, Sb* virgins and always kept in the dark. Flies were left to mate at 25ºC for three days. Parents were discarded and on the 14th day after crossing progeny were sorted against balancers and females were selected.

Gr66a-Gal4/+;pUAS-Chrimson-mVenus/+ (*Gr66a*>Chrimson) females were deprived from protein for 3 days in a vial with a paper towel soaked with a 5mL aqueous solution of 100mM sucrose and 400µM all-trans retinal. To these female flies, 3 males were added to the vials to make sure the females were mated.

After 3 days, flies were tested in the OptoPAD setup with both sources of food containing a 10% yeast solution. In this experiment we changed the structure of the experiment across channels with a periodicity of 5min blocks.

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