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# Behavioural, molecular and cellular bases of ambiguous learning and memory in *Drosophila*

Matthias Durrieu

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# THÈSE

**En vue de l'obtention du  
DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE  
Délivré par l'Université Toulouse 3 - Paul Sabatier**

---

**Présentée et soutenue par  
Matthias DURRIEU**

Le 19 mai 2021

**Bases comportementales, moléculaires et cellulaires gouvernant  
l'apprentissage ambigu et la mémoire chez la drosophile**

---

Ecole doctorale : **BSB - Biologie, Santé, Biotechnologies**

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**CRCA - Centre de Recherches sur la Cognition Animale**

Thèse dirigée par

**Guillaume ISABEL et Antoine WYSTRACH**

Jury

**M. Jean-Christophe SANDOZ**, Rapporteur

**Mme Marie-Laure PARMENTIER**, Rapporteur

**Mme Audrey DUSSUTOUR**, Examinatrice

**M. Philippe LUCAS**, Examineur

**M. Guillaume ISABEL**, Directeur de thèse

**M. Antoine WYSTRACH**, Co-directeur de thèse



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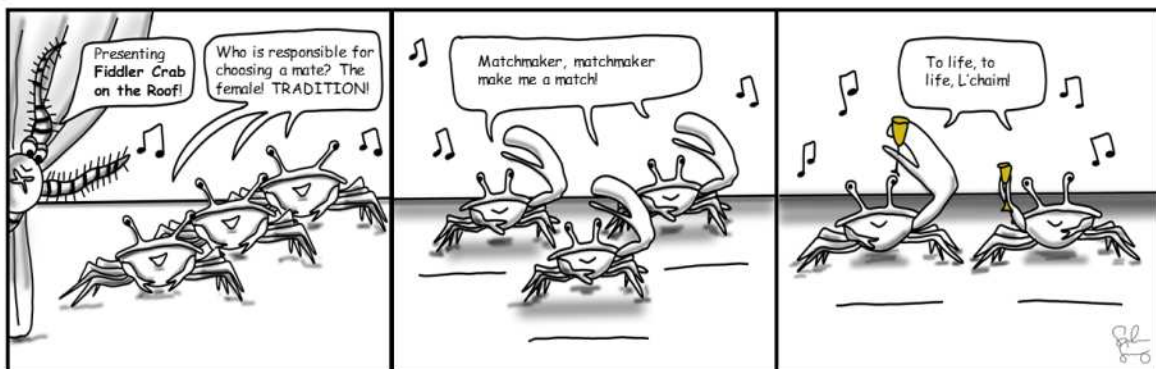
# Introduction

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Living organisms thrive in complex environments in which they need to adapt and survive. Long-term adaptations rely on genetic expression flexibility and promote survival over generations (e.g. genetic adaptation to fairly slow environmental changes like climate change<sup>[1]</sup>). Individuals showing advantageous traits is able to produce more offspring carrying its genetic heritage while disadvantaged beings will produce fewer offspring and so on. This concept is called selection. That way, organisms may evolve beneficial features as in the case of fiddler crabs, a classic example of sexual selection where males show enlarged claws that provide a significant advantage during fights with male conspecifics and courtship<sup>[2]</sup>.

## Interviews with invertebrates **Fiddler crab on the roof**

S. Seroy © 2018



Male fiddler crabs have one very large claw, while females do not. Females are picky and typically only choose mates with the largest claws. Males wave their claw to attract attention, trying to convince the females to pick them. Once successful, the pair reproduces to start the next generation of large-clawed males and picky ladies. These evolutionary traditions, so to speak, keep the fiddler crab populations in balance. L'chaim!

[interviewswithinvertebrates.com](http://interviewswithinvertebrates.com)

Beyond these rather anatomical adaptations, animals may also show internally coordinated responses (including actions but also inactions) to internal or external stimuli, which involve observable features. These responses are defined as behaviour<sup>[3]</sup>, the study of which is the cornerstone of ethology. By studying the mechanisms underlying behaviour, along with their development and adaptative functions, Nikolaas Tinbergen work showed that a large fraction of behaviours can occur without any prior experience. These behaviours, called innate, are shaped over long periods of time, the same way as anatomical adaptations: through selection. Muscular reflexes, taxis (a form of directed movement elicited by external stimuli) and Fixed Action Patterns (which are sequences of coordinated movements involved in various activities such as prey hunting<sup>[4]</sup>) are all examples of innate behaviours. However, the environments in which animals live also involves short-term challenges that require swift adaptations. A food source might be depleted. A previously safe location might be crowded with predators.

Therefore, behaviours also need to be flexible. The ability to change a behaviour following experience is called learning.

## The gradual construction of the tenets of learning processes

Learning results from plasticity at cellular level allowing hard-wired stereotyped behaviours to be reshaped according to experiences. For example, a food related odour usually triggers an innate seeking behaviour. But if this particular odour is encountered in a harmful context, the animal can learn to avoid it instead. Importantly, learning can also lead to the inhibition of specific behaviours. For instance, a dog can be trained not to bark at bystanders. In this case, the absence of the initially displayed response constitutes an alternative, learnt behaviour. The common element of many (although not all) types of learning is the presence of a stimulus of biological relevance (food, pain...) that elicits an innate behaviour.

As many scientific concepts (including behaviour, for which the definition has been discussed and shaped over the years, learning was not explicitly defined at once, but was rather built from the work of several pioneers. Following their discovery, we will now retrace the building of a major learning conceptual framework.

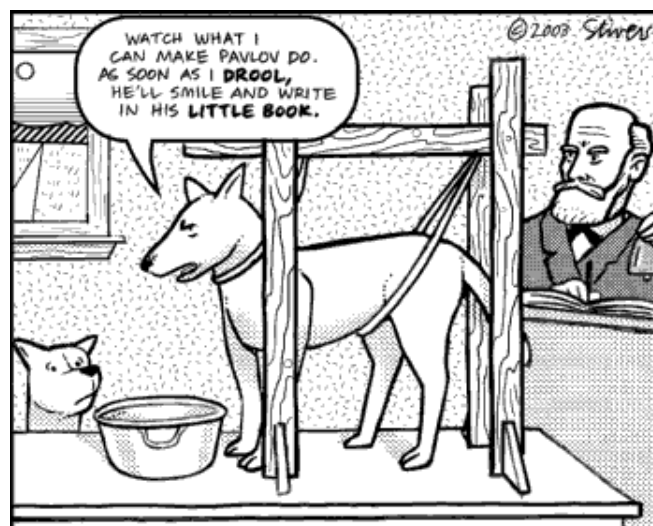
Interestingly, In the early 20<sup>th</sup> century, the term of behaviour integrated both innate (then called reflex) and acquired responses. The general study of behaviour based on measurable responses was called behaviourism and was opposed, in a way, to more theoretical approaches (such as Freudian psychoanalysis) or other experimental approaches such as ethology and naturalism. In fact, behaviourism was grounded in the idea (since contradicted) that there is no such thing as inherited behaviour, the way an individual acts being determined by their overall experiences<sup>[5]</sup>.

## Reinforcement and conditioning – The emergence of an experimental framework for the study of learning

Behaviourist theory was founded by John Watson and supported by human-focused experiments. To demonstrate that behaviour was an acquired process, Watson showed that

he was able to reliably train infants to respond to a given stimulus in a different way from what their response was before training, a method called conditioning. His most famous demonstration was a child named Albert, who was trained to express fear at the sight of a white rat, whereas his spontaneous response before conditioning was petting it. Interestingly, Albert's conditioned response was not only elicited by rats but was also triggered by various white and fuzzy objects, a phenomenon known as generalisation that is critical in our work (see the Discrimination Vs Generalisation part and the chapter III). Overall, Watson approach of psychology represented a significant milestone in the field. Indeed, at a time when concepts like cognition, emotions and mind were mainly explored through philosophical and human based approaches, he chose to emphasise experimental methods to analyse observable behaviour. Although this school of thought was formulated by Watson, many of its inherent concepts came from his deep interest in the work of his contemporaries, one of which gave his name to a major type of learning : Ivan Pavlov<sup>[6]</sup>.

An exploration of learning through physiological processes: How Pavlov's work was a decisive turning point for the field



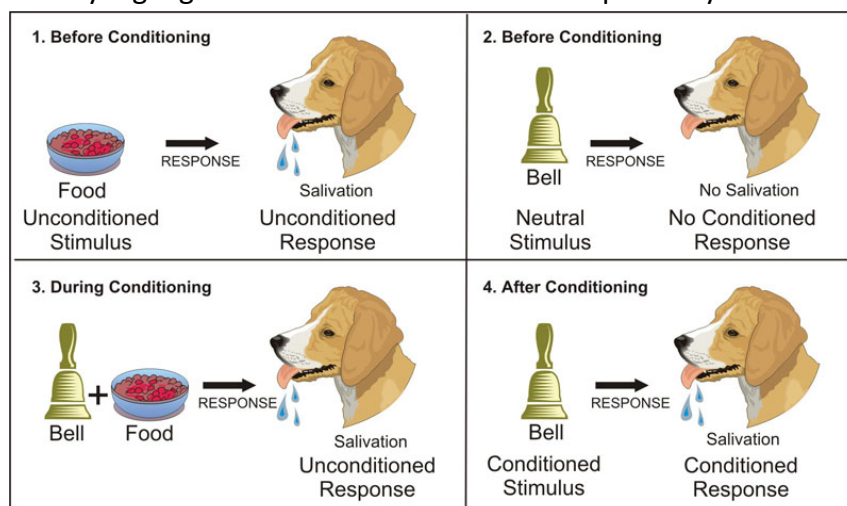
Interestingly, in the beginning, Pavlov was not a trained psychologist, which may have contributed to his innovative approach of cognition. Indeed, as Pavlov was initially working on digestion<sup>[7]</sup>, he had specialized in measuring physiological parameters, mainly gastrointestinal secretions. Among them, salivary secretion was the easiest to quantify and offered the advantage of being completely involuntary, therefore defined as a reflex (or

innate behaviour) elicited by a biologically relevant stimulus: a food source. Pavlov thus called the salivary secretion an Unconditional Reflex (UR), as it was innately elicited by food perception, therefore called Unconditional Stimulus (US).

Pavlov discovered that repeated association between a neutral stimulus and the US presentation led the subject to subsequently start responding to the neutral stimulus as if it was the US. Eventually, the initially neutral stimulus alone elicited a salivary secretion response and was thus described as a Conditional Stimulus (CS) in contrast with the US. Similarly, the learnt response was described as a Conditional Reflex (CR).

Incidentally, as this terminology is still used today, the innate reflex boundary has been extended, the “R” in UR and CR standing now for “Response” to encompass non-reflexive behaviours as well.

Interestingly, if the CS was subsequently presented repeatedly without any following US, the CR gradually faded until it didn’t elicit any response anymore. This phenomenon, known as “Extinction”, already highlighted a remarkable behavioural plasticity.



### Classical Conditioning

Figure 1 Schematic representation of the different steps leading to a conditioned response. 1) Before conditioning, the Unconditioned Stimulus (US) elicits an innate response, which is termed “Unconditioned Response” (UR). 2) In contrast, the neutral stimulus does not elicit the innate response. 3) During conditioning, the neutral stimulus and the US are systematically presented together. The subject displays an UR. 4) After conditioning, the previously neutral stimulus is now a Conditioned Stimulus (CS) and elicits a Conditioned Response (CR) of the same kind of the UR.

Following this discovery, Pavlov proceeded to break-down the basic mechanisms of this form of learning by testing numerous stimuli, timings and protocols. One of them consisted in presenting a first stimulus consistently associated with an US while a second stimulus was always applied without US. The subject learned to respond to the first stimulus but not to the



second, a paradigm known as Differential Conditioning. The goal in adopting such approach is to ensure that the tested subject learns to respond specifically to the reinforced stimulus (called CS+) and not to the non-reinforced ones (called CS-) in a systematic way. Differential conditioning has been since used to explore discrimination abilities in numerous species (including *Drosophila*), as will be described later (see the Discrimination Vs Generalisation part).

His work is amongst the most important contributions to the learning field and the study of behaviour. He developed an innovative way to study cognition by focusing on physiological measures as proxies for nervous activity and his core experiment, now referred to as classical (or Pavlovian) conditioning is still widely used in modern projects. Interestingly, the fact that Pavlov's greatest discovery was collateral to his main research emphasises the importance of general curiosity and flexibility in basic research. While a comprehensive description of Pavlov's work would be of great interest, we shall instead attempt to show how he influenced decades of behavioural science through the description of the work of his successors.

### Edward Thorndike and the puzzle boxes

Classical conditioning trademark is the passive nature of learning, as subjects develop conditioned responses unconsciously. However, from the perspective of many psychologists, learning was viewed as an active process, requiring the conscious involvement of the learner, where conditioning would thus be a lesser form of adaptation.

Incidentally, as biology research has always been plagued by an entrenched anthropocentrism, the psychology field was especially reluctant to endow animals with any form of intelligence, for rather philosophical and theological reasons and despite the fact that Darwin's work was putting humans and animals on equal terms. E. L. Thorndike was no exception, but he was opposed to any form of dogma, seeking instead for objective evidence. That is why he was arguably the first trained psychologist (in contrast with Pavlov) to bring non-human animals in the laboratory to assess their learning ability experimentally<sup>[8]</sup>. Thorndike was investigating whether animals could learn to solve puzzle tasks by acquiring appropriate behaviour. To do so, he designed puzzle boxes that required the restrained

animal to perform a specific response to be opened (e.g., pull a lever, push a button...). (Figure2)

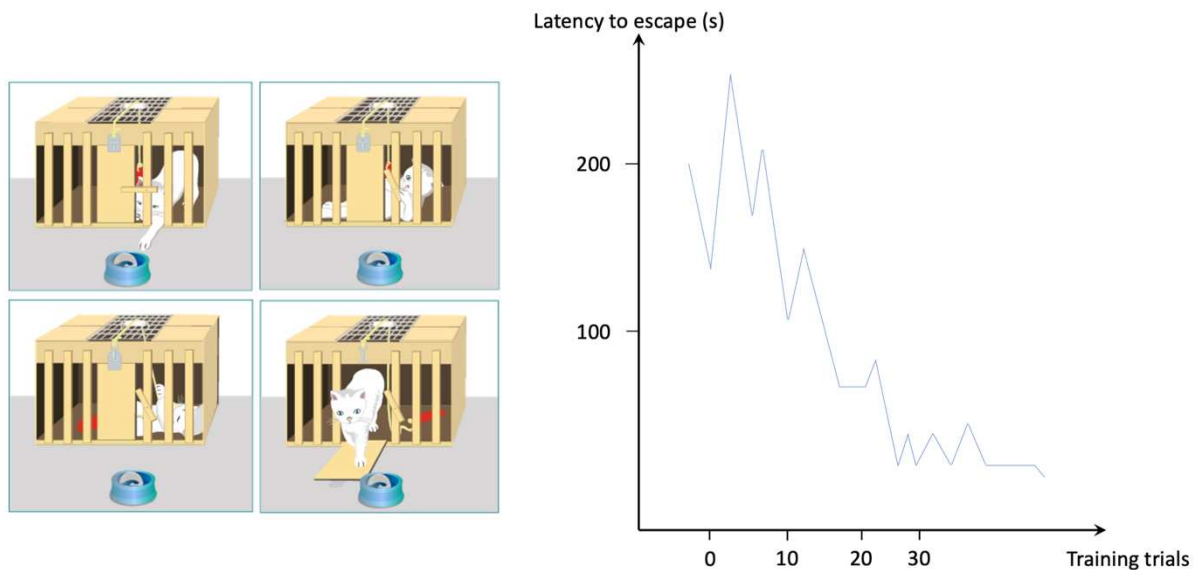


Figure 2 Thorndike's puzzle box. The cat is trapped in the box and motivated by the presence of food. In order to reach the food, the cat has to exit the box by performing a specific action (in this example, actioning the red lever). At first, the cat's actions are uncertain but over the course of trials, the one action always leading to the opening of the box is reliably learnt and repeated, every time with a shorter latency.

The subject was motivated by the presence of food within sight and had to figure out how to reach it. Thorndike observed that the subject first performed various random actions, trying to escape the box. Eventually, one of these actions incidentally led to the unlocking of the box, allowing the animal to get out and access food. Interestingly, when put again several times in the same situation, the time it took to get out gradually shortened. By recording the time for each trial, Thorndike was able to establish a learning curve, demonstrating that rather than an "insight" mechanism where the animal would suddenly find the solution to the problem<sup>[9]</sup>, the puzzle box is solved rather gradually through a trial and error process<sup>[10]</sup> where the successful moves will be reproduced while the unsuccessful ones will not.

Based on these results, Thorndike was able to identify fundamental properties of learning, among which the fact that it gradually acquired in the same way by all animals (although depending on the task and the animal, the learning curve may be slightly different), through repetition. He called this principle "law of exercise". More importantly, he defined the law of effect which stated that responses that produce a satisfying effect (e.g., unlocks the puzzle

box) are more likely to occur again while responses leading to discomfort become less likely to occur again. Moreover, he suggested that this reinforcement of behaviour reflected a reinforcement in associated brain connections, an approach called “connectionism”. This fundamental principle was taken up and expanded years later, supported by actual brain data, ultimately leading to the well-known Hebbian theory<sup>[11]</sup> (i.e. if one neuron stimulates another neuron repeatedly, the strength of the connection between the two neurons will be increased).

In the shorter term, Thorndike’s findings fitted very well within the behaviourism framework, as it once again highlighted the limitations of introspective approaches while laying the groundwork for a more objective way of describing behaviour. That is why he and Watson are often considered as the fathers of this intellectual current. However, it took another scientist to finally establish a conceptual framework assembling the principles of pavlovian conditioning and learning under the same roof: B.F. Skinner<sup>[12]</sup>.

### The emergence of the associative learning conceptual framework: insights from the work of B.F Skinner

Indeed, Pavlov and Thorndike’s work led to the establishment of fundamental properties of learning, but they were regarded as distinct conceptual models. Skinner, on the other hand, developed the central idea that they were two sides of the same coin. Moreover, he took up Thorndike’s experimental paradigm by designing a variation of his puzzle box, which allowed him to push the concept of “law of effect” further and bring it together with conditioning principles. These boxes were equipped with levers that could be operated by the subject. The whole box was surrounded with stimuli generators, some neutral (sound, light...) others carrying a biological value (electric grid, food dispenser). That way, in contrast to Thorndike’s puzzle box, he was able to test the law of effect by associating specific behaviours with significant, controlled outcomes. In addition to another validation of the law of effect, he developed the fundamental concept of reinforcement. Indeed, when an animal pressed the lever and was rewarded with a food pellet in return, it was more likely to repeat that behaviour (which was thus reinforced). In contrast, if the lever activation was followed by an electrical shock (a punishment), the behaviour was decreased. Skinner also discovered that a

behaviour leading to the removal of an aversive stimulus produced the same kind of reinforcement as adding a pleasant stimulus and vice versa. These experiments led to the conceptualization of the reinforcement theory shown in Figure 3, a practical working framework that is still widely used in experimental work, educational psychology and animal training.

	<b>Reinforcement</b> (Increase / maintain behavior)	<b>Punishment</b> (Decrease behavior)
<b>Positive</b> (add stimulus)	<b>Add</b> pleasant stimulus <i>to</i> <b>Increase / maintain behavior</b>	<b>Add</b> aversive stimulus <i>to</i> <b>Decrease behavior</b>
<b>Negative</b> (remove stimulus)	<b>Remove</b> aversive stimulus <i>to</i> <b>Increase / maintain behavior</b>	<b>Remove</b> pleasant stimulus <i>to</i> <b>Decrease behavior</b>

Figure 3 The reinforcement concepts as theorized by B.F. Skinner.

Skinner’s experiments were both influenced by Thorndike’s paradigm and Pavlov’s conditioned reflexes, as the animal was involved in the process through its actions, yet because in that case the reinforcement was controlled and applied very similarly to animals subjected to classical conditioning, he called this procedure “operant conditioning”, producing operant behaviours. Much like Pavlov, he was able to finely characterize the base mechanisms of operant conditioning. For instance, he found that learning was highly sensitive to timing. He showed that for a reward or punishment to affect a given behaviour, it had to be applied right after the behaviour was performed. One of the most elegant experiments he conceived is known as the “superstitious pigeon”, during which he fed the animal on continuous intervals while measuring its behaviour. He showed that the pigeon tended to repeat the actions he was doing right before receiving food, even though these had nothing to do with the reward. Interestingly, the subject had learned causal relationship between these unrelated actions and the reward, something Skinner called superstition, as he interpreted their behaviour as a misguided belief that their actions influenced subsequent events.

## Clark Hull, the concept of motivation and modelling inception

Interestingly, Unconditional Stimuli all share a fundamental feature: they are directly linked to physiological needs, such as avoiding pain or danger, finding sexual partners and so on. Looking back at physiology, in principle a hungry animal will show a stronger response to food presentation than a satiated one, which might also affect associative learning processes. Based on that postulate, Clark Hull introduced the concept of motivation as another driving factor of associative learning, for which he also provided experimental evidence<sup>[13]</sup>. He called this factor “Drive”, now better known as “Motivation”

Interestingly, Hull was not entirely satisfied with the methods used to measure learning and showed a strong interest in quantification. As he also had a strong background in mathematics, this led him to be one of the first if not the first to develop a mathematical formulation of learning acquisition in collaboration with his student, Kenneth Spence (see the “Discriminative vs Generalisation” chapter). Then, he spent most of his career refining his learning equation by designing experiments specifically meant to test its validity and correct it. This methodology is still widely used today using both mathematical models and computational simulations, which are part of the approaches we develop in our work (see chapter II).

Skinner tested discriminative abilities similarly to Pavlov’s experiments, using the surrounding stimuli the boxes were equipped with. For example, pressing the lever only led to a reward if a green light was lit. Moreover, he engaged in a systematic characterization of what he called the “Schedules of reinforcement”, testing various reinforcement intervals and ratios. Most notably, he investigated variable, sometimes pseudo-random schedules, showing that depending on the quality and strength of the reinforcement, animals do not necessarily need to be rewarded or punished at every trial to learn a given association. These experiments are the source of numerous following studies on gambling and addiction<sup>[14]</sup>. Furthermore, Skinner coined the concept of shaping, which is used to train animals by reinforcing successive approximations of a target behaviour. For instance, he was able to train pigeons to orient to and strike a target in a box by reinforcing each building block of the sought response (turning in the right direction, moving toward the target, raising the head and finally striking the target). Thus, the term “shaping” suggests a step-by-step process refining and building up new behaviours that would be very unlikely to be shown by a naïve animal.

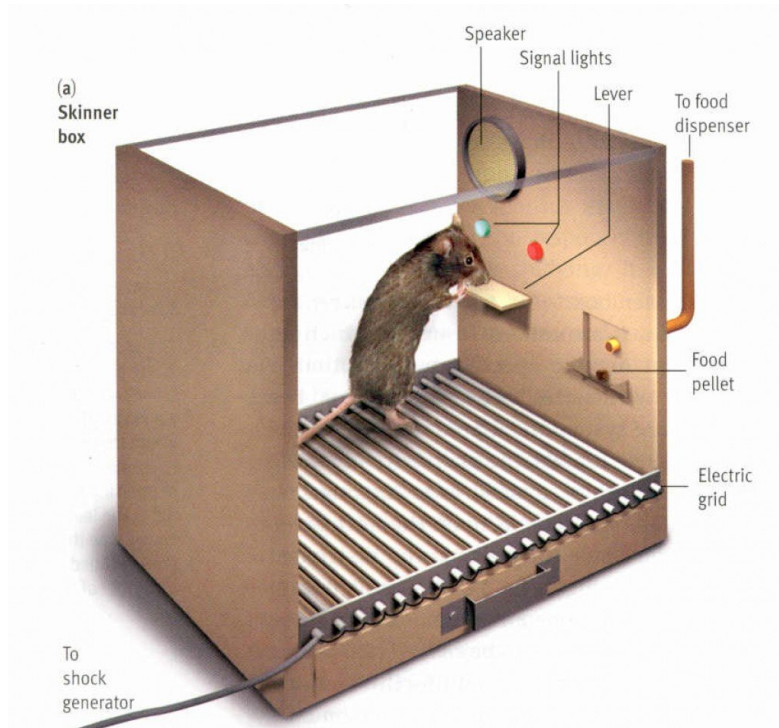


Figure 4 Schematic representation of the Skinner box. The animal is trapped in a box equipped with easily distinguishable features (levers, lights, food dispenser). The skinner box allows tightly controlled operant conditioning experiments.

Beyond his remarkable experimental work, Skinner greatly contributed to the growth of experimental psychology and behaviourism, of that he developed a radical approach throughout his career. As a prolific author, he generalized and completed the concepts developed by his predecessors, and extended them through potential applications in numerous fields, such as education, economics and behavioural therapy. He eventually faced intense criticism for extending operant conditioning concepts to every cognitive process, ultimately leading to quite extremist ideas. Nevertheless, his influence enabled the generalisation of experimental approaches to behaviour and psychology.

To sum up, the conceptual framework in which our work takes place was built from the idea that brain functions such as learning can be studied experimentally using paradigms meant to measure their outward manifestations. In other terms, learning abilities may be assessed by looking at changes in behaviour. Animals exhibit various innate behaviours in relation to biologically significant stimuli (US), such as shelters, food sources, predators or preys. Throughout their lives, they also learn which cues are reliable predictors of said stimuli and which are not.

Eventually, animals start responding to these cues in anticipation of the associated outcome, for example by avoiding a location often associated with the presence of a predator. That is why this aptitude is called associative learning.

### Associative vs non associative learning

In the most basic forms of learning, only one stimulus is required and its presentation, by itself, can modulate future behaviour. These forms of learning are therefore called “non-associative”. Repeated exposure to a stimulus may result in a gradual inhibition (a process termed “habituation”) or amplification (termed sensitization) of the initial behaviour exhibited by a subject toward it. These phenomena are well depicted in the classical case of the *Aplysia* gill and siphon withdrawal reflex carried by Eric Kandel<sup>[15]</sup>(Figure 5).

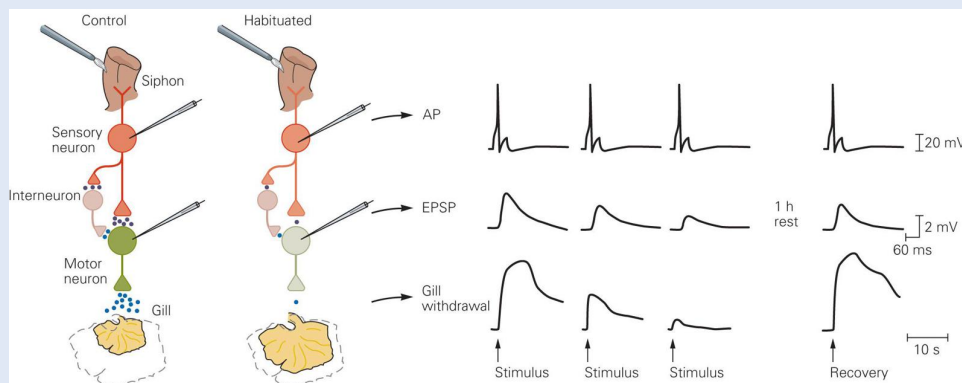


Figure 5 Short-term habituation of the gill withdrawal reflex of the marine snail *Aplysia* - *Cellular Mechanisms of Implicit Memory Storage and the Biological Basis of Individuality*. The initial behaviour of the *Aplysia* upon mechanical stimulation is the gill and siphon withdrawal, underlain by mechanoreceptor neurons projecting onto motor neurons. Repeated stimulation reduces synaptic transmission from the sensory neuron to the downstream motor neuron, inducing an inhibition of the withdrawal behaviour.

Interestingly, non-associative learning cases have been described in numerous species, vertebrate<sup>[16][17]</sup> and invertebrates<sup>[18]</sup> alike, and even, more recently, in single-celled organisms<sup>[19]</sup>. Non-associative learning allows swift behavioural adaptation, reducing one’s response (and thus allocated resources) to inconsequential stimuli while enhancing responsiveness to meaningful ones. Thus, cellular plasticity, whether at the level of a cell or an entire neural network, modulates pre-existing behaviour.

In most above-mentioned experiments, laboratory conditions enabled reproducible association with simple, unequivocal stimuli, at least in theory. However, in every situation, experimenters were interested in the specificity of the conditioned response: as described before, Watson’s little Albert did not only learn to fear rats but any white and fuzzy object (he mentioned testing unrelated items such as a Santa Claus mask with fake beard that triggered



Albert's response). Similarly, Pavlov conducted alternative studies where he conditioned dogs by pairing food presence with a tactile stimulation (a leg rub). He observed that trained dogs not only responded to the leg rub but also to various tactile stimulations (such as scratching instead of rubbing). Thus, the question of stimuli perception quickly became a prolific topic in the behaviourist community.

## Discrimination vs Generalisation

Looking at conditioning from the learner point of view, how the world is perceived is indeed a critical issue to better understand the mechanisms underlying associative learning. At this point, of course, modern neuroscience techniques were not yet available. Thus, one could almost only rely on behavioural observations, which might have inspired a lot of researchers in establishing crafty behavioural experiments. Following Watson observations about Albert reacting to stimuli carrying similar features to the one he was conditioned with, one of his students, Karl Lashley hypothesized that animals show a generalisation response to a given stimulus when they are not able to distinguish it from a previously reinforced one. In other words, generalisation and discrimination are both opposing and complementary concepts.

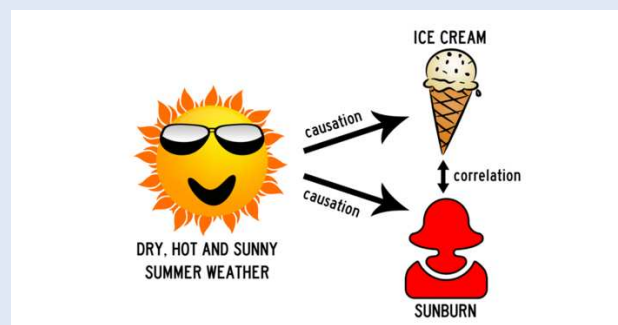
In theory, the more similar two stimuli are, the more likely they are to be mistaken for each other. If an animal was conditioned to respond to green circles, it might also respond to various shades of green or other geometrical figures filled with green. Kenneth Spence was particularly interested in quantifying generalisation. Based on previous experiments combined with his own observations on primates, Spence developed a theoretical framework encompassing learning acquisition and generalisation process<sup>[20]</sup>. The principle was to focus on one sensory modality in order to operate on one dimension measurement scale. Typically, sounds can be measured in frequency, colours in wavelengths, etc. That way he was able to compare perceived similarity with physical similarities. He hypothesised that upon conditioning, animals do not learn in a discrete but rather continuous way. If an animal is taught to respond to a 2000 Hz sound, it will learn to respond to an interval of frequencies centred on 2000 Hz. The closer to 2000 Hz the sound will get, the more likely to respond the animal will be. By recording the behavioural response to a panel of sounds (e.g., how many



times the animal responds to a 1500 Hz sound compared to a 1200 Hz sound), it would be possible to draw response probability curves that he called “generalisation gradients”. Incidentally, there would be excitatory and inhibitory gradients depending on positive and negative reinforcement. Spence theory was remarkably accurate as numerous behavioural studies successfully showed the presence of generalisation gradients in various species (example provided Figure 7). The variability in response depends on the animal acuity for the sensory modality of interest, as an animal able to distinguish very similar shades of colours should be able to respond with more accuracy than an animal with limited colour vision.

### Classical approaches in Neuroscience and correlation / causality bias

After his theoretical work on discrimination, Lashley focused on finding brain regions involved in memory storage. Although memory is not our main topic, his approach is worth mentioning. Indeed, like other famous scientists such as Paul Broca, Lashley believed in the cerebral localization theory, suggesting that particular brain areas were dedicated to specific functions, including memory. That is why his experiments consisted in training rats to solve mazes, before inducing brain lesions in different areas in the hope of finding the one involved in memory, which he called the “engram”. Although he was unable to find a unique region specifically involved in memory, the approach based on impairing specific brain regions while measuring learning (or memory) performances in a controlled behavioural task was reliably implemented as a classical methodology in neuroscience.



Such approaches have been described in numerous species using, inter alia, surgical lesions, pharmacological agents, mutagenic treatments. In any case, the assumption is that if a brain region is involved in learning, its impairment should lead to a learning performances loss. This is the first step in deciphering neural networks underlying learning, the next step always involves a battery of controls for all the factors previously described that affect learning, such as motivation or perception. Indeed, gouging the eyes of an animal impairs visual learning but not the brain circuitry of learning itself. Similarly, when working on appetitive learning, altering the brain circuits involved in food consumption may alter learning performances as well.

Moreover, Spence’s model predicted a crucial collateral effect of generalisation gradients: If we consider the visual environment of an animal as a continuum along which each wavelength is associated with a response probability, Spence postulated that each learning event that includes a given wavelength would modify its associated response probability. In other terms, he suggested that excitatory and inhibitory strengths associated with conditioning trials are additive. Thus, the behavioural output displayed by a trained animal would be driven by the resultant sum of its past experiences. In the example shown below, an animal is trained with a positive reinforcement associated with a visual stimulus at 500 nm (shown in red), while being trained with a negative reinforcement associated with a stimulus at 540 nm (shown in blue). The resulting behavioural responses of the trained animal are displayed in green. Interestingly, although the animal was trained to respond to 500 nm stimuli (and thus is expected to show a maximum response rate at 500 nm), the actual maximum response rate (or peak response) is shifted toward 480 nm instead, as the response probability associated with 500 nm stimuli has been inhibited by the previous negative reinforcement associated with the 540 nm stimuli. Spence called this phenomenon “Peak Shift”.

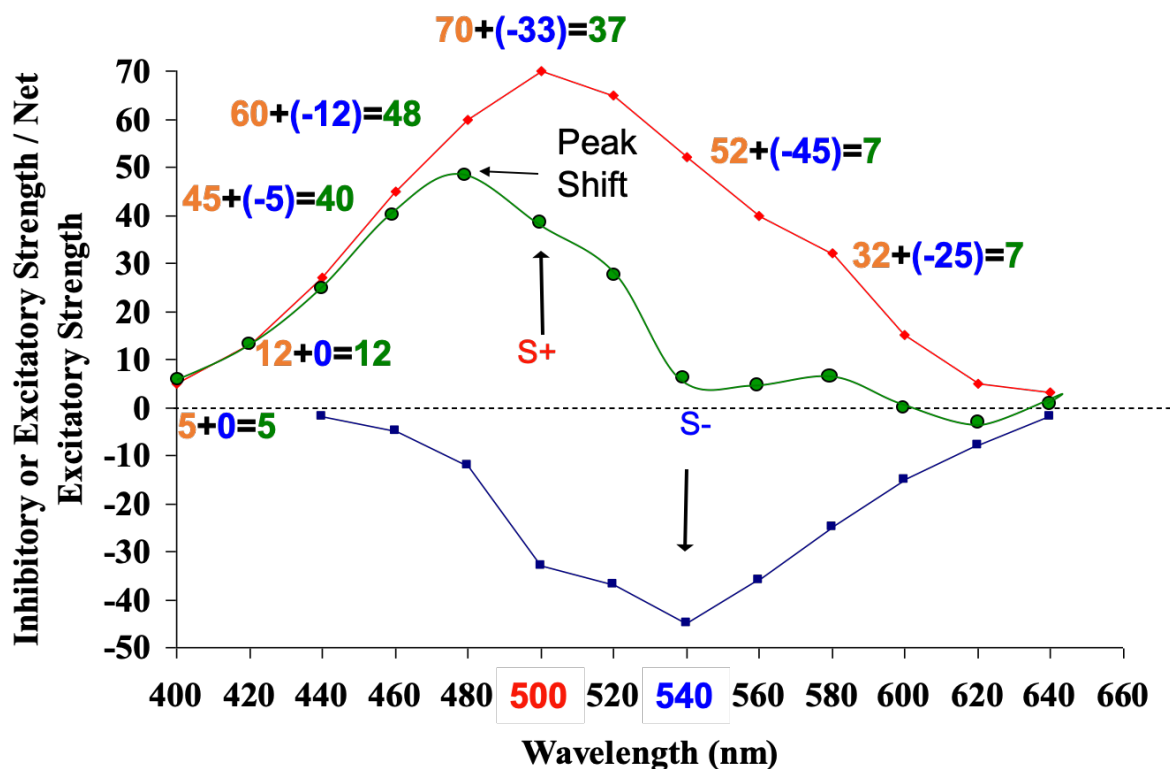


Figure 6 Illustration of the peak shift concept (adapted from: [21])

Peak shift is an ideal scenario, as it is a clear-cut emerging property of the model that can be easily tested in vivo in order to verify or invalidate Spence's theory. Years later, Spence's peak shift was effectively reported for the first time in pigeons<sup>[22]</sup>, but also later in insects such as bumblebees<sup>[23]</sup> and honeybees<sup>[24]</sup>, all of which using visual (pigeon and bumblebees) and olfactory (honeybee) modalities. The concept of peak-shift is particularly relevant in the scope of our work as it formalizes how the interaction of similar stimuli representation from the same sensory modality shapes resulting learnt response.

Along with his mentor Clark Hull, Spence was a pioneer in learning formalisation and modelling, demonstrating that it is possible to build a mathematical framework based on already existing data in order to drive the emergence of unforeseen predictions. By isolating testable properties of such a model, it is then quite straightforward to set the limits of what it can explain and what is still missing. Incidentally, as the experimental psychology field was growing, the mathematical approaches aimed at modelling associative learning phenomena followed closely. In operant conditioning case, the most prominent one is the Bush & Mosteller model<sup>[25]</sup>, which is often considered as the ancestor of its counterpart in pavlovian conditioning : The Rescorla & Wagner model, on which we will focus as our work is entirely based on pavlovian conditioning.

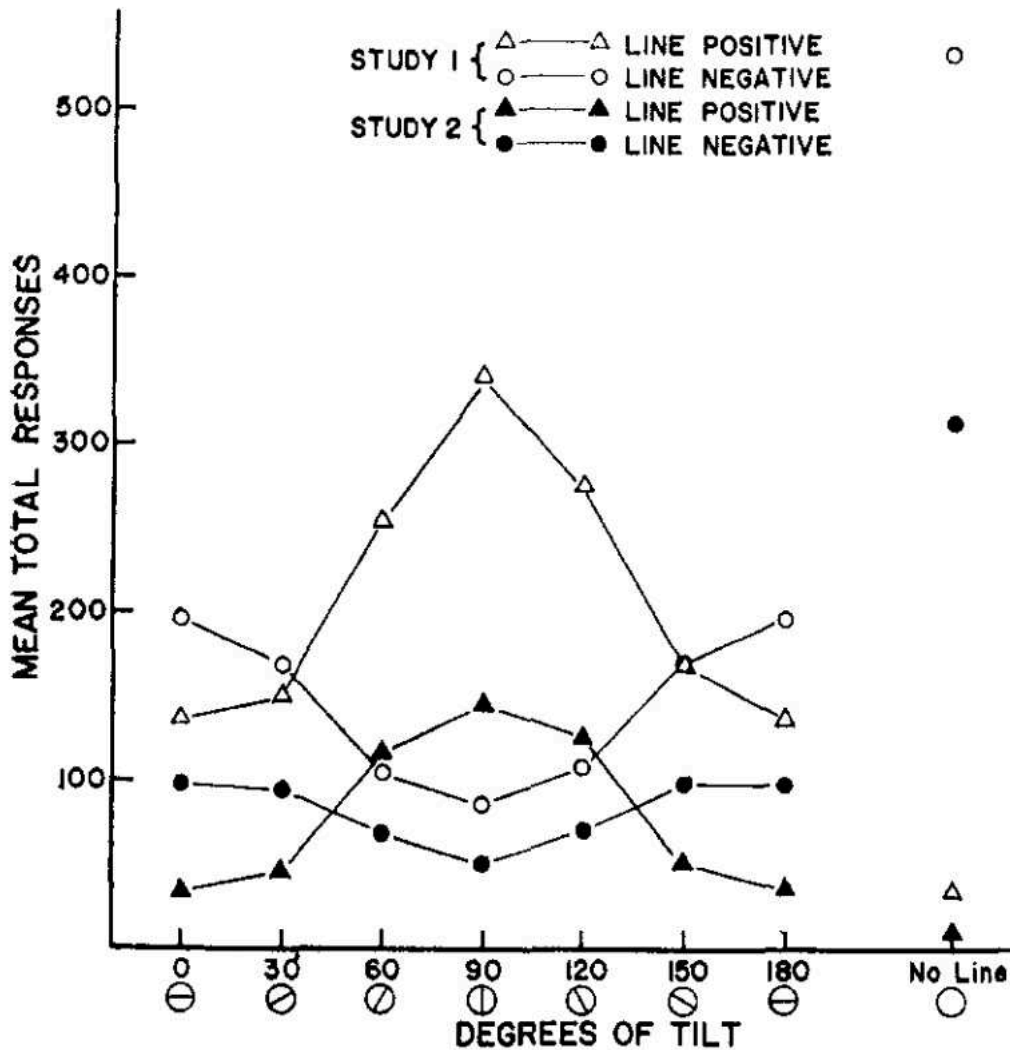


Figure 7 experimental validation of Spence generalisation gradients<sup>[26]</sup>. Here pigeons were conditioned to respond to a circle with a vertical bar in it by giving them food whenever this stimulus was presented while showing them a hollow circle without reinforcement as control. Afterwards, different stimuli presented on the x axis were presented and pigeons responses (attempts to peck) were measured (termed "line positive"). A second group was trained not to respond to the vertical bar by reinforcing the hollow circle while presenting the vertical bar without reinforcement (termed "line negative"). Animals in the line positive group showed a maximum response rate for the actual previously reinforced stimulus, but also responded strongly to slightly tilted lines, then showed a lower response to more tilted lines and displayed the lowest response rate to horizontal lines. Similarly, animals in the line negative group showed a maximum inhibition for the vertical line, with gradually decreased inhibition as the line approach the horizontal position.

## Classical models of associative learning: How to put numbers on learnt associations?

### Elemental & Non-Elemental learning (Rescorla & Wagner)

In the previous chapters, we defined the CS/US association as the heart of associative learning. It is not surprising then that a large part of the research about associative learning focused on the relationship between CS and US. In the 1960's most of the conducted experiments focused on the training repetition, based on the assumption that the more the conditioning procedure was repeated, the better the association between CS and US would be. In contrast, Robert Rescorla focused on the consistency of the CS/US association, already suggesting that rather than absolute, intrinsic links, the CS/US association was a matter of likelihood for the CS to predict the US (e.g., the likelihood for a given visual cue to predict the presence of food). In 1968, Rescorla tested this theory, that he called contingency theory, by conducting an experiment known as the "truly random control". During this experiment, rats were exposed to electric shocks associated with the presentation of tones. While in a first experimental group tone and shocks were always presented together, in a second group the tone was followed by shocks only half of the time. In other terms, the probability for the rats to receive a shock after a tone was 50%. Importantly, the overall number of stimulations was consistent, so that only the contingency between CS and US varied. Rescorla showed that while the rats in the first group easily learnt the association between CS and US, the rats in the second group were unable to learn it, suggesting that from the perspective of the rats, the tone was not a predictor of shocks<sup>[27]</sup>.

Later, Rescorla became associated with another experimental psychologist, Allan Wagner, who happened to be a former student of Kenneth Spence (see the "Peak Shift" part above). Together, they built a conceptual model of associative learning aiming at merging the already known aspects underlying its acquisition. Based on their respective work combined with previous models<sup>[13,25]</sup>, the Rescorla & Wagner (RW) model integrated stimuli salience, a maximum value of associative strength (that is, a theoretical situation where the CS would perfectly predict the US and thus would trigger the appropriate behavioural response with the same efficiency as an US presentation. Last but not least, Rescorla & Wagner expressed

learning acquisition as a variation of associative strength over the course of trials driven by the discrepancy between what is expected and what occurs, a concept known as “prediction error”.

Formally expressed, the model can be described as two assumptions:

$$\Delta V_X^{n+1} = \alpha_X \beta (\lambda - V_{total}^n) \quad (1)$$

$$V_X^{n+1} = V_X^n + \Delta V_X^{n+1} \quad (2)$$

Where  $\Delta V_X^{n+1}$  is the change in the associative strength (V) between the CS X and the US on trial n+1,  $\alpha$  is the associability of the CS X, that is, how well the CS is associated with US (which depends on numerous factors, including the CS intensity, its perception, etc., and ranges from 0 to 1). Conversely,  $\beta$  is the associability of the US.  $\lambda$  is the maximum associative strength that the US may drive.  $V_{total}^n$  is the total amount of associative strengths of all CS present on trial n+1. The second equation simply states that the associative strength of the CS X after trial n+1 is the associative strength before trial n+1 summed with the change in associative strength of the CS during the trial n+1 as computed in equation 1.

As stated before, the central principle driving associative learning according to Rescorla & Wagner is concept of prediction error, that can be reworded as the effect of surprise. At first, the CS is completely unrelated to the US and shouldn't elicit any US related behaviour. The first explicit association between CS and US is therefore unexpected which maximize learning acquisition. Over the course of trials, the animal is increasingly aware of the CS-US relationship and is able to predict the US event from the CS perception alone, which diminishes the impact of subsequent learning trials on the association. In other terms, the animals learning state matches his experiences and does not need to be updated. Eventually, associative strength between CS and US reaches a plateau, as the CS/US predictive link reaches 100%. The resulting learning curve takes a logarithmic form:

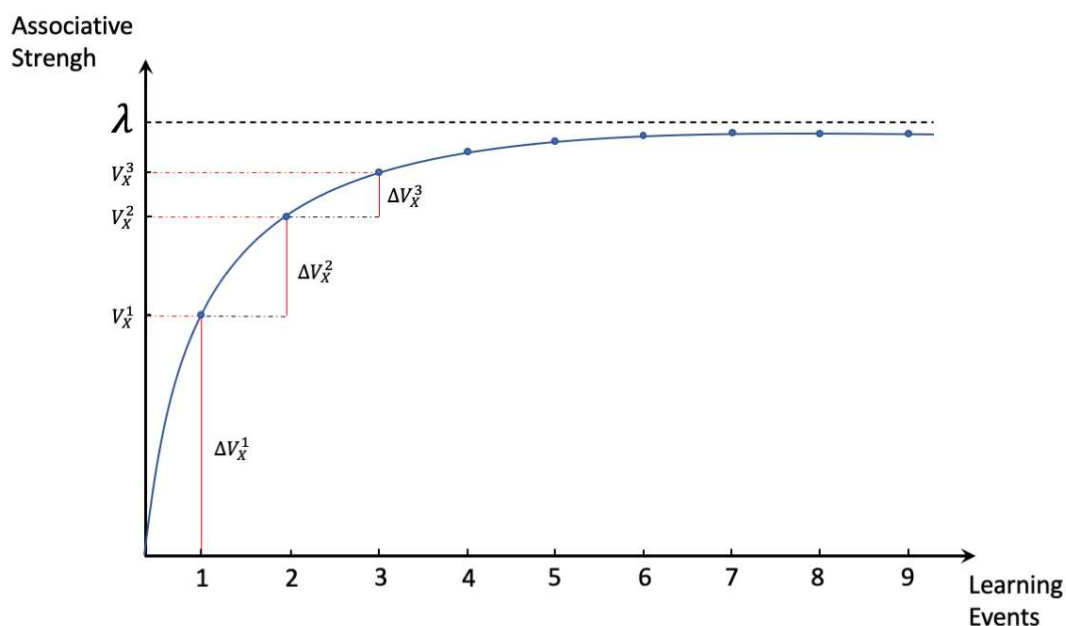


Figure 8 Associative learning acquisition curve according to the Rescorla & Wagner model.

This model, although quite simple, is still one of the most efficient attempts to account for many observed learning-related behaviours : First, the logarithmic acquisition curve fits very well with the observed acquisition of conditioned response, shown for instance in rats and rabbits<sup>[28]</sup>. Second, the model also accounts for the extinction phenomenon first described by Pavlov<sup>[6]</sup>: indeed, should the CS presentation not be followed by the US anymore, it would constitute a discrepancy with previously learnt information and lead to gradual extinction of the acquired learning. The extinction principle implies that learning acquisition goes both ways, taking on a positive value when the experience reinforces the measured behaviour and a negative value when it suppresses it. Formally, when the CS is not reinforced, the  $\lambda$  parameter is not the maximum associative strength anymore but the minimum associative strength, 0, which is why the  $(\lambda - V_{total}^n)$  term takes on a negative value. In addition, extinction is a fairly slower process than acquisition, and the  $\beta^{\text{extinction}}$  parameter is thus smaller than its 'acquisition' counterpart.

Based on the central tenet that associative strength is additive, Rescorla and his collaborators developed the concept of elements to explain more elaborate forms of learning, involving several stimuli. Indeed, outside of laboratory conditions, in principle animals are not exposed to isolated stimuli but rather to complex sceneries. According to the RW model, each

stimulus should thus become individually associated with the US and the total acquired learning strength would be the linear sum of the components' associative strength. Formally,  $V_{total}^n$  encompass every stimulus involved. Let A, B and C be the stimuli involved; A, B and C are considered as elements, which is why this model of learning is called elemental learning. In that case, the RW model states that  $V_{total}^n = V_A^n + V_B^n + V_C^n$ .

### Conditioning paradigms

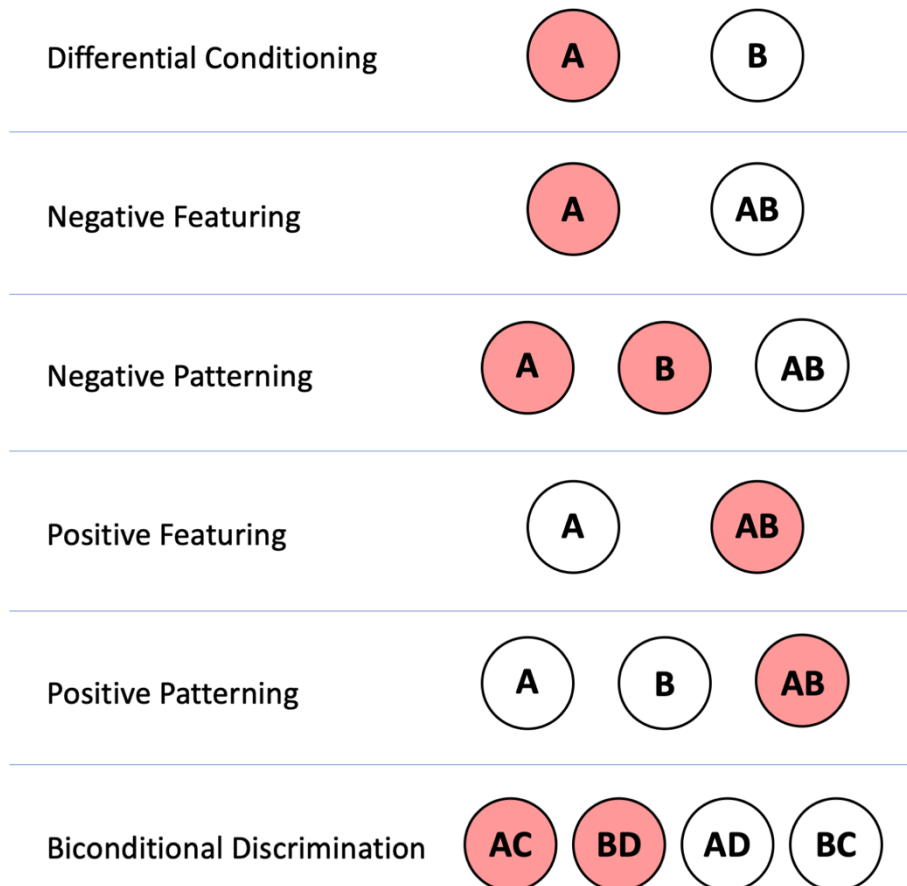


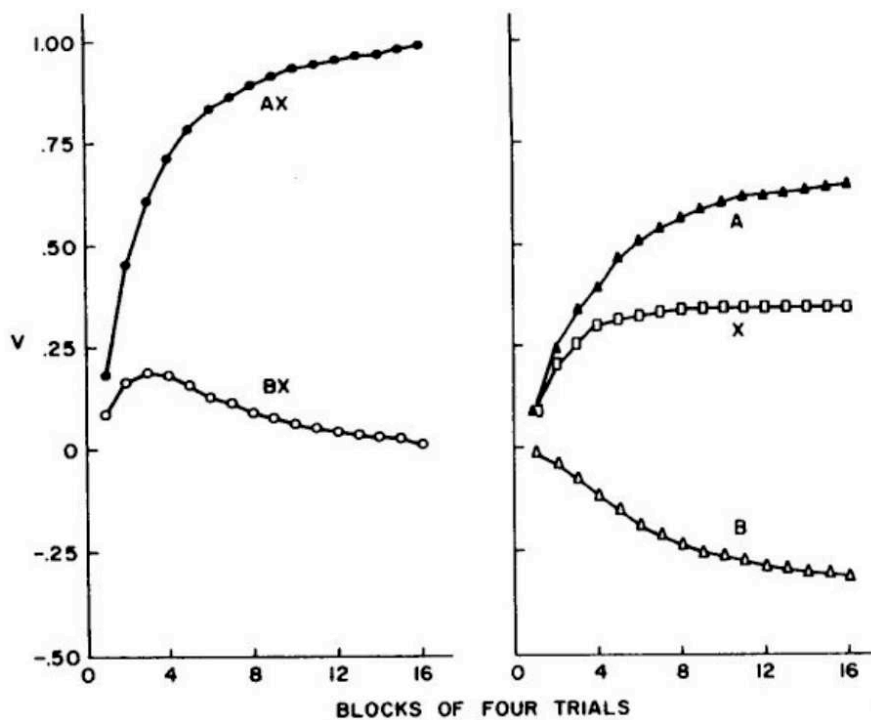
Figure 9 Illustration of the most commonly used behavioural paradigms in associative learning. Red shadowing indicates that the stimulus is reinforced.

Interestingly, Rescorla and Wagner used this characteristic to explain generalization, assuming that any CS could be broken down in smaller elements and suggesting that two similar stimuli shared common elements. For instance, let X be a learnt stimulus and Y another one that elicits a generalization response; the RW model suggests that X and Y could be translated into X=AB and Y=BC. When reinforcing X, both the A and B elements are reinforced, and because B is also a part of Y, the trained animal would also respond to Y. An interesting prediction resulting from this interpretation states that reinforcing X, which means



reinforcing AB will eventually lead to  $V_{total}^n = V_A^n + V_B^n = \lambda$ . In principle, the associative strength has reached the asymptote and cannot be further improved. However, if A (or B) is isolated and reinforced on its own, it should overcome the aforementioned limitation because  $V_A^n < \lambda$ . Rescorla later provided experimental confirmation of this characteristic<sup>[29]</sup>. Moreover, although purely theoretical at this point, the possibility of breaking down stimuli perception into smaller elements proved surprisingly accurate, fitting well with the neural architecture of perceptual regions in various animal models (including *Drosophila*, as discussed later).

In a discrimination task, if Y is now presented without reinforcement (making it the CS-, in contrast to X, the CS+), A and B would be reinforced through X, while B and C would be inhibited through Y, leading to an overall better discriminability between X and Y. Because the reinforcement is generally stronger than inhibition through lack of reinforcement, the RW model expects an initial rise of associative strength (and thus behavioural response) for both X and Y, followed by a gradual decrease in response to Y, which here again closely match experimental data<sup>[30]</sup>.



**Figure 6.** Mean Vs computed from a sample learning run in which AX reinforced trials were alternated with BX nonreinforced trials. The left panel depicts the compound theoretical values, the right panel the corresponding component values.

*Figure 10 Theoretical acquisition curve during a discriminative learning involving stimuli sharing an element<sup>[31]</sup>*

## How to define complex?

Learning tasks are often classified in terms of complexity. In fact, one of the first versions of this Thesis work was called “complex learning in *Drosophila*”. However, how complexity is defined, hence its relevance as a qualifier is not a trivial matter. It is indeed not enough that a behavioural task has been designed to be complex to be able to objectively assert that it is complex from the perspective of the animal. A task could be defined as complex depending on the proportion of animals able to solve it. For instance, while non-associative learning has been found in numerous species (see the “non associative-learning box), insight learning has only been found in few species, mainly birds<sup>[32]</sup> and primates<sup>[33]</sup>. But this definition is inevitably flawed, as it is impossible to formally prove the nonexistence of something. Indeed, in the case of Insight, experimental paradigms designed to test for it mainly rely on tool use, which is challenging to implement in many models.

Some behavioural tasks are considered complex because subjects need more trials to solve them successfully. Negative Patterning has been shown to require more trials than typical differential conditioning to be learnt<sup>[34]</sup>. Whether the task of interest takes more trials to be learnt or is only learnt by some species, it raises the central question of the underlying neural mechanisms, which constitute another way to define complexity. Indeed, one can make the assumption that the more complex a task is, the more processing needs to be achieved to solve it, thus involving additional neural functions. This idea is supported by experimental evidence, both in mammals and insect, as non-linear discriminations requires brain regions that are not essential for linear forms of learning in both cases<sup>[35,36]</sup>.

Finally, all of the above stances are focused on the animal perspective. However, an alternative, interesting stance would be to focus on the stimulus itself. Indeed, regardless of how complex the experimenter wants a stimulus to be, particular attention should be paid to the question of which physical features of the stimulus are truly significant, in relation to the animal perceptual system. Apparently complex stimuli might be ‘reduced’ to simple features by the perceptual system, whereas a seemingly simple stimulus, for instance an image, might be very difficult to decipher for an animal used to live in a 3D world.

In a review article on this very subject, J.G Fetterman wrote: “Everyone is familiar with technological advances that have afforded ever more realistic depictions of the world. Renaissance painters created more realistic works once they gained knowledge of perspective and principles such as shading, haze, and relative size. The motion picture industry has changed from black and white silent films, to Technicolor, to wide-screen colour films with surround sound, making the movie experience more realistic. [...] These advances have come about from increased knowledge of the relevant stimuli, not from internally generated changes in perception.”<sup>[37]</sup> Indeed, the physical features of stimuli and what parts of it are relevant for the animal perceptual system constitute a fascinating research field, in which the researcher would benefit from an approach similar to that used by painters and filmmakers to make the spectator feel immersed in the story.

A consequence of the elemental learning is that two stimuli may be discriminated if – and only if – they present distinct elements that can be differentially reinforced (or suppressed). That is why, while exploring the potential limits of the RW model, its authors became interested in behavioural tasks in which the stimuli to distinguish share all their elements. Interestingly, the idea that learning is additive was already brought up in anterior work<sup>[38]</sup> where a behavioural task designed to conflict with this principle was developed: The Negative Patterning (NP). During a NP task, two stimuli, A and B are individually reinforced. In parallel, they are presented to the subject as a compound AB without reinforcement. Interestingly, the task is considered as learnt if the animal responds mostly to A and B but not to AB. According to the RW model,  $V_{total}^n = V_A^n + V_B^n - V_{AB}^n$  which can be translated as  $V_{total}^n = V_A^n + V_B^n - V_A^{n'} - V_B^{n'}$  where  $V_A^{n'}$  and  $V_B^{n'}$  are inhibitory associative strength resulting from the presentation of AB without reinforcement. The model states that  $V_A^n < V_A^{n'}$  and  $V_B^n < V_B^{n'}$ . When presented with A, B, and AB, the RW model thus predicts that the subject should respond mostly to AB compared to A and B that contains only a part of the overall associative strength. In that case, the model and experimental evidence diverge, as in latter animals are able to learn the task and respond to the single elements rather than to the compound. The RW model in its original form was thus inconsistent with Negative Patterning solving (along with other tasks like Biconditional discrimination where no distinct element enables an elemental solving<sup>[39]</sup>). To sort out this discrepancy, two parallel theoretical explanations were developed for these tasks, henceforth referred to as “non-elemental”.

### The unique cue theory

To explain non-elemental task solving, Rescorla & Wagner stucked to the initial idea that every stimulus may be broken down in elements, suggesting that somehow the compound AB was not only composed of its constituents A and B, but also generated an additional compound-specific element that he called “unique cue”. In that case,  $V_{total}^n = V_A^n + V_B^n + V_u^n$ . By gradually suppressing their response to u while reinforcing their response to A and B, animals should in principle be able to solve the Negative Patterning task. Rescorla provided experimental evidence supporting this explanation<sup>[40]</sup>. To do so, the authors conceived an elegant experiment using another interesting feature of associative learning first

demonstrated by Pavlov<sup>[6]</sup>: Once a CS has been reliably learnt as predictor of a given US (thus becoming a CS+), it is able to act as a US by itself when presented jointly with another CS. Let A be the initially learnt CS, upon repeated association with X, a CS that was never explicitly paired with the US, X gain some of A's associative strength. This mechanism is called 'second order conditioning'. In their experiments, Rescorla et al. first conditioned pigeons with a Negative Patterning protocol (A+ B+ AB-). Then, they paired A with another stimulus, X using a second order conditioning procedure. They also paired B with Y and tested the pigeons' response to X, Y and XY. They showed that not only pigeons responded to X and Y as if it was A and B, but they also responded to XY as if it was AB and not A+B. They concluded that XY was generating the unique cue in the same way AB did, which was an experimental demonstration that beyond the physical properties of stimuli, joint representation of AB or their respective associates could generate a specific, learning-related stimulus.

### The configural theory

However, there were still experimental data that could not be explained by the unique cue theory. An alternative explanation, steering away from the initial additive model was also developed<sup>[41,42]</sup>. Although there are earlier evidence of similar theories<sup>[43]</sup>, Pearce benefited from a vast panel of experimental data to fully develop his model, including Rescorla & Wagner experiments. As stated before, the RW model dominant feature is that performances in any form of discrimination are determined by the algebraic summation of all the individual associative strengths of the stimuli coming into play (whether they are explicitly involved in the process or emerging from joint stimuli presentation such as the unique cue). Not unlike Clarke Hull, Pearce was interested in generalization events, arguing that in natural settings, learning restitution was rarely occurring in the exact same conditions as learning acquisition took place. Thus, generalization is an essential ingredient for animals to be able to adapt to the inevitable fluctuating settings they encounter. Fluctuations also arise from the interaction of encountered stimuli with each other; for instance, Pavlov described a procedure called 'Overshadowing' in which he showed that animals response elicited by a stimulus B is reduced if B was presented in a compound AB during conditioning compared to a more conventional situation where B would be presented alone. Hence the term 'Overshadowing' of the B

associative strength by the compound. Interestingly, the RW model account for overshadowing events, but requires multiple learning trials to be detected. In contrast, experimental data show that overshadowing already occurs at the first trial<sup>[44]</sup>. Another example of stimuli interaction is the Positive Featuring (PF) case. During a Positive Featuring procedure, AB is reinforced while A alone is not. At first, animals respond both to AB and A, but gradually learn to respond only to AB. B is never explicitly presented outside of the compound and is thus regarded as a ‘feature’. Interestingly, in a set of experiments using a Positive Featuring conditioning, authors found that B elicited a very weak response by itself, compared to the response elicited by the compound AB<sup>[45]</sup>. According to the RW model though, because animals do not respond to A anymore, response to AB should rely on B (in other terms,  $V_A^n = 0$  and  $V_B^n = \lambda$ ). In that case, the observed results may be interpreted given a unique cue produced by the joint presentation of A and B, although it does not explain why B would elicit a weak response while being repeatedly reinforced unless it is overshadowed, which comes back to the overshadowing problem aforementioned. Lastly, another mechanism involved in stimuli interaction was described in anterior work called ‘external inhibition’<sup>[6]</sup> or ‘blocking’<sup>[46]</sup>. In that case, A is reinforced alone as a pretraining before being reinforced in the compound AB. Subsequently, B is found to elicit a weaker response than when the pretraining is omitted.

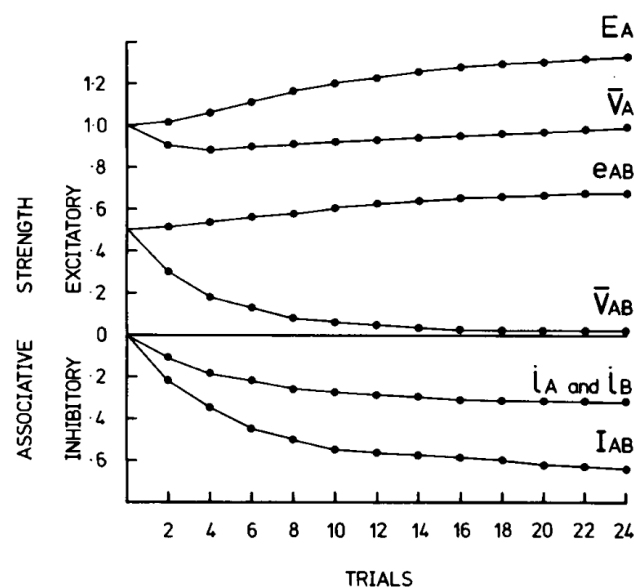


Figure 1. The trial-by-trial changes in associative strength that are predicted by Equation 10 to occur during discrimination training of the form  $A^+AB^0$ .

Figure 11 Pearce's account for Negative Featuring learning acquisition<sup>[41]</sup>

These results can be interpreted suggesting that by reinforcing A during the pretraining, its relative significance compared to B is augmented. In any case, the idea that adding a neutral stimulus may inhibit the observed response is inconsistent with the RW model where associative strengths are summed and addition of a neutral stimulus should not impact the animal response. Instead, Pearce presented an alternative model, a configural theory suggesting that the CS being associated to the US by a trained animal is in fact the entire set of perceived stimuli as a whole. Any change to the scenery would lead in principle to a new CS-US association. Pearce introduced a “s” parameter, standing for “similarity”. In his model,  $s \in ]0;1]$ . The close s is to 1, the more similar the compared CS are. Interestingly, s is never 0 because every sensory perception is taken into account in the CS definition, including the experimental setting (the testing box, light conditions, temperature, etc.) which brings a base level of similarity. The s parameter is used to determine to what extent the effects of a conditioning on a CS1 will generalize to a CS2. In Pearce’s configural model though, the amount of information an animal can store during a conditioning event is limited and each stimulus will be associated with a relative significance depending on their perceived intensity compared to the total perceived intensity. This feature explains well overshadowing and blocking occurrences as the way an animal is trained may reorder the salience of each stimulus relative to the others. For instance, if A is pretrained in an external inhibition protocol, its significance will be enhanced. Afterwards, when reinforced as a compound AB, AB will most likely elicit a representation that is closer to A than it would be without pretraining, which will also block out the representation associated with B. Formally, as for the RW model, two equations describe the evolution of associative strength for a given CS A that is reinforced :

$$V_A = E_A + e_A - (I_A + i_A) \quad (1)$$

$$\Delta E_A = \beta(\lambda - V_A) \quad (2)$$

In the first equation,  $V_A$  is the net associative strength mobilized by A. It depends on  $E_A$  and  $I_A$  which are respectively the direct excitatory and inhibitory strength acquired by A through conditioning but also  $e_A$  and  $i_A$  which are the indirect excitatory and inhibitory strength acquired by A through generalization.  $e_A$  and  $i_A$  are computed as the sum of every other

stimulus' associative strength weighted by the  $s$  parameter. The second equation is very similar to the RW model. It states that the increment in excitatory associative strength,  $\Delta E_A$  depends on  $\beta$  which is a learning rate parameter taking a value between 0 and 1 depending on the nature of the reinforcer.  $\lambda$  is the maximum associative strength that can be acquired. The  $(\lambda - V_A)$  integrates the RW concept of gradual prediction error.

Interestingly, over time both theories accurately predict a significant proportion of situations encountered during behavioural experiments, while being actually supported by behavioural data. For instance, blocking has been shown to require more than one training trial to be observed<sup>[47]</sup>, but was also measured with one-trial in other experiments<sup>[48]</sup>.

Overall, the R-W unique cue theory and the Pearce theory are both good models of non-elemental learning, actually very similar in their description of learning acquisition. The main distinction lies in how stimuli are defined and their modularity. However, behavioural experiments alone are insufficient to identify their relative validity in relation to animal cognition. Indeed, understanding how learning is actually implemented in animals' brains is crucial to characterize how they solve Negative patterning discriminations.

## Non elemental learning in vertebrates

In mammal brains, hippocampus is the subject of numerous experiments. Many functions have been associated to this region, including memory (following the study of the notorious 'patient H.M'<sup>[49]</sup>). Over the years, hippocampus has arguably become one of the most if not the most studied region in mammal brain, including a very rich literature on rodent learning and memory. As described in the "part on Lashley and cerebral localization theory", the most frequently used methodology was to cause lesions to the hippocampal region and test animals' abilities to solve various behavioural paradigms. Of course, non-elemental tasks resolution was amongst the tasks of interest and several papers proposed that hippocampus was involved in various non-elemental forms of learning despite being dispensable for elemental learning<sup>[35,50,51]</sup>. Interestingly, tasks described in the cited papers are multi-faceted but they all have one common feature: all involve multiple stimuli that need to be learnt in relation to each other. For instance, in the Place-Learning paradigm, animals need to rely on

a set of distal-cues to retrieve a previously reinforced target. In the Morris pool paradigm, one form of Place-learning, the tested animal has to swim its way to the location of a previously placed rescue platform. With no proximal cue available and an everchanging starting location, the animal can only rely on distal cue, which require to be able to extrapolate current location based on the relative position of said cues. Place-learning has been shown to be dependent on hippocampus function<sup>[52]</sup>.

During a contextual fear conditioning, the animal is placed in a box and receives an electric-shock paired with an auditory cue. Interestingly, it has been shown that the animal not only learns the “CS/US” association but also the context (unrelated perceived stimuli) in which the association occurred<sup>[51]</sup>. Additional work on contextual fear conditioning suggests that much like place-learning, it requires a conjunctive representation of surrounding stimuli to be learnt<sup>[53]</sup>. Hippocampus has already been shown to be necessary for Negative Featuring learning Finally, stimulus ambiguity may also come from temporal discrepancy, as is the case in the Reversal-Learning task. Animals are trained in a differential conditioning fashion, with a A stimulus associated with US and B stimulus presented without reinforcement. Afterwards, B is associated with the US and A is not anymore. The animal has to update its learnt information to reverse its conditioned response. Here again, the task has been shown to rely on hippocampus<sup>[54]</sup>. The fact that according to this theory, hippocampus role goes beyond sensory modalities or temporal associations is remarkable. Rather, the theory suggests that Hippocampus is necessary to link events together into a coherent scenery.

This concept implies a very important assumption for our work: non-elemental learning should not only rely on discrimination abilities. Indeed, in the case of contextual fear conditioning for instance, two contexts are easy to discriminate but the obstacle in the task is the fact that a specific cue has been reliably associated with an US and is present in both contexts. It is thus a matter of which stimuli (reinforced or not) are relevant clues for the animal to solve the task, that is, cues that allows reliable predictions.



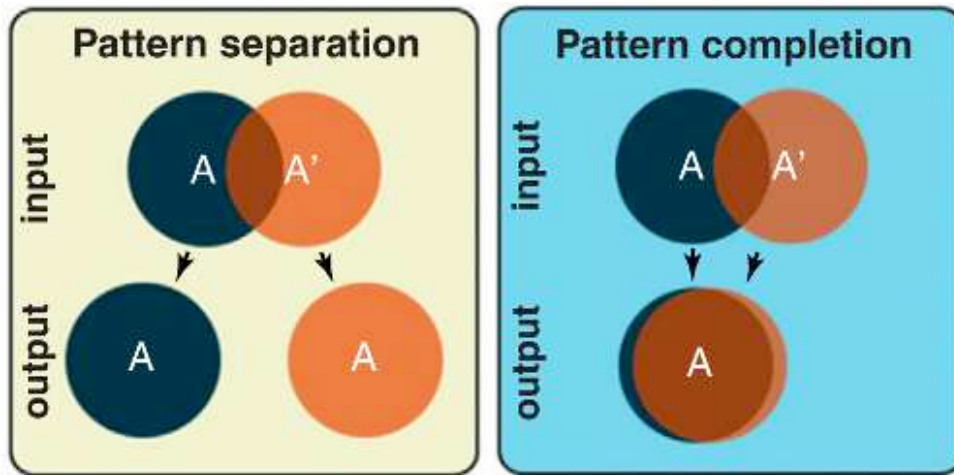


Figure 12 Schematic representation of Pattern separation and Pattern completion concepts.

Incidentally, because many aforementioned experiments include both contextual and non-contextual, multi-sensory stimuli, rodent literature is strongly oriented towards Pearce's configural account of non-elemental learning where the animals whole surrounding environment is taken into account to acquire associative learning. Configural learning in rodent is still an active topic, with an emphasis on how elemental association may enter in association with each other to form configural representations<sup>[55]</sup>. Indeed, current theories suggest that every distinguishable element in a given scenery is learnt individually upon reinforcement and linked to each other within the hippocampus. Interestingly, this implies the existence of two antagonistic phenomena: Pattern completion and Pattern separation. Pattern completion concept suggests that presenting one salient element of a given learnt scenery may lead to the response associated with the entire scenery. In contrast, pattern separation suggests that two overlapping reinforced sceneries may be distinctly encoded in order to be distinguished<sup>[56]</sup>. The latter is very similar to Negative Patterning events, where A, B and AB are overlapping but need to be learnt as different sceneries. What about other animal models?

## The case of insects in behaviour and neuroscience

As previously mentioned, non-elemental learning is regarded as a "high-level" cognitive task, although very little is known about the underlying neural mechanisms and while involved neural regions can be identified in mammals, said regions often remain black boxes as the

kind of computation they perform is unclear. This issue has much to do with mammals' brain architectural complexity (making it tricky to model it).

On the other hand, insect models also show remarkable behavioural abilities. Thus, Jean-Henri Fabre, often considered as the father of entomology, unveiled the exceptional proficiency displayed by male giant peacock moth (*Saturnia pyri*) as he observed it finding its way to its female counterpart<sup>[57]</sup>. As he put it:

“What are the organs of information that direct the rutting Moth on its nightly pilgrimage? [...] One suspects the antennae, which, in the males, do in fact seem to be questioning space with their spreading tufts of feathers. [...] Are there, in point of fact, effluvia similar to what we call odour, effluvia of extreme subtlety, absolutely imperceptible to ourselves and yet capable of impressing a sense of smell better endowed than ours?”

Indeed, his work highlighted both insects' olfaction and communication capacities. Years later, biochemist Peter Karlson demonstrated that the effluvia Fabre suspected were indeed elaborate communication substances he called pheromones<sup>[58]</sup>.

In a rather anthropocentric mindset, the idea that such small creature would display such incredible aptitudes was puzzling. Even more baffling was the fact that insects performed complex behaviours while carrying a fairly small amount of processing power.

Interestingly, Charles Darwin was among Fabre's collaborators and was also interested in insect behaviour. Indeed, he produced extensive reports on experiments he carried out with honeybees. For instance, Darwin studied how bees were able to build perfect hexagonal prisms, a challenge that puzzled even human geometers. Indeed, while honeycombs remarkable structure was often thought to be an expression of God's plan, Darwin suggested that it was instead the product of thousands of years of evolutionary shaping. He also emitted the hypothesis that honeycomb structure could be the result of the cooperation and coordination of hundreds of bees, each performing simple, repeated actions eventually leading to complex structures. His theory was supported by similar observations in various species, like spiders building their webs or birds building their nests. Darwin's hypothesis was further reinforced by the work of Pierre-Paul Grassé who developed the concept and coined

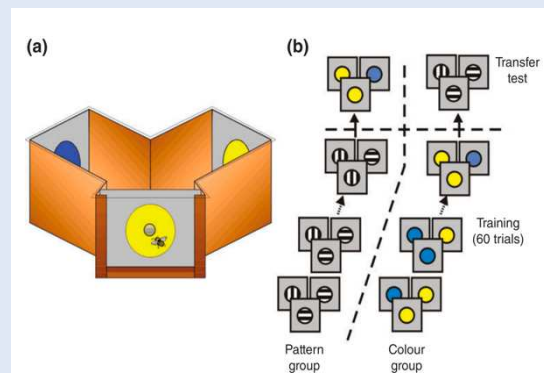
the term “stigmergy”, showing that rather than being the result of “blueprints” of the honeycombs each bee would have stored, hives emerged from the independent work of bees following simple reflexive rules based on the IFTTT (If This Then This) pattern. Each worker would produce precise responses according to their perception, gradually building the honeycombs. Darwin also initiated the fascinating field of insect navigation as he observed how bees always followed the same foraging route when looking for flower patches. Thus, insects challenged our way of tackling cognition, looking for arguably simple explanations for complex behaviours instead of sticking to the brain size hypothesis<sup>[59,60]</sup>.

Whether or not it was regarded as a true form of learning, Pavlov’s conditioned reflexes gripped a worldwide attention. It is therefore not surprising that early entomologists looked for evidence of conditioned reflexes in insects. Charles Henry Turner, who was the first to show that insect (moths in his case) could perceive airborne sounds, also observed that they were able to associate specific sounds to an aversive treatment (rough handling), thus providing one of the first evidences of classical conditioning in insect<sup>[61]</sup>. Modification of innate behaviour through reinforcement was also shown in cockroaches, using an apparatus very similar to the fear conditioning protocols in mice<sup>[62]</sup>. Such apparatus is composed of a light and a dark compartment ; the naïve animal tends to seek the safety of darkness, but this innate behaviour can be reversed by associating the dark compartment with a punishment (electric shocks).

What about more elaborate forms of learning? In 1957, Kuwabara presented a way to perform classical conditioning on tethered honeybees: the proboscis extension reflex (PER) conditioning<sup>[63]</sup>. Bees, as other insects use their antennae to probe their environment, looking for food sources such as flowers nectar. Upon sensing sugar, they show an innate response, the PER (illustrated in the “The wondrous abilities of honeybees” panel). If an experimenter systematically presents an initially neutral odour followed by sugar, the bee rapidly learns the association and starts extending its proboscis at the smell of the conditioned odour, even when there is no more sugar presentation. These tightly controlled experimental conditions allowed to test for various learning and memory abilities. For instance, in differential conditioning protocols, one odour is reliably associated with a sugar reward while another is not. Thus, the bee learns to respond specifically to the reinforced odour.

## The wondrous abilities of honeybees

Honeybee (*Apis mellifera*) holds a special position within the insect cognition field, as it enabled some of the most astonishing discoveries in insect's cognition. Indeed, Karl Von Frisch paved the way to decades of fruitful experiments when he demonstrated that bees were endowed with color vision, along with the ability to perceive UV and polarized light<sup>[64]</sup>. Using this sensory information, bees are able to navigate in search of good quality flower patches, establish and memorize foraging routes between their nest and the patches, and even communicate patches' locations to their conspecifics through an elaborate dance communication. Once brought to the lab, bees exhibit remarkable learning and memory abilities that can be measured using PER



protocols (Left picture), which also allows the use of neuroscience tools to unveil neural correlates of both elemental and non-elemental forms of learning<sup>[36,65,66]</sup>. Finally, ingenious free flight experiments were developed, combining controlled conditions with animals behaving in semi-natural conditions. Using this paradigm, pioneer works demonstrated bees ability in social learning, concept learning and even numerosity<sup>[67,68]</sup>. For instance, in the right picture is displayed a typical “delayed matching-to-sample” task (from: [69]) ; bees enter a Y-maze through a door ornated with a sample. Within the Y-Maze, two choices are possible, each ornated with a different sample. The bee is rewarded when choosing the arm associated with the same sample found at the entrance, regardless of any other feature. In other terms, the bee has to always chose “the same” sample it saw when entering the maze. After the conditioning, the bee is subjected to a transfer test where it has to perform the same tasks with completely unrelated stimuli. Thus, bees are able to learn abstract rules such as “always choose what you encountered upon arrival” or “always choose what you didn't encounter upon arrival”.

Using this procedure, pioneer work identified non-elemental learning abilities in bees<sup>[70]</sup>. Intriguingly, while various forms of non-elemental learning were also identified in other insects<sup>[71–74]</sup>, bee is the only insect model where negative patterning abilities were demonstrated so far. Following the path of vertebrate neuroscientists, bee researchers subsequently proceeded to identify neural correlates of elemental and non-elemental

learning in bees by altering brain regions, either using physical (cold shock) or pharmacological (injections) procedures<sup>[36,65,66]</sup>.

Because the insect brain is arguably simpler in terms of architecture, researchers were able to follow the olfactory pathways to identify potential regions involved in associative learning. That is how they identified the Mushroom Bodies (MB) as a major brain region involved in learning and memory, especially for non-elemental forms of learning. Indeed, injections of local anesthetics (Procaine) showed that functional MB are necessary both for negative patterning<sup>[36]</sup> and reversal learning<sup>[75]</sup>, which establish striking similarities with vertebrates hippocampus.

Thus, honeybee is a key model in insect neuroscience, showing remarkable cognitive abilities while being the source of numerous discoveries about how such small animals are able to learn arguably very complex tasks (see “The wondrous abilities of honeybees” insert). However, bees research comes with its share of shortcomings. Bees are raised in hives, which makes it challenging to control the environment of the subjects (genetic background, nutrition, weather-impact...). Moreover, access to the brain is restricted to pharmacological approaches, although recent breakthroughs based on CRISPR protocols might significantly broaden the panel of available tools to explore bees neural networks<sup>[76,77]</sup>. Developing a reliable negative patterning paradigm in an insect model more easily handled in lab conditions is therefore of high interest, especially if it enables access to powerful tools to dissect the underlying neural mechanisms.

## Learning and memory in *Drosophila*

Fruit flies (*Drosophila melanogaster*) is one of the most extensively used models in biology, along with mice (*Mus musculus*) and worms (*Caenorhabditis elegans*). It occupies a sweet spot, exhibiting a remarkable behavioural repertoire and benefiting from decades of research in genetics. Indeed, *Drosophila* history as a model organism took its rise with the pioneer work of Thomas Hunt Morgan. Indeed, looking to reproduce Mendel results on beans genetics in animals, Morgan looked for an affordable model, easily handled in the lab and that shows rapid generation rate. Following the footsteps of colleagues such as Charles William Woodworth and William Ernest Castle, Morgan started working on *Drosophila* and eventually

discovered and studied the first white-eyed mutants. By careful breeding and phenotypic observations, Morgan established the chromosome theory of heredity. Indeed, he showed that identifiable hereditary traits are not transmitted separately. Rather, they form physically distinguishable clusters that are almost always transmitted together to the offspring. His life work laid the foundations of modern genetics and as basically every aspect of an organism functioning is somehow influenced by genetically encoded mechanisms, Morgan's findings were also applied in numerous fields, such as developmental biology, physiology, behaviour and neuroscience<sup>[78]</sup>.

### Seymour Benzer and the first learning mutants

Neurosciences is a particularly rich field as it brings together many disciplines, such as molecular and cellular biology, physics, behaviour and even, as mentioned above, genetics. Seymour Benzer embodied this diversity; coming from a physics background, he first got interested in bacteriophage genetics where his contributions were many<sup>[79]</sup>. Building on this experience, Benzer then moved on to behaviour where he pioneered the field of behavioural genetics.

However, at this point animal behaviour (especially human wise) was believed to stand beyond genetics, suggesting that single-gene mechanisms were too low-level to actually direct complex behaviours. That perspective was driven by another influential scientist, Jerry Hirsch. Interestingly, this ideological opposition was extremely healthy as it led to distinct but complementary approaches in the study of behaviour.

Both approaches, focused on *Drosophila*, required to isolate behaviours of interest. On the one hand, Hirsch then proceeded on artificial selection of animals displaying the behaviour of interest over many generations. On the other hand, Benzer proceeded to mutagenesis screens to produce mutants for said behaviour. For each behaviour of interest, Benzer had to develop experimental devices designed to quantify and compare animals' response (e.g., phototaxis measurement device, Figure 13-Top Left). Then, flies were exposed to mutagenic agents (in this case, exposure to ethyl methanesulfonate) and selected according to their displayed behaviour. For instance, vision mutants could be isolated by selecting flies that displayed altered phototactic behaviour after being exposed to mutagenic agents (for

instance, it is the case of the non-phototactic flies, carrying a mutation of the eye absent (*eya*) gene<sup>[80,81]</sup>).

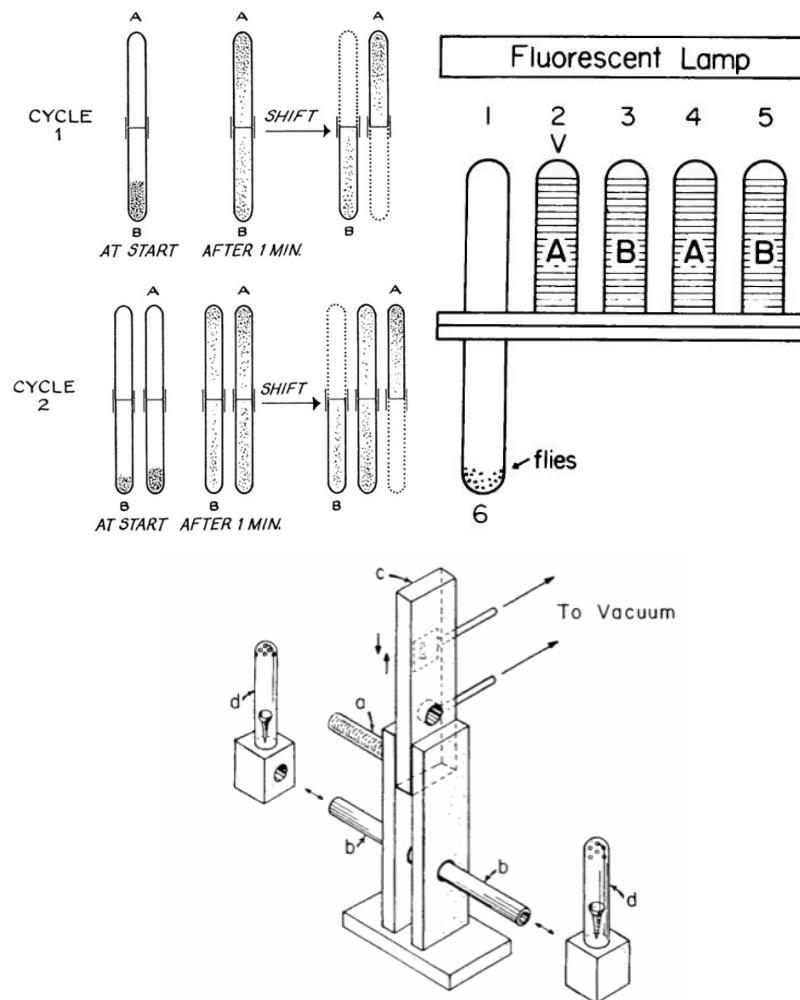


Figure 13 Up-Left: Benzer's phototaxis measurement device. Flies are placed in the device and left to distribute freely within the two tubes exposed to different lighting conditions. After 1 min, tubes are separated and the resulting fractions of flies are subjected to the same procedure again. Procedure is repeated 15 times and ensures selection of flies based on their phototactic behaviour.

Up-Right: Olfactory conditioning device: This device is very similar to the phototaxis device but in that case, the enlightened compartment is covered in copper and flies entering said compartment associate any encountered features (odours, colours, etc.) with electric shocks. A fluorescent lamp is used to lure flies inside the reinforcement compartment. Tube 1 is a rest tube, 2 and 3 are used for training, during which odour A or B is reinforced while the other is not. Tubes 4 and 5 are test tubes where post-training responses are recorded. Learning performances is assessed by counting how many flies avoided A and B despite the attraction provided by the fluorescent lamp.

Bottom: T-Maze for training and testing flies in a differential conditioning assay. A group of flies is placed in the training tube (a) that is coated with an electrifiable grid. Two odours (d) are subsequently supplied using a vacuum system, one of which is associated with electric shocks (CS+) while the other not (CS-). After training, flies are transferred to a choice point (b) via an elevator (c), where both odours are presented simultaneously. Each fly can move freely between the two odourants and learning performances are computed as the relative proportion of flies found in the tube filled with CS- compared with CS+.

Similarly, Benzer got interested in associative learning abilities of *Drosophila*. First, he used a modified version of the phototaxis device (Figure 13 up-right) and demonstrated that flies were indeed able to avoid an odour that was previously associated with electric shocks<sup>[82]</sup>. In that seminal work, he also showed extinction and reversal learning, along with evidence of memory of the learning events. Importantly, this first paradigm was an operant procedure, as flies were freely moving in the apparatus. In contrast, Benzer's former collaborators next conceived a device to quantify associative learning based on a Pavlovian conditioning procedure : The T-Maze (Figure 13-Bottom)<sup>[83]</sup>. This conditioning apparatus is designed for differential conditioning experiments involving two odourants that are to be discriminated in a binary choice manner (detailed procedure is described in the Methods part).

#### Individual vs. group performances

In the original work of Benzer's team, as in most of subsequent studies using T-Mazes, flies are tested in large groups, ensuring a robust statistical assessment of learning performances<sup>[82,83]</sup>. However, associative learning is often regarded as an individual process, and as such, cannot be reduced to the performance of a group of animals. In addition, the classical method assesses learning based on a single snapshot of flies' location after a 1 to 3 min test, which blinds the observer to behavioural variations during test phase. Incidentally, flies are gregarious animals whose behaviour can be modulated by conspecifics. That is why Claridge-Chang's team proposed an alternative test, where olfactory preference was measured by the time a fly spent in each arm of the T-maze<sup>[84]</sup>. This continuous measure allowed fine observations of individual flies and constitute a robust alternative to the classical procedure. In addition, *Drosophila* is well suited to procedure automation and animal's time spent in each arm of the T-Maze can be objectively assessed using video-tracking.

Although that kind of paradigm is inevitably an oversimplification of actual learning events encountered by animals, it enables a wide variety of tightly controlled parameters to be tested in perfectly controlled conditions, which is why it remains a major associative learning paradigm in *Drosophila*. In addition to the proof of concept that T-Mazes provide a reliable associative learning paradigm in *Drosophila*, Tully & Quinn also finely characterized learning dynamics related to various parameters that were directly linked to the Rescorla & Wagner model : US strength was modulated by varying applied voltage, CS salience was modulated by varying odour intensity, and learning acquisition was measured over multiple CS-US associations, leading to learning curves nicely matching the R&W predictions. Moreover,



diverse temporal CS-US pairing timings and even non-associative protocols were tested, leading to an extremely robust experimental framework for subsequent investigations. Next, Benzer's procedure already used for the screening of vision mutants was adopted by several teams and applied using the T-Maze protocol to identify associative learning mutants, that is, mutant that would not display the characteristic learnt avoidance of previously punished odours. The most notorious learning mutants include *dunce*, *rutabaga* and *amnesiac* mutants, which all show defect either in associative learning acquisition or retention<sup>[83]</sup>. Following mutants' identifications, the next step of such procedure is to understand why these mutants show learning defects. For instance, the gene responsible for *rutabaga* mutants phenotype was shown to encode a CA<sup>2+</sup> Calmodulin-responsive Adenylate Cyclase (CAM) later shown to be involved in coincidence detection between the perceived odour (CS) and the reinforcement (US) within the Mushroom Bodies<sup>[85–90]</sup>. In conclusion, to this day, Tully & Quinn's T-Maze remains one of the most widely used protocol to study *Drosophila*'s learning and memory, and while various alternative protocols have been proposed since<sup>[91,92]</sup>, it is the one we used in our study. Moreover, as Benzer's pioneer work was performed using olfactory learning, and because insects heavily rely on olfaction on a daily basis, a crushing majority of literature on associative learning in *Drosophila* is focused on olfactory learning. This is why we will focus on olfactory associative learning, even if remarkable examples of associative learning involving visual<sup>[93]</sup>, mechanosensory<sup>[94]</sup> modalities and even combinations of sensory modalities<sup>[95]</sup>.

## Drosophila and the genetic toolbox

Flies rapid generation rate and convenient rearing conditions already make them an interesting research model. However, in the 90s, the *Drosophila* field reached a whole new level with the work of Andrea Brand and Norbert Perrimon. In 1988, two studies show how a yeast transcription factor called Gal4 is able to also activate transcription in mammal cells through its binding with a genetic region called UAS<sup>[96,97]</sup>. The interesting idea that a foreign transcription factor can be expressed in a model organism and specifically activates a region that is almost never found in wild-type animals makes it a remarkable tool for targeted gene expression. Brand and Perrimon adapted this concept in flies by producing so-called "Gal4

lines” expressing Gal4 in small cell subsets. In parallel, they constructed a version of the development gene “even-skipped” under the control of an UAS promoter. Thus, they were able to target even-skipped expression in specific cell subsets such as photoreceptor cells<sup>[98]</sup>. Thus, the UAS/Gal4 system was conceived, reproduced and adapted for a huge array of experiments, as it allows to basically express any gene in any type of cell, provided the appropriate Gal4 and UAS lines are constructed. Beyond normally expressed genes, the UAS/Gal4 system enables the expression of tools such as Green Fluorescent Protein (GFP) in the Gal4-targeted cells, making them visible with a fluorescence microscope<sup>[99]</sup>, or, more recently, channelrhodopsin, an ion channel that can be activated with light and be expressed in subsets of neurons in order to activate them using a light beam<sup>[100]</sup>, a technique called optogenetic. In the last decades, entire institutions dedicated to the construction of stock centres collecting and maintaining numerous Gal4 and UAS fly lines (among others), used in almost every Drosophila research area (e.g., [The Bloomington Drosophila Stock Centre \(BDSC\)](#)).

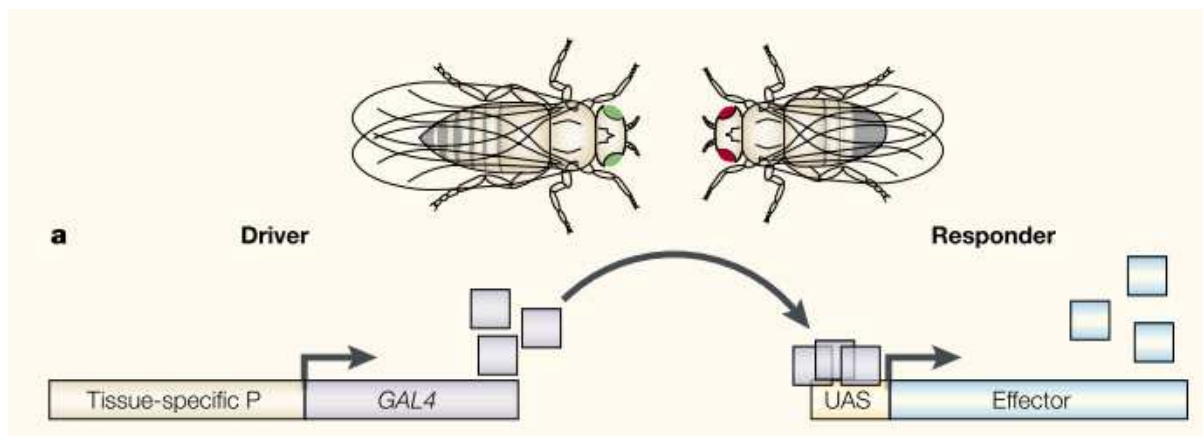


Figure 14 Illustration of the UAS/Gal4 system for targeted expression of genes of interest (adapted from: [101])

In addition to the cell-specificity brought by the Gal4 targeted expression, its combination with a thermo-sensitive repressor, Gal80<sup>ts</sup>, also allows temporal-specificity, as Gal4 expression is repressed at permissive temperature (25°C)<sup>[102]</sup>. Here, the designer authors exploited the ectothermic nature of insects, so that when placed at 30°C (the restrictive temperature), flies’ body temperature is also shifted to 30°C leading to the inactivation of Gal80<sup>ts</sup> and the expression of Gal4. Such mechanism allows to avoid any development effect of a given UAS/Gal4 construct.

In sum, besides the already attractive characteristics of flies, the UAS/Gal4 system makes them a unique research model, especially in behaviour and neuroscience, where they display complex behaviour that can be finely manipulated using genetically encoded tools controlled in space and time. That is why *Drosophila* constitute an ideal model for the study of neural networks underlying behavioural processes. Moreover, flies' brain displays a fairly simple architecture compared to mammal brains. Thus, systematic mapping of regions involved in various behaviour, including learning and memory has been a fruitful field of research over the last decades, which provided a robust background to select specific candidate neurons that might be necessary for non-elemental learning.

### Neural Pathways supporting olfactory learning in *Drosophila*

Olfactory associative learning involves two main steps: the subject has to properly perceive the stimulus to learn and the reinforcement. Then, association between the odour (CS) and the reinforcement (US) has to be encoded (and sustainably stored, which is what is called memory and won't be specifically addressed in this study as we focused solely on learning). Using classical neuroscience tools such as pharmacological treatments and electrophysiology, along with *Drosophila*-specific tools involving genetic targeting of neural subsets, the main brain regions and neurons involved in simple olfactory learning in flies have been gradually described and characterized.

#### *Olfactory perception: the sensilla, primary sensory organ in insects*

Olfaction relies on the perception of airborne chemical compounds through specialized receptors. In *Drosophila*, these olfactory receptors are located at the level of antennae and maxillary palps (see figure 15, left), where they are expressed at the surface of sensory neurons called Olfactory Receptor Neurons (ORNs), which are embedded within functional units called sensilla (Figure 15, right). Basically, sensilla contain the dendrites of ORN surrounded by liquid lymph called sensillar fluid that helps the capture of odour molecules. ORN come in ~50 different subtypes<sup>[103]</sup> based on the olfactory receptors they express<sup>[104]</sup> as in principle, each ORN expresses only one type of odorant receptor<sup>[105]</sup>. Apart from the

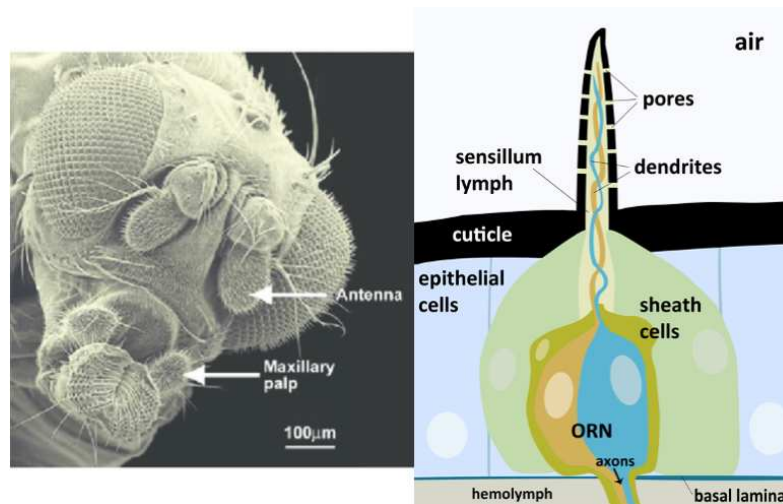


Figure 15 Left: Olfactory organs location in *Drosophila* (adapted from J. Scott, 2006) Right: Schematic organization of *Drosophila* olfactory unit, the sensilla (source: [106])

olfactory receptors that vary from one neuron to the other, all ORNs share remarkably common molecular mechanisms, to such an extent that swapping receptors between two ORNs neatly swaps the corresponding odour responses<sup>[107]</sup>. Interestingly, ORNs show spontaneous, basal activity<sup>[108,109]</sup>, which can be raised or inhibited dynamically upon odour/receptor binding in a concentration-dependent manner<sup>[110]</sup>. OR signal relies on ionotropic transduction<sup>[111]</sup> and may be broadly tuned, narrowly tuned or in between. Thus, each ORN type usually respond to multiple odours that do not necessarily share structural similarities<sup>[112]</sup>, but some are also specialized in the detection of biologically significant cues such as pheromones<sup>[113,114]</sup>. Finally, ORN responses are fast and dynamic. Upon activation, a peak of response (i.e. spike rate) is usually observed in 30 ms (for comparison, in vertebrates, the time to reach a response peak is around 400 ms<sup>[115]</sup>), and decreases rapidly after odour offset<sup>[110]</sup>. In sum, binding of an odourant results in the selective opening of a subset of olfactory receptors which cause a shift in the corresponding ORNs spike frequency. That way, the perception of an odour is translated into nervous signal.

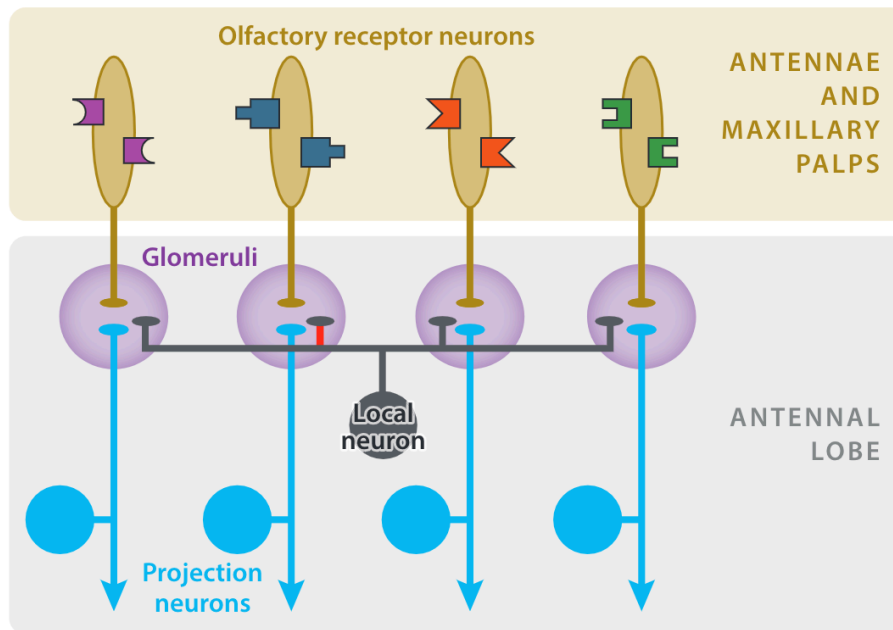


Figure 16 Schematic anatomy of the *Drosophila* olfactory system (from: [116])

### *Combinatorial odour code in the Antennal Lobes*

Once an odourant has been detected, elicited olfactory signal is carried to a second encoding region called Antennal Lobes (AL). While ORN of the same subtype are generally scattered across the surface of flies' sensory organs, their projections within the ALs are regrouped in functional units called Glomeruli. As there are roughly 50 subtypes of ORN, ~50 glomeruli are characterized in the ALs<sup>[103]</sup>. Interestingly, each odour produces a specific glomerular activity pattern that is also conserved across individuals<sup>[117]</sup>. Thus, it is possible to establish an odour map for each olfactory stimulus. In fact, some researchers in the *Drosophila* field created a "Database Of Odorant Responses" (DoOR) where anyone in possession of glomerular recording associated with specific odourant(s) may upload the recording results<sup>[118]</sup>. This crowd effort led to an ever-growing library of odour responses which can be accessed here: <sup>[119]</sup>. As an illustration, two of the odours we used in the present study are represented as glomerular patterns in Figure 16. Importantly, olfactory signal does not only depend on ORN activity, as glomeruli are also composed of lateral inhibitory interneurons that produce a normalization activity scaled with the total activity of the ORNs<sup>[120]</sup>. In other terms, glomerular interneurons act as a filter, dynamically modulating ORN responses, which leads to a more uniform and reliable odour code<sup>[121]</sup>. Animals may encounter a vast range of concentrations of the same odourant throughout their life. Interneurons inhibition has also been shown to

ensure proper response scaling across various odour concentrations<sup>[122]</sup>. In our study, we focus on behavioural paradigms involving odour mixtures. How are such compound stimuli encoded in the ALs? Compelling evidence shows that mixtures of two odourants are encoded as the sum of their components on which normalization is applied<sup>[123]</sup>. Thus, mixtures display strong similarities with their components, but compound-specific glomerular activity also arises from the normalization process, which is consistent with observations in honey bees<sup>[124]</sup>. How two odours are discriminated within the ALs? Previous studies showed that given two odourants, their behavioural similarity may be computed as their distance (or lack thereof) at the level of glomerular patterns<sup>[125,126]</sup>.

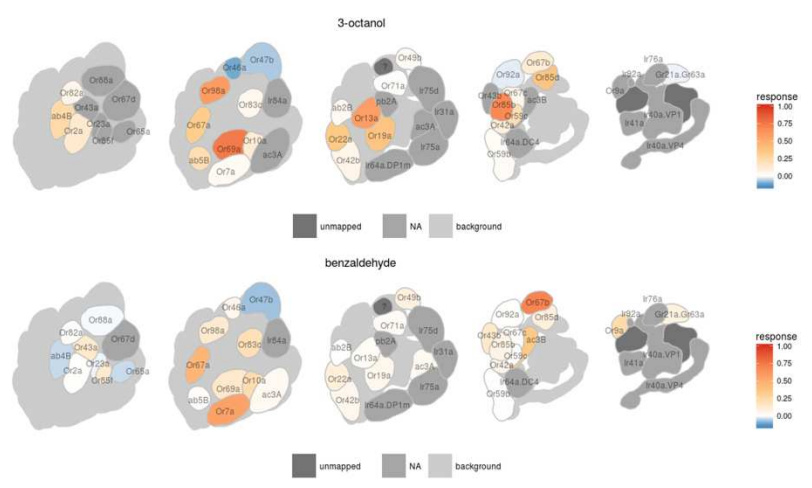


Figure 17 Glomerular pattern elicited by two odourants used in our experiments, 3-Octanol and Benzaldehyde. Each image is a layer of the glomerulus, responses are normalized and may be excitatory or inhibitory. Images were generated on the DoOR database.

In other terms, odour similarity is correlated with how many glomeruli are activated by both odourants compared to how many are specifically activated by one odourant or the other<sup>[127]</sup>. Interestingly, such similarity at the perceptual level is a close match with generalization experiments presented in previous chapters. In addition, discrimination / generalization seems to be two extrema of a continuous scale, which depends on the distance between odours. This was emphasized in a study where odour distance was artificially reduced by genetically silencing subsets of neurons within the AL<sup>[127]</sup>. Resulting behaviour was shifted

toward generalization behaviour. The more similar odour pairs were before silencing, the more dramatic the effect of AL neurons silencing on odour discriminability (Figure 17).

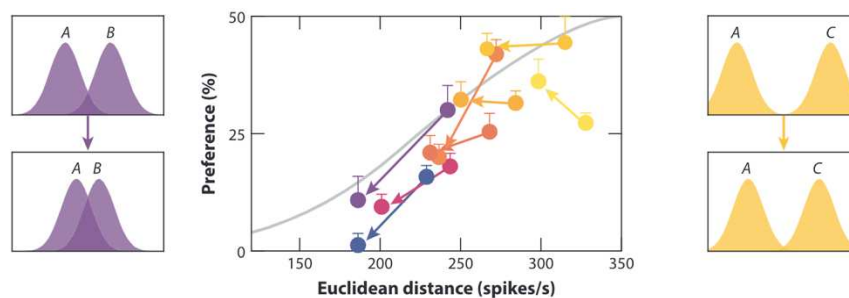


Figure 18 Schematic representation of the relationship between distance between odours representations and discriminability, adapted from: [128]. Odours A, B and C elicit differential naive preference in wild-type flies. Each coloured dot represents an odour pair (of which only A Vs B and A Vs C are represented left and right). Upon artificially increasing their similarity, flies show impaired naive discrimination, as shown by the arrows. Data are represented as the differential activity displayed toward one odourant compared to the other in terms of spikes/second. Behavioural preference was assessed using a modified T-Maze as presented in a previous study[84].

Thus, the AL constitutes the first olfactory integration centre. Each odour is encoded as a specific chemotopic pattern. Filtering operation occurring within the AL already provide significant information about odour perception and part of insects' discrimination abilities. Interestingly, glomerular organization seems to be a general architecture for olfactory systems, as numerous insects display the same structure and *Drosophila* AL show remarkable similarities with the vertebrate olfactory bulb<sup>[129,130]</sup>. Nonetheless, in a sense, AL is a relay region, as glomeruli are located at the synapses between ORNs and AL output neurons called Projection Neurons (PN). Indeed, each glomerulus activity is transmitted to the next brain regions through an average of 3 PNs<sup>[131]</sup>. PNs then project onto two main regions, the Lateral Horn (LH), mainly involved in innate behaviour (which is why we won't further discuss its function in this study) and the Mushroom Body (MB), mainly involved in learnt response (although it is important to stress the fact that the distinction may not be so absolute, as highlighted by recent studies<sup>[132–135]</sup>). In any case, in the next chapters we will focus on MB, as this region is deeply involved in learnt processes as already mentioned in the chapter describing honeybee non-elemental learning abilities, and learning mutants in *Drosophila*.



## The Mushroom Bodies as integrative centres of associative learning

### Mushroom Bodies anatomy and first ties with olfactory learning in *Drosophila*

In 1850, Felix Dujardin describes for the first time an hymenopteran brain region that he compares to mushroom-like structures (*corpore pedunculata*)<sup>[136]</sup>. He postulates that these structures, currently known as Mushroom Bodies (MB) are the site of intelligence. Although that assumption would eventually be proven to be an overstatement, the characterization of MBs constituted a major discovery as they have been demonstrated to be involved in numerous processes, such as associative memory in bees<sup>[137]</sup>, cocoon spinning<sup>[138]</sup>, navigation<sup>[139]</sup> and even courtship memory<sup>[140]</sup>. MBs are composed of functional units called Kenyon Cells (KC, named after their discoverer<sup>[141]</sup>), which differ in number depending on the species (in cockroaches, MBs contain approximately 200 000 KCs while flies MBs contain ~2000 KC in each hemisphere). MBs show a specific anatomy with an input region called the Calyx that contains KCs' cell bodies which project axons through the pedunculus and branching into three regions, called the lobes that are disposed in a horizontal and vertical fashion, as shown in Figure 18.

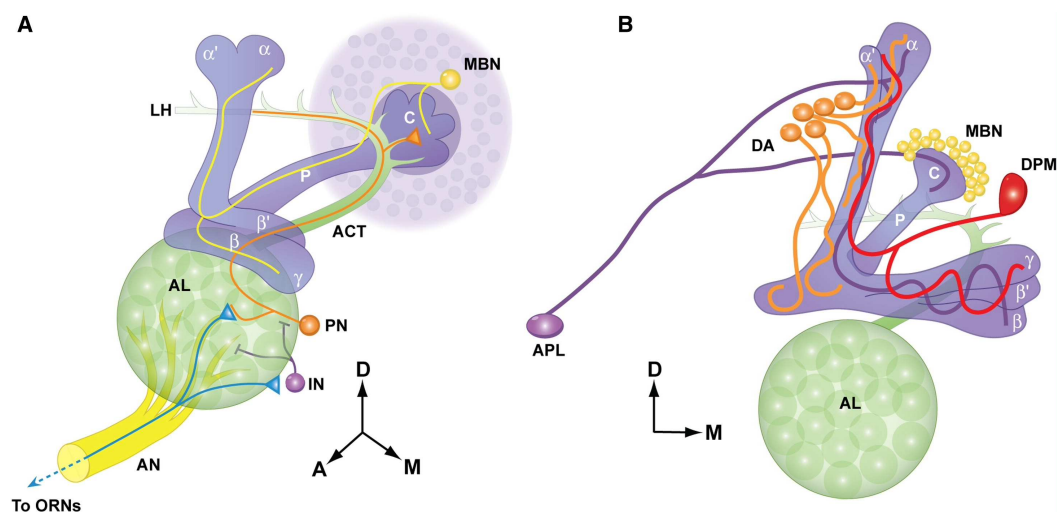


Figure 19 Schematic representation of the Mushroom Bodies (adapted from: [142]). A) General organization of the MB. Antennal Lobes (AL) are shown in green where glomeruli are visible, along with the lateral inhibitory neurons (IN). Olfactory information is conveyed from the AL to the MB through Projection Neurons (PN) which show a bifurcation and also project onto the Lateral Horn (LH). Kenyon Cells are represented as MBN (MB neurons). Their cell body is located in the Calyx (C) and their axons are projected through the Pedunculus (P) as MB lobes, which are classically divided in 5 subsets,  $\alpha$   $\alpha'$   $\beta$   $\beta'$   $\gamma$ . B) Representation of input and output neurons showing direct connectivity with the MB. Here are represented the DPM neurons in red, the APL neurons in magenta, the Dopaminergic Neurons (DA) in orange.



In 1985, Martin Heisenberg explored the role of MBs in associative learning in *Drosophila*<sup>[143]</sup>. To do so, he retrieved specific MB mutants obtained using the very same methodology used by Seymour Benzer (Mutagenic treatment), but instead of selecting the lines based on their learning abilities, he selected them based on highly localized MB defects (for instance, the mushroom body miniature mutant displays only small Calyx and almost non-existent MB lobes in female flies). Then, flies were tested in various learning paradigm, including the T-Maze protocol but also other associative learning like the arena paradigm described Figure 19. Heisenberg showed that MB impaired flies showed a deficit in olfactory learning, which could not be attributed to non-specific defects as mutant flies showed normal US (electrical shocks and sucrose) sensing, and CS (odourants) perception<sup>[144]</sup>. From this discovery onwards, MB and their direct neural partners have been thoroughly studied, in learning and memory, both appetitive and aversive.

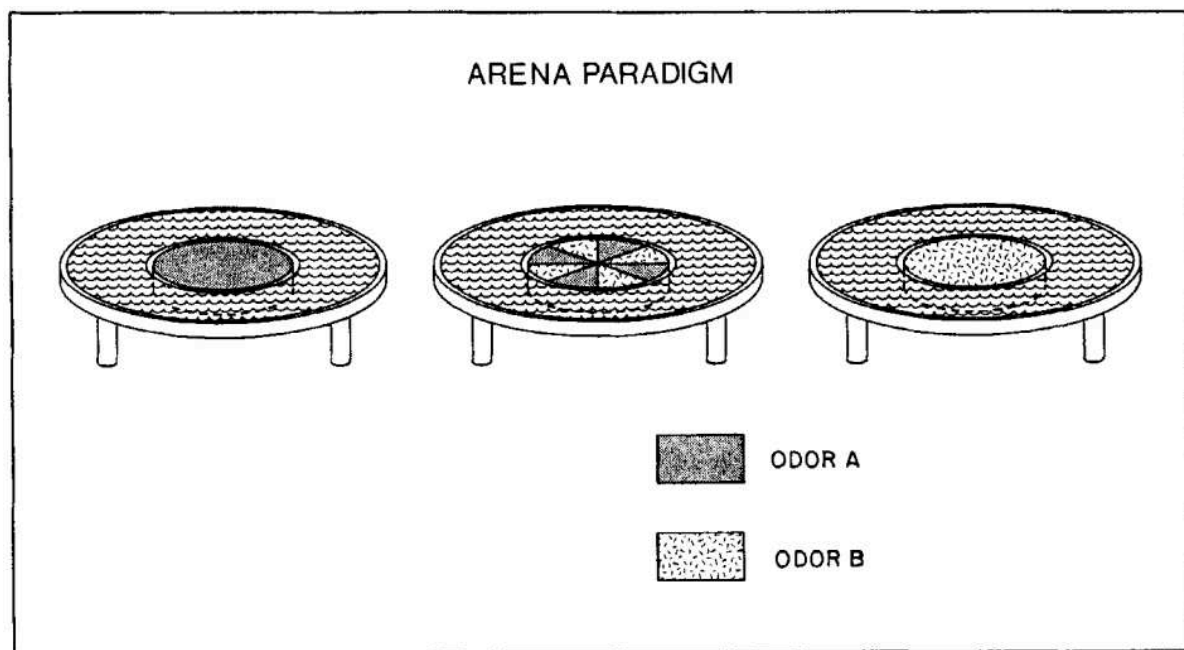


Figure 20 Schematic representation of the arena paradigm. two conditioning arenas are displayed left and right, each associated with a specific odourant. On one of these arenas, flies are exposed to a sugar reward, while in the other arena flies are left unrewarded. After the conditioning phase, flies are placed in the middle arena where they may navigate across 8 quadrants, alternatively displaying either the odourant previously encountered in the rewarded arena or the one that was never associated to a reward. Flies' time spent on each quadrant is recorded and compared between the two odourants to compute a learning index<sup>[143]</sup>.

## Kenyon Cells as convergence point between CS and US pathways

After a first processing in the ALs, olfactory signal is transmitted to the MB KCs via the PNs. Therefore, as each odourant may be characterized as a specific glomerular pattern within the ALs, it may also be characterized as a KC activity pattern within the MB. Importantly, whereas ORNs connectivity within the AL is deterministic and thus conserved across individuals, PN to KCs connectivity is suggested to be random<sup>[145]</sup>. This way, while odour representation in the MB is conserved within the same individual, it varies, in contrast, across individuals.

For any associative learning to occur, coincidence between CS and US must be detected. What mediates US transmission within the MBs? In seminal work, aversive and appetitive learning were shown to depend on two distinct neurotransmitters, Dopamine and Octopamine respectively. Indeed, selective knockdown of Tyramine $\beta$ -Hydroxylase, an enzyme required for Octopamine synthesis, resulted in impaired appetitive but not aversive learning performances while silencing of Dopaminergic neurons (DANs) impaired aversive but not appetitive learning<sup>[146]</sup>. However, more recent studies showed that octopamine signalling is also involved in aversive learning<sup>[147]</sup>, while appetitive reinforcement is also eventually mediated by DANs<sup>[148]</sup>. Indeed, MBs are surrounded by several subsets of DANs. One of these subsets, belonging to the PPL1 neurons cluster, has been demonstrated to mediate aversive reinforcement signalling to the MBs, while another subset, the PAM neurons, has been shown to mediate appetitive reinforcement signalling<sup>[149]</sup>. Thus, during olfactory associative learning, CS information is processed and mediated by the ORN, AL and produces a specific cell activity pattern within the MB. In parallel, US information is transmitted to the MB through subsets of DANs.

## About the not-so-powerful neurogenetical tools of fly scientists

The discovery of dopaminergic neurons as US transmission pathways provides a good example of the limits and caveats associated with *Drosophila* neuroscience. Why Dopamine was not shown to be involved in appetitive learning in the first place? In the first study mentioned<sup>[146]</sup>, Dopaminergic neurons were targeted using a Gal4 line called “TH-Gal4”. While TH-Gal4 marks a vast proportion of Dopaminergic neurons, its expression pattern does not include the PAM neurons. Thus, the authors concluded that dopamine was not involved in appetitive learning, without actually investigating the neurons required for appetitive reinforcement. While neurogenetical tools provide an unmatched precision in the exploration of neural circuits, one should always keep in mind that they are not perfect and few Gal4 lines target only the structures of interest (although this problem has largely been addressed with the conception of the “Split” gal4 lines<sup>[150]</sup>).

## Rutabaga, the molecular coincidence detector of CS / US association

In a previous chapter, we mentioned one of the first discovered associative learning mutants, rutabaga. We also brought up the idea that Rutabaga was, in fact, encoding a  $Ca^{2+}$  Calmodulin-responsive Adenylate Cyclase (CAM) within the MBs. Interestingly, the rutabaga encoded CAM (or Rut AC), has been shown to be sensitive both to CS and US stimulation: The CS pathway leads to an increase of intracellular  $Ca^{2+}$ , which induces Rut AC stimulation through a calmodulin-dependent signalling<sup>[85,89,90]</sup>. In contrast, the US pathway mediates Rut AC stimulation via the activation of G-protein coupled with Dopamine receptors<sup>[86]</sup>. Upon CS or US exposure alone, Rut AC is mildly stimulated. However, CS and US joint presentation causes a synergistic activation of Rut AC which secures associative learning.

What are the effects of Rut AC recruitment? First, as an adenylate cyclase, Rut AC produces cyclic Adenosine MonoPhosphate (cAMP), a well-known cellular messenger (in fact, Rut AC activity is measured by the amount of cAMP generated in response to its stimulation). cAMP is generally involved in plasticity related processes along with its target, the Protein Kinase A (PKA), both in vertebrates and invertebrates<sup>[152]</sup>, as highlighted by the remarkable work of Eric Kandel, which we already briefly mentioned in the box dedicated to non-associative learning. More specifically, cAMP/PKA pathway is involved in the modulation of synaptic connectivity, either strengthening them (a process called Long-Term-Potential, LTP) or weakening them (a process called Long-Term Depression, LTD).

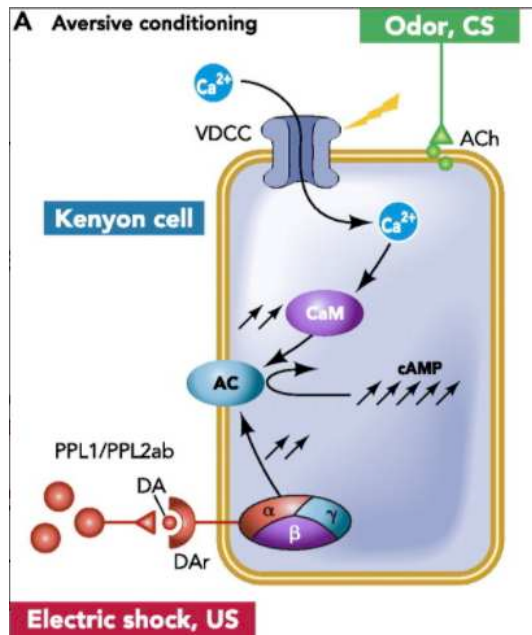


Figure 21 Schematic representation of CS + US synergistic activation of Rut AC through Calmodulin and G-protein stimulation (here shown in the case of aversive conditioning), adapted from: [151].

In a way, LTP and LTD may be seen as the molecular expression of the Hebbian rule. Back in *Drosophila*, once KCs associated with the CS+ have been reinforced, their synaptic connectivity has been modulated, which allows for a conditioned response even without the US.

*The Mushroom Body Output Neurons and the approach/avoidance balance*

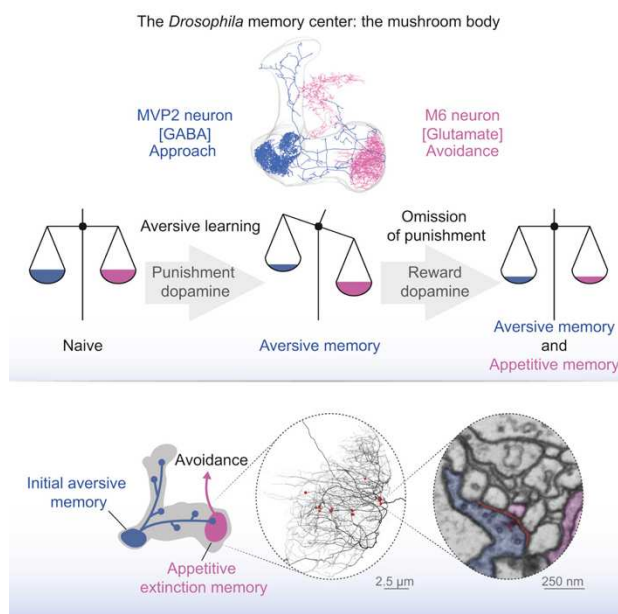


Figure 22 Integration of parallel and opposing memories during an extinction experiment. Figure from: [153]

Which synapses are modulated by olfactory learning? KCs axons project onto a small subset of neurons (34 neurons identified, classified in 21 types) called Mushroom Body Output Neurons (MBONs). MBONs have been shown to encode valence, as their optogenetic activation elicits either avoidance or approach<sup>[154]</sup>. Moreover, olfactory learning has been shown to induce synaptic plasticity at the KC-MBON synapses<sup>[155]</sup>. Interestingly, in a somewhat counterintuitive way, aversive learning do not drive synaptic potentiation of KC synapses with aversive MBONs, but rather leads to a depression of KC synapses with attractive MBONs<sup>[156,157]</sup>. Thus, MBONs collectively drive behaviour: naive flies show no learnt response as attractive and appetitive MBON activities are initially balanced. However, upon associative learning, MBON activity balance is skewed which leads to a learnt behavioural bias<sup>[158]</sup>. In a particularly inspiring work, authors nicely emphasize the parallel nature of *Drosophila* learning by training flies in an aversive learning paradigm (thus depressing approach MBONs and producing an aversive memory), which is followed by an extinction experiment (repeated presentation of the previously reinforced stimulus without reinforcement). Their results show that rather than erasing the previously formed memory, extinction leads to the formation of a parallel, opposing memory by depressing avoidance MBONs through the activation of reward DANs<sup>[153]</sup>. An illustration of this mechanism is displayed in Figure 21.

### Consolidated memories in *Drosophila*

Upon conditioning trials repetitions, an initially labile association may be consolidated through complex and interacting cellular processes. Interestingly, distinct MB compartments are involved in different phases of memory consolidation. For instance, early memory traces are detected in the MB  $\alpha'/\beta'$  lobes<sup>[159]</sup>. In contrast, long-lasting memory is shown to be rather located within the  $\alpha/\beta$  lobes<sup>[160]</sup>. In addition, distinct forms of memory are elicited depending on the conditioning paradigm applied. Training without breaks forms a memory called "Anesthesia Resistant Memory" (ARM) while training including 15 min breaks forms "Long Term Memory" (LTM)<sup>[161]</sup>. Interestingly, these distinct forms of memory have been demonstrated to be mutually exclusive<sup>[162]</sup>. ARM and LTM also depend on distinct characteristics. For instance, as LTM requires de novo protein synthesis, its formation is linked to sugar consumption<sup>[163]</sup>. Incidentally, ARM has been shown to be better retrieved when flies are tested in large groups, which is referred to as "social facilitation of memory"<sup>[164]</sup>. Finally, recent studies show that, similarly to learning processes, memory involves parallel and opposing valence memories enabling flies to store previously learnt information both about the CS+ and the CS- over fairly long periods of time<sup>[165,166]</sup>.

### *The APL neurons: A major actor in complex forms of learning?*

Beside the DANs involved in reinforcement coding and the MBONs steering behaviour based on learning events, the MB are also contacted by other neural populations involved in MB Input/Output interactions. For instance, the Dorsal Paired Median (DPM) neurons are required for memory consolidation<sup>[167]</sup>, and were recently found to be involved in the social facilitation of memory<sup>[168]</sup>.

In 2009, Liu & Davis identified another important subset of MB Input/Output neurons called the Anterior Paired Lateral (APL) neurons, which are found in only one copy per hemisphere, much like DPM neurons (Figure 18). APL were found to be GABAergic and showed an antagonist relationship with KCs. More specifically, Liu & Davis demonstrated that reducing GABA transmission from the APL to the KCs induced learning facilitation and in return, they showed that olfactory learning suppressed APL activity<sup>[169]</sup>. More recently, APL activity suppression was shown to be mediated by the DAN involved in aversive reinforcement signalling (PPL1) through D2-receptors located at APL membrane<sup>[170]</sup>.

Moreover, another team published a fundamental study showing that APL neurons provide an inhibitory feedback to the KCs, which leads to a normalization process not unlike what has been described regarding the lateral Inhibitory Neurons in the ALs<sup>[171]</sup>. Upon KCs activation by an odourant, APL neurons (which take input at the MB lobes) apply a graded GABAergic inhibition at the level of the calyx. As a result, only an average of the 8,4% most activated KCs remain active. This function is called “Sparse coding”, as each odourant is represented by a small subset of KCs. Hence, referring to how odours are discriminated (see the chapter on combinatorial odour code in the ALs), sparse coding increases the distance between odour representations at the level of KCs, ensuring a minimal overlapping of the KCs activation patterns associated with discriminated odours. The authors provided an experimental demonstration of this by silencing APL neurons during differential conditioning involving either similar or dissimilar odours. They showed that while APL silencing does not prevent discrimination of dissimilar odours, it does impair flies’ ability to discriminate similar odours (Figure 22).

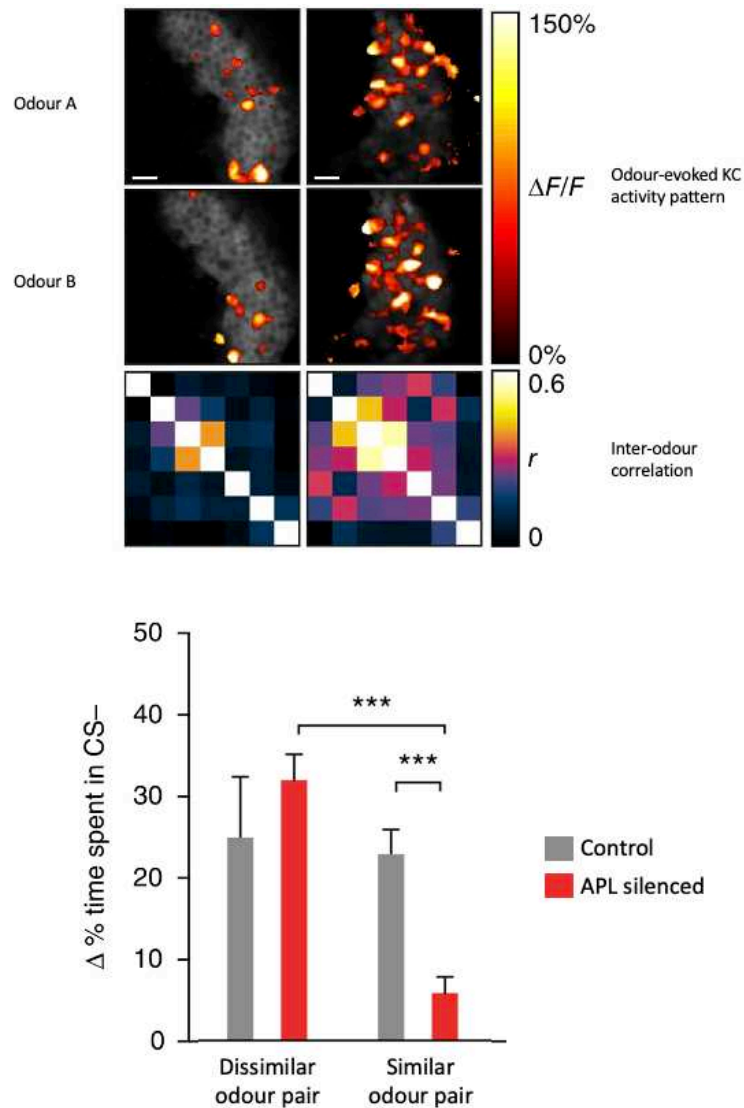


Figure 23 Effect of APL silencing on odour representation in the MB, their distance and associated discriminability (adapted from: [171]). Top: Imaging of KC activity patterns elicited by the presentation of two odour, A and B. Activity has been measured using calcium imaging (see methods for more details). Inter-odour correlation has been computed (which is inversely related to the distance between odour patterns). Bottom: Flies learning performances computed as the relative time spent in the CS- T-Maze arm compared to the time spent in the CS+ arm. Performances using dissimilar and similar odours were compared as well as data with functional and silenced APL neurons.

Importantly, MBs architecture and random connectivity with the PNs already provide a huge coding space for olfactory stimuli, compared to ALs. Indeed, each KC receives, in average, input from 7 PN. As there are 2000 KCs, this results in  $\sim 14\,000$  PN-KC contacts. As mentioned above, APL neurons ensures that each odourant activates  $\sim 8.4\%$  of all KCs (168 KCs)<sup>[172]</sup>. The range of possible resulting combinations is tremendous. However, assuming that discrimination performances are correlated with odour distance, how to reconcile APL sparse coding activity and its suppression by olfactory learning? One explanation is that flies

displayed behaviour is also correlated with stimulus perceived salience, regardless of the animals' discrimination abilities. In other terms, for a given stimulus, flies response is correlated with the number of KCs that will drive the MBONs activity. Thus, optimal learning performances requires a delicate balance between producing a robust response to the CS+ and being able to distinguish it from the CS-

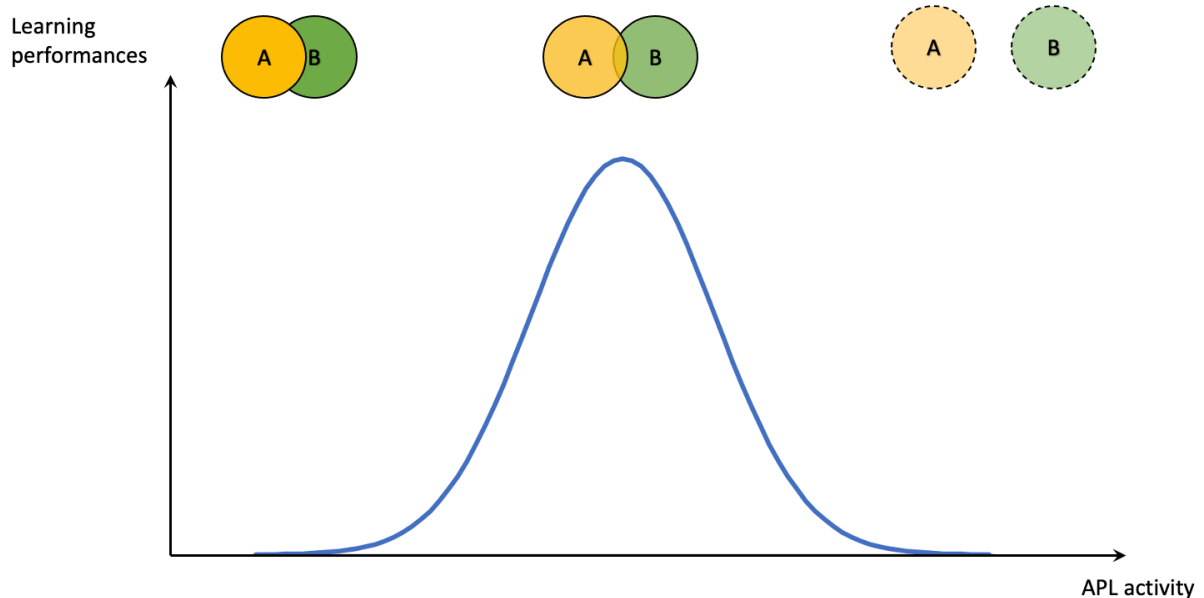


Figure 24 Illustration of the APL-mediated sensitivity / discriminability balance. A and B stimuli are represented as coloured circles. Colour transparency reflect sensitivity while circles overlapping level represents discriminability. Low APL activity leads to higher stimulus-elicited KC activity, but stimuli evoked patterns show significant overlapping, preventing discrimination. In contrast, high APL activity leads to an optimal discriminability of A and B at the expense of response strength.

In the work describing suppression of APL activity by olfactory learning, odours tested are significantly dissimilar. One may assume that in that case, inhibiting APL neurons should not impair their discriminability while facilitating the resulting stimulation of MBONs through higher KC activity. However, little is known about APL activity modulation during a learning task involving similar stimuli. We addressed some aspects of it in the chapters II and III of the results parts. As already mentioned, when generalization experiments were presented, another important part of learning in animals is their ability to display learnt behaviour outside the context in which learning occurred. From that perspective, if an animal encounters a stimulus that is fairly similar to one already associated with reinforcement, there is a fairly high probability that these stimuli are one and the same, with small distance, concentration and context variations. Thus, the appropriate response toward this stimulus is the generalization response. In that case, excessive discrimination might even be detrimental.



Finally, APL neurons may provide a baseline suppression of associative learning in order to prevent the animal from learning irrelevant associations. In that case, APL neurons suppression upon actual CS/US pairing would lift the baseline inhibition on the KC and enable associative learning.

APL neurons seem to be a highly-conserved neural structure among insects. In fact, first depictions of such neuron occurred in locusts, where it is called “Giant GABAergic Neuron” (GGN), referring to the large size of the GGN projections onto the MB<sup>[173]</sup>. Interestingly the GGN has also been shown to be crucial for sparse coding through a GABAergic feedback<sup>[174]</sup>. Moreover, an APL equivalent is also found in honeybee, where it is called “A3v” neurons<sup>[175–177]</sup>. Previous studies in the lab showed that A3v neurons are specifically required for non-elemental tasks learning, as their pharmacological ablation was shown to impair Reversal learning (A+ B- => A- B+) and Negative Patterning (A+ B+ AB-) abilities in bees<sup>[36,178]</sup>. This constitutes another similarity with *Drosophila*, as APL neurons have also been demonstrated to be required for reversal learning, both in olfactory and visual modalities<sup>[179,180]</sup>. APL requirement for reversal learning suggests that APL activity might mediate the suppression of the first associative learning in order for the animal to learn the new rule, which is consistent with previous observations about APL inhibitory activity. Furthermore, as A3v neurons are required for Negative Patterning, APL neurons is also suspected to be involved in non-elemental learning in *Drosophila*.

## En route to the study of non-elemental learning in *Drosophila*

The present overview of associative learning and its neural correlates in insect and more specifically in fruit flies constitute a good proof that *Drosophila* is a particularly powerful experimental model for the study of learning and memory processes on multiple scales. However, in order to explore the neural mechanisms of a given behaviour, said behaviour has to be characterized in the model of interest. As mentioned before, so far, Negative Patterning in insect had only been identified in honeybee. In contrast, previous attempts at showing Negative Patterning in flies were proven unsuccessful<sup>[181]</sup>. Interestingly the same authors built an elegant computational model of non-elemental olfactory learning in *Drosophila* and

showed that, in principle, flies should be able to solve Negative Patterning<sup>[182]</sup>. Indeed, in their model, the compound AB representation closely matches the one described in imaging studies<sup>[123]</sup> and follows a kind of unique cue processing, displaying similar features with its components, A and B, but also compound specific patterns. The authors suggest that the compound specific patterns, although in minority, could be sufficient for flies to discriminate AB from A and B. Are flies able to solve the so-called complex learning task of Negative Patterning?

In this study, we explore the question of non-elemental learning in *Drosophila* on multiple scales, from behaviour to neural networks and molecular correlates. Our work is divided in three independent projects, two of which are either published (chapter I) or about to be submitted to a peer-reviewed journal (chapter II).

In a first part, we develop a variation of the classical T-Maze protocol designed to implement a reliable Negative Patterning training in flies. Using this protocol, we demonstrate for the first time that flies are able to solve Negative Patterning discriminations and proceed to explore how flies process the compound during such task and the behavioural specifics associated with Negative Patterning (and an alternative paradigm, the Negative Feature discrimination).

In a second part, we build a minimal computational model of olfactory learning in *Drosophila*. Initially based on already established attributes of flies learning circuits, we take advantage of the modular nature of our model to test potential neural mechanisms that could qualitatively explain the behavioural results obtained on wild-type flies solving ambiguous forms of learning tasks. In particular, we explore the possible role of APL neurons in non-elemental learning acquisition. Next, we manipulate APL activity using neurogenetical tools and test the validity of our model's predictions *in vivo*.

In a third part, we explore APL role beyond non-elemental learning, in learning paradigms involving stimuli of varying similarities. We discuss the fundamental role of differential conditioning in the modulation of stimulus representation, depending on the task at hand.



# General methodology for the exploration of Drosophila learning and memory

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As each part of our work is independent and represent a separate project, detailed methodology specifically related to each part can be found in a separate Material & Methods part in each chapter. Yet, core methodology we use is the same throughout the three projects and thus, the basic tenets of the behavioural experiments and neurogenetic tools used are described below.

## Fly lines stocks holding conditions

Flies are kept at constant temperature of 25°C and relative humidity of ~60%, under a 12: -12h light: -dark cycle. Flies are kept in 36x82 mm plastic tubes containing approximately 20mL of medium, the composition of which is detailed in Annex4.

Fly lines used are outcrossed to a Canton-Special (CS) genetical background, so that wild-type and transgenic flies all share the same background. The principle of outcross is as follows: all transgenic fly lines are in a white-eyed genetical CS background. Eye pigments are restored by a sequence directly provided by the transgene of interest. That way, it is possible to identify flies carrying the transgene based on their eyes colour. One copy of the transgene usually (although not always) leads to orange eyes while two copies lead to the wild-type red eyes. Newly received transgenic lines are crossed with white-eyed flies that display a CS genetical background. That way, the next generation of flies inherit from both the transgene and some of the CS genetical background. By crossing again flies carrying the transgene with white CS flies multiple times, we generate fly lines of increasing CS genetical background that also carry the transgene of interest. That way, we ensure that any measured behavioural variability between control and transgenic flies can only be attributed to the transgene. This process is illustrated below:

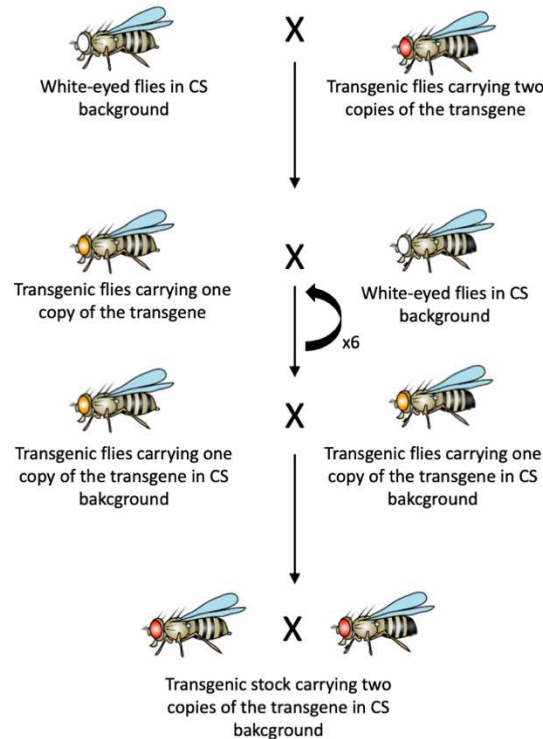


Figure 25 schematic of the outcross procedure. Flies phenotypes were generated using the Roote & Prokop open package<sup>[183]</sup>.

## High-throughput olfactory conditioning using a semi-automated procedure

All of our experimental work is based on pavlovian conditioning using an aversive modality. The goal of our protocol is to lead flies to associate one odour (CS) with an aversive reinforcement (US) and test their discrimination ability when given a choice between the reinforced odour (now CS+) and another odour that has been consistently presented without reinforcement (CS-). One of the benefits of *Drosophila* is its rapid generation rate, which enables a high-rate of experimental data production. However, all experiments have to be performed in tightly controlled conditions. That is why we use a barrel-shape device adapted from previous studies, which is specifically designed for simultaneous training of up to 6 groups of animals<sup>[184]</sup>.

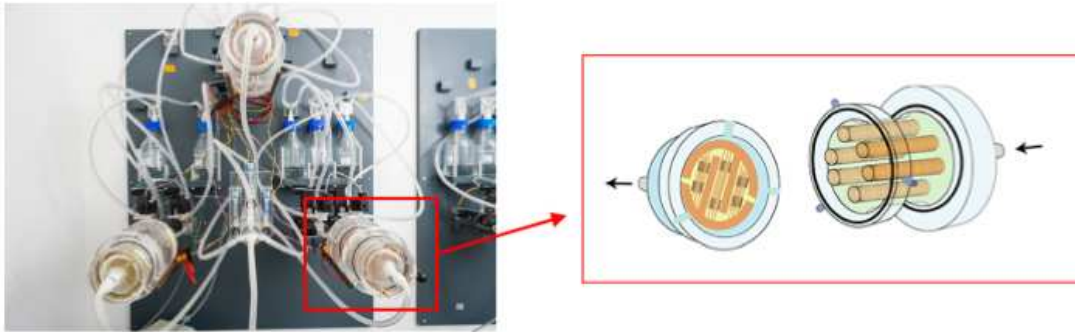


Figure 26 Real (left) and illustrated (right) view of the semi-automated conditioning barrels. Left picture: ©Cyril FRESILLON/CRCA /CNRS' photo library , right: adapted from: [184].

US are 60V electric shocks applied through copper or gold grids. CS are odours diluted in mineral oil. Odour stimulations are continuous, 2L/min air flow delivered using vacuum pumps. A conditioning protocol typically includes 1.30min of acclimatation followed by 1min of one CS associated with 12 pulses of 1.5s electric shocks every 5s. After 1min break, the second CS is presented without shocks for 1 min. The whole procedure is automated using a custom generator designed in the lab by P. Arrufat. The same procedure may be adapted for an appetitive conditioning by replacing the metallic grids with partly sugar-coated tubes in which flies are alternately exposed to and moved away from the food source by stainless steel rotating mechanisms (adapted from: [185]). Conditioning cycles may be repeated, and every parameter modulated. However, in this study, US and CS exposure durations and intensity are always the same.

## Learning performances assessment: the T-Maze test

After conditioning, flies are placed in a T-Maze where they may freely move between two compartments filled with air flows of 0.8 L/min of either the CS+ or the CS-. After 1min, flies in each compartment are isolated and counted. Learning performances are computed as follows:

Flies in CS- compartment – Flies in CS+ compartment

Total number of flies

Thus, flies Performance Index (PI), ranges from -1 to 1, with a chance level at 0. An example of test result is displayed below:

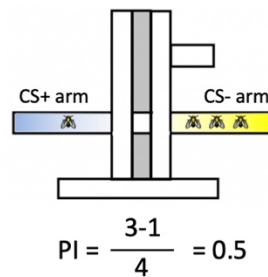


Figure 27 Example of T-maze post-test flies' distribution and associated Performance Index (PI).

Importantly, the statistical robustness of the protocol is ensured by the large number of flies tested together. Typically, a group of flies consists of ~30 flies that come from the same stocks, in the same tubes, are the same age. Each group is trained and tested together.

## Experimental bias control: Classical vs. Paired/Unpaired procedure

The procedure described above only enables reliable learning performance assessment under a crucial assumption: outside of the scope of an associative learning, flies shouldn't show any preference for one side or the other within the T-Maze. To make sure that is the case, all experiments are performed under red light (which flies are not supposed to see) in order to avoid phototaxis effects and odour concentrations are balanced so that any innate preference is neutralized. Despite these precautions, a perfect balance is almost never obtained in everyday conditions. Thus, an additional control is performed to account for this experimental bias.

In the original procedure, Tully & Quinn controlled for experimental bias by performing half the experiments with one of the odours as CS+ (A+B-) and the other half with the second odour as CS+ (B+A-). All trained flies were tested in the same conditions so that any bias underestimating a learnt avoidance toward A would be balanced by an underestimated learnt avoidance toward B<sup>[83]</sup>. A single PI would be computed as the average PI of two opposing PIs. This historical method was subsequently used in a vast majority of olfactory conditioning papers. However, that kind of experimental layout can only be considered if A and B are analogous (in terms of attraction/repulsion) so that conceptually, learning to avoid one is the



same as learning to avoid the other. In contrast, our study is focused on learning paradigms such as Negative Patterning which involves non-equivalent stimuli. Indeed, A+B+AB- is not equivalent to AB+ A- B-, which is a distinct behavioural paradigm called Positive Patterning with different features<sup>[186,187]</sup>. In addition, while A vs B concentrations are balanced, AB vs A or B is not, which justifies even more the need of a bias control. Adjusting AB concentration to A and B would be possible as it didn't seem to change Negative Patterning in bees<sup>[70]</sup>, but it would introduce a supplementary and unnecessary variable (odour concentration influence on NP resolution constitutes, in fact, a question per se, which is out of the scope of our work). Moreover, as explained above, it does not fully prevent experimental bias even in simple differential conditioning. This is why we developed an alternative procedure called Paired/Unpaired protocol.

The first part of this protocol, called the Paired procedure, is the exact same conditioning procedure detailed above (A+ B-). In order to assess experimental bias, for each group of flies exposed to a Paired procedure, we train another group of flies from the same stocks and lines in an Unpaired procedure during which they were exposed to the US and CS, all of which in an unsynchronized way to prevent any association between them (+ A- B-). After training, flies from Paired and Unpaired procedures are tested sequentially on the same T-mazes in the same conditions. This time, each replicate consists of a relative PI computed as the difference between a Paired PI and its Unpaired control. Paired and Unpaired procedures are summarized below:

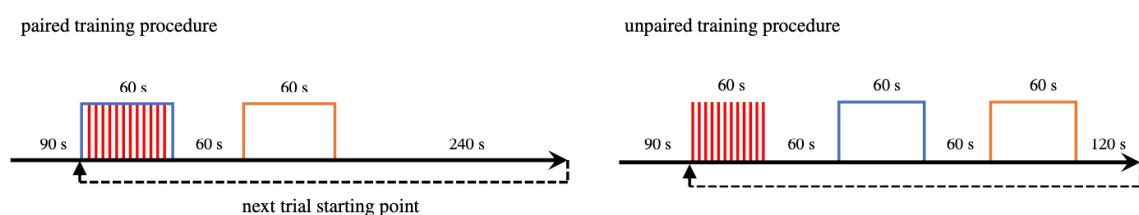


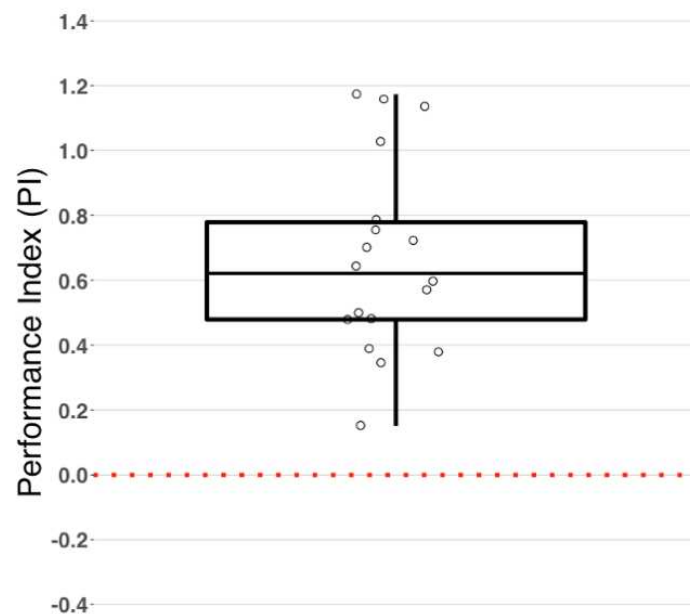
Figure 28 Typical Paired (left) and Unpaired (right) procedures used throughout this study. Orange and blue boxes are the two odours whereas red bars represent electric shocks. Adapted from: [188].

Paired/Unpaired procedure is specifically designed to quantify the effect of an associative learning experience on flies' behaviour in both balanced and imbalanced settings. While the first chapter is focused on behaviour of wild-type flies, we combined this protocol with the UAS/Gal4 system described in the Introduction to target subsets of neurons potentially involved in ambiguous learning and their molecular machinery in the second and third

chapters. Although numerous techniques were developed in drosophila, we will only focus on those that are exhibited in this study.

## Statistical analysis and representation of learning performances data

All data are analyzed and represented using R-software (version 4.0.2). All behavioural data are represented as boxplots, in which the middle line represents the median, while the upper and lower limits of the box are the 25 and 75% quantiles. The whiskers are the maximum and minimum values of the data that are, respectively, within 1.5 times the interquartile range over the 75<sup>th</sup> percentile and under the 25<sup>th</sup> percentile. Raw data are always superimposed as jittered dots. Data are plotted using the following packages : ggplot2, ggsignif, ggpubr, ggthemes, gridExtra. An example of typical boxplot is represented below :



*Figure 29 Relative performance indices of flies trained with a Differential Conditioning paradigm. Red dotted line represents chance level.*

It is important to note that as they are computed as the difference between two performance indices, each ranging from -1 to 1, the relative performance indices range from -2 to 2, which is why some data points exceed 1.

Datasets are analysed using the following packages: AICmodavg, lme4, Emmeans. Different statistical strategies depending on how many groups are compared and what is the statistical question. Generally, it is important to stress the fact that we mainly performed parametric statistical tests, the validity of which depend on two critical assumptions : the error between the computed statistical model and actual data have to follow a normal distribution and samples' variance should be homogeneous (which is called homoscedasticity). It is often recommended to formally test these assumptions using, for instance, the Shapiro-Wilkinson's normality test or the Bartlett's homoscedasticity test. However, this methodology is often contested as it is proven ineffective and logically flawed<sup>[189,190]</sup>. Indeed, inferential statistics are built to detect deviations from null hypothesis but not the other way around. In other words, failing to find a significant difference between experimental data and a theoretical normal distribution does not prove that the data are normally distributed. Thus, in this study, we mainly rely on graphical validation of normality and variance assumptions, although we also check the output of aforementioned test as an additional precaution.

One-sample analyses are performed to compare learning performances with chance level (0). To do so, data normality is assessed by fitting a normal distribution on experimental data distribution. Normality is also check using the Shapiro-Wilkinson's normality test.

Two sample analyses are performed to test for instance the effect of training repetition within a given learning paradigm. In that case as well, data normality is tested the same way as for one sample analyses. Then data are compared using two-samples two tailed T.tests. In addition, potential heteroscedasticity is tested using Bartlett's test and accounted for, if necessary, using a Welch's adjustment.

Statistical analysis of more than two samples within the same paradigm are performed to test for instance the effect of a given transgene. Indeed, UAS/Gal4 targeted transgenes effects are always compared to the effect of the UAS and Gal4 constructs alone, using fly lines, the genotype of which includes either UAS, Gal4, or both. which leads to 3 groups to be compared together. In that case, we use Analyses of Variance (ANOVA). First, we build several relevant statistical models including various explanatory variables we gather during experiments (e.g., odorants used) and their interactions. Then these models are compared using the Akaike information criterion (AIC) which is an estimator of the relative quality of statistical models

based on their goodness of fit with the experimental data and their simplicity. AIC of each model are ranked and the best model is the one displaying the lowest AIC. Next, we further test the validity of the selected model using diagnostic plots that compare actual and model-fitted data including distribution and variance. An example of diagnostic plot is displayed below.

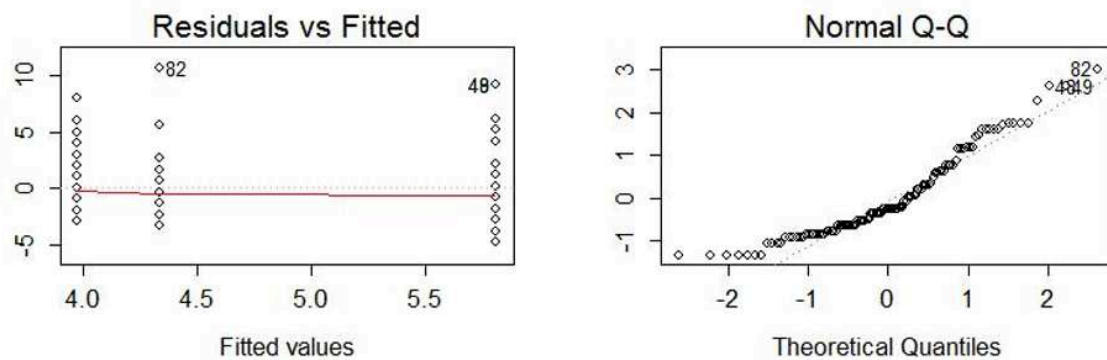


Figure 30 Typical diagnostic plots for linear models' validation.

For instance, the left plot shows the variance of residuals vs. fitted values. Each series of vertically disposed dots represents a statistical group. Here we can check if all groups show homogeneous variances. Similarly, normality can be assessed using the right plot, called Q-Q plot. Data are considered as normal if there is little deviation between dots representing experimental data and a perfect normal distribution represented as the dashed line. In addition, Normality and variance may also be tested using Shapiro-Wilkinson's normality test and Bartlett's tests, respectively. The ANOVA results are checked to identify global effects statistics, for instance the effect of genotype. If a significant effect is detected, the next step is to investigate which groups differ from which. Two strategies are possible :

If all groups are to be compared, the ANOVA results are checked. If a significant effect of flies' genotype is detected, we perform a Tukey Honestly Significant Difference test (or Tukey's HSD), which is a post-hoc test designed to assess pairwise comparisons significance between all groups compared.

In other cases, some comparisons are irrelevant while other are crucial. Performing a post-hoc test in such case is not appropriate as adjustments for multiple comparisons come at the expense of statistical power. In such situation, relevant comparisons are planned before the analysis, which reduces the weight of adjustment needed and mitigates its detrimental effect.

In all cases, a significance level of  $p < 0.05$  is set, although p-values along with relevant statistics are always reported, for more parsimonious interpretations.

## Shibire: a temperature controlled neural inhibitor

Neuronal chemical signalling through synapses is sharply timed. To ensure that, neurotransmitters need to be rapidly released within the synaptic cleft, but also to be efficiently cleared after exerting their function. One of the mechanisms responsible for neurotransmitters removal is their endocytosis back into the pre-synaptic neuron. In 1991, two studies describe the role of a specific Dynamin called Shibire in neurotransmitters endocytosis<sup>[191,192]</sup>.

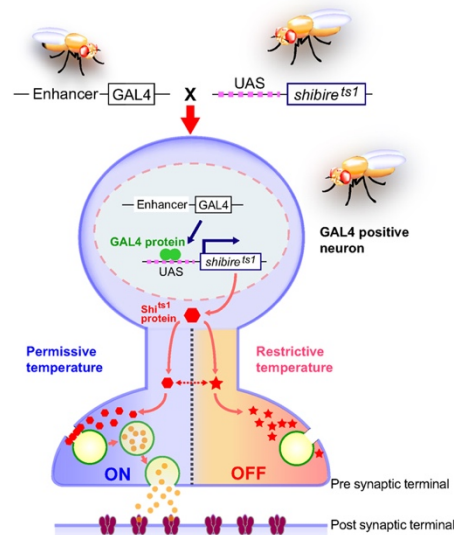


Figure 31 Schematic representation of *shibire<sup>ts</sup>* mediated manipulation of synaptic neurotransmission, adapted from: [193]

Ten years later, Toshihiro Kitamoto develops a methodology using a temperature-sensitive variant of Shibire to manipulate synaptic transmission<sup>[194]</sup>. Indeed, the ectothermic nature of flies makes it possible to alter *shibire<sup>ts1</sup>* function simply by placing flies at the restrictive temperature (in our case, 33°C) for ~10 min (specific timings are detailed in the chapters). Importantly, moving flies back at the permissive temperature of 25°C for ~10 min causes *shibire<sup>ts1</sup>* to recover a normal function. By combining *shibire<sup>ts1</sup>* with the UAS/Gal4 system, it is possible to selectively inhibit neurotransmission of small subsets of neurons in a time-controlled and reversible way.

## Molecular targeted knockdown through RNA interference

Shibire targeted neural activity inhibition is a powerful way to pinpoint neural subsets involved in the behaviour of interest. However, complementary approaches are required for the characterization of the underlying molecular pathways, such as which neurotransmitters are released, or whether a particular molecular messenger is involved. One possible strategy is to induce a selective knockdown of the candidate expression through RNA interference. Indeed, proteins are built based on molecular blueprints, the messenger RNA (mRNA) sequences directly translated from DNA coding regions. Interestingly, mRNA regulation involves, among other, the expression of other RNA molecules that bind to mRNA, triggering RNA degradation pathways. Here again, it is possible to take advantage of an existing natural molecular machinery to manipulate the expression of a targeted protein. By driving an interferent RNA in a subset of neurons using UAS/GAL4 system, the expression of the corresponding protein may be knocked-down. In addition, a thermosensitive control may be added using Gal80<sup>ts</sup> already described in introduction in order to restrict the RNAi expression to the adult stage and thus, avoid potential developmental effects due to a constitutive knock-down. It is important to note that Gal80<sup>ts</sup> has a reduced temporal resolution compared to Shibire<sup>ts</sup>, as RNAi induction requires flies to be placed at 30°C for 5 days before the experiment.

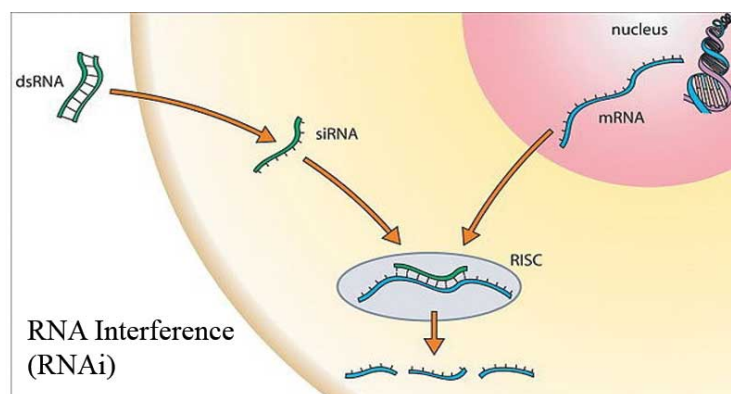


Figure 32 Illustration of RNA interference mechanism, adapted from: [195]



# Chapter I: Fruit flies can learn non-elemental olfactory discriminations

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## Research



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**Author for correspondence:**

Guillaume Isabel

e-mail: [guillaume.isabel@univ-tlse3.fr](mailto:guillaume.isabel@univ-tlse3.fr)

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# Fruit flies can learn non-elemental olfactory discriminations

Matthias Durrieu<sup>1</sup>, Antoine Wystrach<sup>1</sup>, Patrick Arrufat<sup>1</sup>, Martin Giurfa<sup>1,2,3</sup> and Guillaume Isabel<sup>1</sup>

<sup>1</sup>Research Centre on Animal Cognition, Centre for Integrative Biology, CNRS, University of Toulouse, 118 route de Narbonne, F-31062 Toulouse, France

<sup>2</sup>College of Animal Science (College of Bee Science), Fujian Agriculture and Forestry University, Fuzhou 350002, China

<sup>3</sup>Institut Universitaire de France (IUF), Paris, France

MD, 0000-0003-3675-1140; AW, 0000-0002-3273-7483; MG, 0000-0001-7173-769X; GI, 0000-0003-1312-9826

Associative learning allows animals to establish links between stimuli based on their concomitance. In the case of Pavlovian conditioning, a single stimulus A (the conditional stimulus, CS) is reinforced unambiguously with an unconditional stimulus (US) eliciting an innate response. This conditioning constitutes an ‘elemental’ association to elicit a learnt response from A<sup>+</sup> without US presentation after learning. However, associative learning may involve a ‘complex’ CS composed of several components. In that case, the compound may predict a different outcome than the components taken separately, leading to ambiguity and requiring the animal to perform so-called non-elemental discrimination. Here, we focus on such a non-elemental task, the negative patterning (NP) problem, and provide the first evidence of NP solving in *Drosophila*. We show that *Drosophila* learn to discriminate a simple component (A or B) associated with electric shocks (+) from an odour mixture composed either partly (called ‘feature-negative discrimination’ A<sup>+</sup> versus AB<sup>-</sup>) or entirely (called ‘NP’ A<sup>+</sup>B<sup>+</sup> versus AB<sup>-</sup>) of the shock-associated components. Furthermore, we show that conditioning repetition results in a transition from an elemental to a configural representation of the mixture required to solve the NP task, highlighting the cognitive flexibility of *Drosophila*.

## 1. Introduction

The ability to form a link between meaningful events is the cornerstone of associative learning. One of the most studied forms of associative learning is Pavlovian conditioning, where animals learn a conditional relation between a conditioned stimulus (CS) and an unconditioned stimulus (US) [1]. Several protocols can be used to study Pavlovian conditioning. One of them is differential conditioning (DC), which enables the study of how animals learn to associate different CSs with different outcomes (e.g. a reinforced stimulus A<sup>+</sup> from a non-reinforced stimulus B<sup>-</sup>) [2]. In this case, each CS is associated with a distinct US through a simple, non-ambiguous link so that they are learnt independently of each other. In consequence, this type of learning is also referred to as ‘elemental learning’ [3,4].

However, stimuli in nature may not appear as isolated, distinct elements. Usually, they are compounds constituted of multiple elements. For the elemental learning theory, a compound AB is the linear sum of its elements. In other words, an animal presented with AB would learn the independent relation of A and of B with reinforcement. Yet, several compounds with shared elements and different outcomes could coexist, creating thereby ambiguity at the level of the single elements. This possibility led to the proposal of the configural

(non-elemental) theory, which states that a compound is not learned as the linear sum of its components but as a distinct configuration in which elements would not be fully recognized [5–7]. The validity of this hypothesis has been studied using a discrimination task termed ‘negative patterning’ (NP) in which a subject has to learn to respond to two single elements reinforced ( $A^+$ ,  $B^+$ ) and to inhibit its response to their non-reinforced compound ( $AB^-$ ). For the elemental learning account, solving this task is impossible as animals reinforced on A and B should respond twice as more to AB. By contrast, for the configural account, NP learning is possible as AB would be perceived as a configuration different from A and B, thus facilitating differentiation [5]. Other studies explored alternative explanations for NP solving and argued that the compound AB would be perceived as the sum of A and B plus a unique cue specific to the AB compound (therefore termed ‘unique cue theory’) [8]. Within this framework, joint presentation to the animal may result in perceptual interferences such as overshadowing, which suggests that in that case, only part of A and B are perceived within the compound [9]. In either configural or unique cue theory, animals would learn the NP task by focusing on an unambiguous compound-specific cue.

Another task that has received wide attention among learning scholars is the ‘feature-negative discrimination’ (NF) in which subjects learn to respond to a single element reinforced ( $A^+$ ) but not to a non-reinforced compound  $AB^-$  [10]. NF is interesting as it admits both elemental and configural explanations: for the elemental theory, focusing on B (the negative feature) brings an elemental solution to the problem. As stimulus A has an ambiguous valence, being as often reinforced and non-reinforced, B alone suffices as a conditioned inhibitor to respond appropriately. For the non-elemental theory, discrimination is straightforward as the compound AB is a configuration that is unrelated to its elements [10,11].

Studying if and how animals solve the NP and NF discriminations is important to access the mechanisms of associative learning in the animal brain. Given the importance of invertebrates for studies on learning and memory [12,13], research on lobsters [14] and honeybees [15–17] has focused on the capacity of these animals to learn these discrimination problems. In other insect species, other forms of non-elemental learning such as multimodal NF [18], biconditional discrimination [19,20] or contextual learning [21] have been described, yet the solving of NP has remained circumscribed to the honeybee. Attempts to study these phenomena in the fruit fly *Drosophila melanogaster*, an insect model that is used recurrently for studying elemental learning forms [22,23], have failed [24]. This lack of success is regrettable as the fruit fly offers a vast spectrum of neurogenetic tools, which would allow comparing circuits and mechanisms underlying elemental and non-elemental forms of learning. This approach has allowed the olfactory pathways and learning circuitry of the fly to be identified, both from an anatomical (a full connectome of these circuits has been published recently [25]) and from a functional perspective [23]. Robust computational models based on said circuits have been developed and predict that non-elemental learning should be achievable by fruit flies, as normalization events at the perceptual level support the emergence of configural representation of compound stimuli [26]. Thus, studying non-elemental learning from a mechanistic perspective in the fly represents an attractive and accessible goal.

Here, we achieved the first demonstration of the fruit fly’s capacity to solve NP and NF in the olfactory domain, thus

showing that beyond simple discrimination learning, flies can also solve non-elemental discriminations. In showing this capacity, we determined the associative strategies used by the insects and demonstrated that the processing of olfactory compounds moves along a continuum between elemental and configural processing. Increasing the number of conditioning trials promoted configural processing, enabling flies to solve the NP task.

## 2. Material and methods

### (a) Fly rearing

The wild-type line used in this study was a *Canton-Special* (*Canton-S*) strain. Flies were raised on standard medium at 25°C, approximately 60% humidity and a 12:–12 h light:–dark cycle. The flies were kept in 36 × 82 mm plastic tubes containing approximately 20 ml of medium.

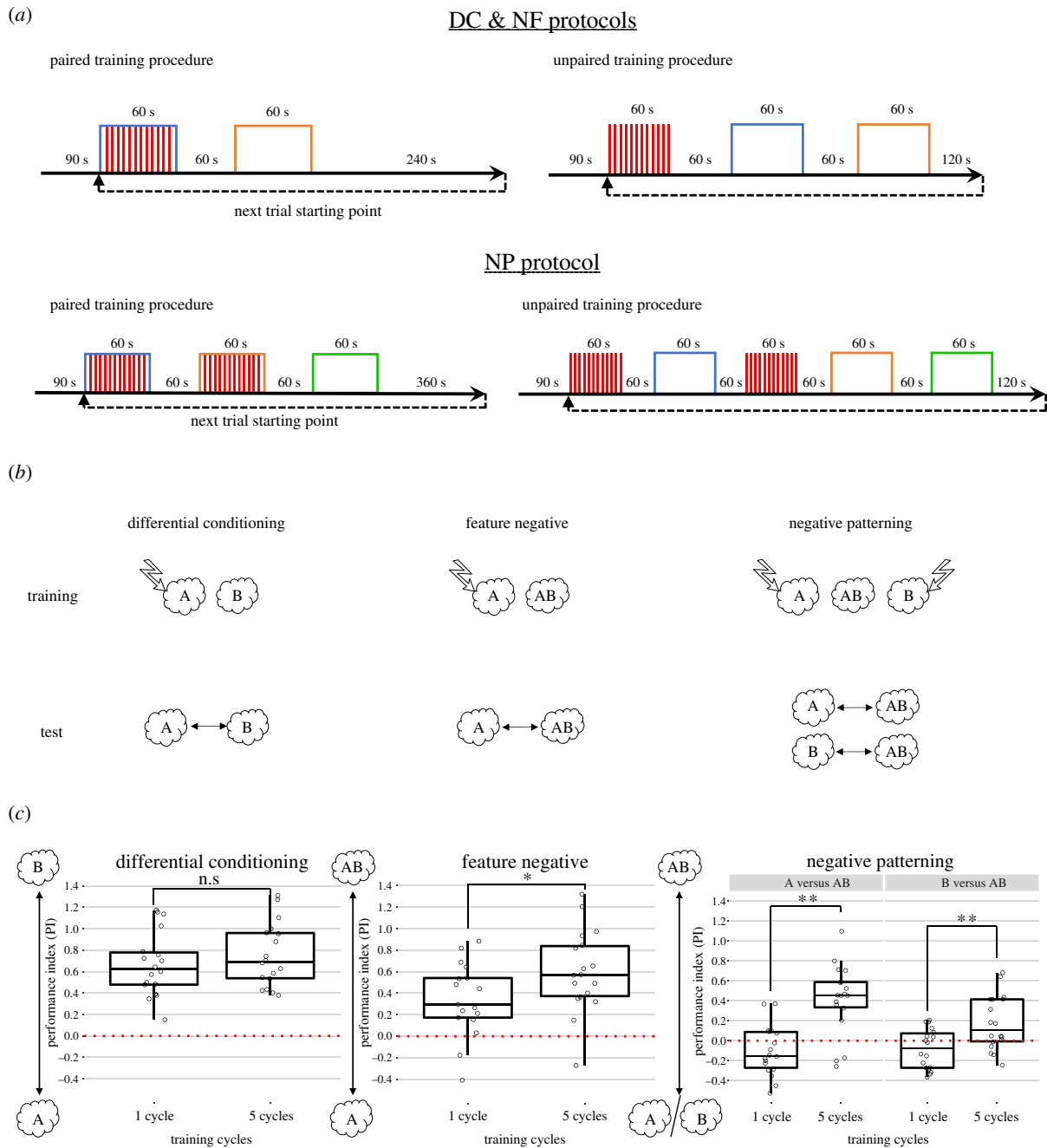
### (b) Olfactory conditioning

Odours were diluted in bottles of mineral oil. Odours used were 3-octanol (termed ‘A’ for the sake of simplicity, 2.27 mM) and 4-methylcyclohexanol (‘B’, 2.62 mM) (figures 1 and 2). Benzaldehyde (‘C’, 1.89 mM) was used as a novel odour in some conditions (figure 2). Isoamyl acetate (D) and ethyl butyrate (E) were used as alternative odours (electronic supplementary material, figure S2) to determine if discrimination solving was independent of the pair of odours used. Odours were delivered at the same concentration whether as components (A, B, D or E) or as mixtures (AB or DE). In the case of mixtures, odours were diluted together in the same bottle of mineral oil. All odours and the solvent were from Sigma Aldrich (France). The US consisted of 12 pulses of 1.5 s of 60 V electric shock every 5 s delivered through a metallic grid. Each experiment included two groups of approximately 30 flies (2–4 days old) and was performed using a semi-automated device based on a previous work [27]. In a T-maze, two main phenomena drive the preference of flies towards a compartment or another: on the one hand, the learnt information about the stimuli acquired during conditioning and on the other hand, the fact that odours are rarely completely neutral; at the concentrations used in our work, they are in fact repulsive to naive flies. When two odorants are opposed in the absence of punishment in the T-maze, repulsion balances; yet, if one odorant is opposed to a compound, which is more repulsive, a bias towards the less repulsive stimulus is visible. To disentangle learning from non-learning behavioural components, one of the groups experienced an explicit pairing of CS and US (paired group), while the other group experienced both stimuli unpaired to prevent their association (unpaired group).

### (c) Training

Each training trial consisted of 90 s of acclimatisation, after which flies were subjected to their respective conditioning protocol. Each odour (CS) was presented once for 1 min with an intertrial interval of 1 min. For the paired group (figure 1a), one or two of the olfactory stimulus ( $CS^+$ ) was paired with the US, while the other stimulus ( $CS^-$ ) remained unpunished. In the unpaired group, flies were exposed to 1 min of either shocks or odours, separated by an interval of 1 min. This sequence formed one conditioning cycle.

Flies were subjected to one of three training protocols (figure 1b): a DC in which they had to learn to discriminate a punished from a non-punished odour ( $A^+$  versus  $B^-$ ), an NF discrimination in which they had to learn to discriminate a punished odour from a non-punished odour compound ( $A^+$  versus



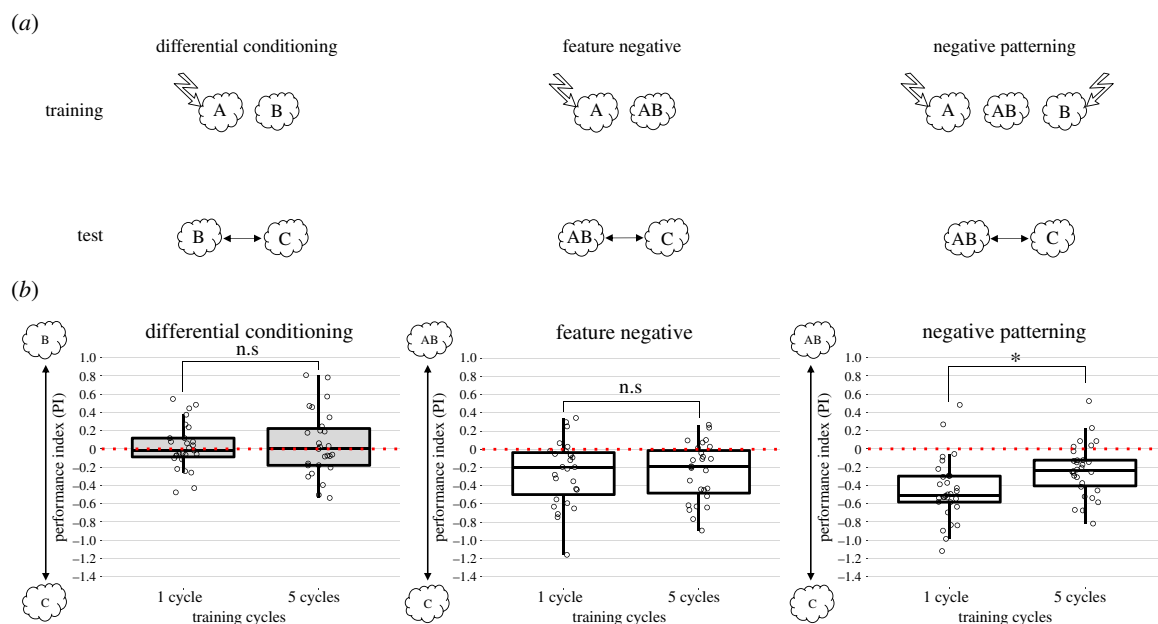
**Figure 1.** (a) Schematic of a typical training cycle. Blue and orange boxes show CS presentation, while red bars show US delivery. (b) Schematic of the conditioning protocols. Clouds represent the CS odourants while lightning bolts indicate the delivery of electric shock during training. A, 3-octanol; B, 4-methylcyclohexanol. (c) Relative PIs computed as the difference between paired and unpaired scores. Performances were compared within the same protocol (i.e. one cycle versus five cycles) but not between protocols. Data are plotted as boxplots. The middle line represents the median, while the upper and lower limits of the box are the 25 and 75% quantiles. The whiskers are the maximum and minimum values of the data that are, respectively, within 1.5 times the interquartile range over the 75th percentile and under the 25th percentile. Raw data are superimposed as jittered dots. 'n.s.' stands for 'non-significant', \* $p < 0.05$ , \*\* $p < 0.01$  after a  $t$ -test (DC and NF) or after a two-way ANOVA (NP).

AB<sup>-</sup>) and an NP discrimination in which they had to learn to discriminate two punished odours from a non-punished odour compound (A<sup>+</sup>, B<sup>+</sup> versus AB<sup>-</sup>). 3-Octanol was always used as the CS<sup>+</sup> for the DC and NF protocols (with 4-methylcyclohexanol (DC) and 3-octanol + 4-methylcyclohexanol (NF) as CS<sup>-</sup>). For the NP protocol, both 3-octanol and 4-methylcyclohexanol were used as CS<sup>+</sup> when presented alone and as CS<sup>-</sup> when presented as a compound. Thus, flies subjected to DC training faced a pure elemental discrimination. On the contrary, flies trained in the NP protocol could only solve the problem if they adopted a non-elemental strategy. Finally, flies subjected to NF training could solve the problem using either an elemental or a non-elemental strategy. For each protocol, training consisted of either one or five cycles, to enable the study of whether the

amount of experience gathered by flies promoted a particular discriminations strategy.

### (d) Test

After training, flies were transferred to a T-maze [28] where they could choose between the CS<sup>+</sup> and the CS<sup>-</sup> in the absence of shock during 1 min. In the case of NP, where flies faced two CS<sup>+</sup>, half of the tests were performed using one of the CS<sup>+</sup> while the other half was performed using the other CS<sup>+</sup>, both presented against the CS<sup>-</sup>. Flies from paired and unpaired protocols were sequentially tested. At the end of the test, flies in each arm of the T-maze were counted. If paired flies learned the discrimination, they should be mostly located in the CS arm,



**Figure 2.** (a) Schematic of the tests performed after the three conditioning protocols to determine the nature of the CS representation. A, 3-octanol; B, 4-methylcyclohexanol; C, benzaldehyde. (b) Relative PIs computed as the difference between paired and unpaired scores. Performances were compared within the same protocol (i.e. one cycle versus five cycles) but not between protocols. Data are plotted as boxplots. The middle line represents the median. The upper and lower limits of the box are the 25 and 75% quantiles. The whiskers are the maximum and minimum values of the data that are, respectively, within 1.5 times the interquartile range over the 75th percentile and under the 25th percentile. Raw data are superimposed as jittered dots. 'n.s.' stands for 'non-significant',  $*p < 0.05$  after a  $t$ -test. Grey shading indicates performances that were not significantly different from chance level while white filling indicates a significant difference from chance level ( $t$ -test against zero). (Online version in colour.)

that is, the arm presenting the odour stimulus that was not associated with the shocks during the training. A performance index (PI) was calculated as: (number of flies in the CS<sup>-</sup> arm – number of flies in the CS<sup>+</sup> arm)/total number of flies. To control for any experimental bias, each replicate consisted of a 'paired group' PI (reflecting associative learning+bias) from which an 'unpaired group' PI (reflecting bias only) was subtracted (electronic supplementary material, figure S1).

### (e) Statistical analysis

PI data were plotted and analysed using R software (3.5.0 v.). Group distributions were tested for normality using the Shapiro–Wilkinson test; homoscedasticity was tested using Bartlett's test. All our experiments met the requirements for parametric statistics. For one or two-sample analyses, we applied one sample or two samples two-tailed Student's tests, respectively. For comparisons involving more than two samples, we used analysis of variance (ANOVA) followed by Tukey's (HSD) *post hoc* tests. A significance level of  $p < 0.05$  was set for every experiment.

## 3. Results

Figure 1c (left panel) shows that flies trained in the DC protocol learned the discrimination and preferred the non-punished odour B<sup>-</sup> to the punished odour A<sup>+</sup>. Comparing their PIs against 0 by means of a one-sample  $t$ -test yielded significant differences after both one training cycle ( $t = 9.49$ , d.f. = 17,  $p = 3.33 \times 10^{-8}$ ) and five training cycles ( $t = 10.83$ , d.f. = 17,  $p = 4.76 \times 10^{-9}$ ). Both PIs did not differ from each other (two-sample  $t$ -test:  $N = 18$  and 18,  $t = -0.87$ , d.f. = 34,  $p = 0.39$ ), thus providing no evidence for an effect of amount of experience on learned preference.

Flies trained in the NF protocol (figure 1c, middle panel) also learned the discrimination between the single odour punished A<sup>+</sup> and the non-punished odour compound AB<sup>-</sup>. Note

that AB is innately more repulsive than A, but the relative PI shows the expected effect of learning (figure 2b; electronic supplementary material, figure S1). Performance was significantly different from zero after both one cycle of training ( $t = 4.13$ , d.f. = 16,  $p = 7.78 \times 10^{-4}$ ) or after five cycles ( $t = 6.69$ , d.f. = 17,  $p = 3.77 \times 10^{-6}$ ). In contrast with DC training, performance improved significantly with the amount of training ( $N = 17$  and 18,  $t = -2.11$ , d.f. = 33,  $p = 0.042$ ). We also tested the flies' performance after the DC or NF protocols balancing odour contingencies, i.e. using B as CS<sup>+</sup> and A as CS<sup>-</sup> (electronic supplementary material, figure S3).

In the case of flies trained in the NP protocol (figure 1c, right panel), the type of odour used during tests had no significant effect on performance (i.e. 'A versus AB' or 'B versus AB'; two-way ANOVA:  $F_{1,69} = 2.72$ ,  $p = 0.10$ ). Yet, the PI varied significantly depending on the number of training cycles ( $F_{1,69} = 34.92$ ,  $p = 1.18 \times 10^{-7}$ ). After one training cycle, the PI was negative, i.e. biased towards the single odours associated with the shocks during training (A<sup>+</sup> or B<sup>+</sup>) ( $t = -2.47$ , d.f. = 35,  $p = 0.018$ ). However, after five training cycles, preference was reversed and flies preferred the non-punished compound AB<sup>-</sup> over the single punished odorants A<sup>+</sup>, B<sup>+</sup> ( $t = 5.37$ , d.f. = 35,  $p = 5.16 \times 10^{-6}$ ; figure 1c, right panel). These findings show that training repetition is crucial for NP solving as it improved the ability of flies to discriminate the odours with different outcome. To determine if NP solving is independent of the pairs of odours used, we repeated the experiment using different odours (D<sup>+</sup>, E<sup>+</sup> versus DE<sup>-</sup>; electronic supplementary material, figure S2). The flies also learned the NP discrimination also in this case, showing that the type of odour used during the test phase had no significant effect on performance (i.e. 'D versus DE' or 'E versus DE'; two-way ANOVA:  $F_{1,69} = 3.005$ ,  $p = 0.088$ ). After one trial, flies were not attracted by



the single odours ( $t = -0.73$ , d.f. = 35,  $p = 0.47$ ). After five trials, they were able to solve the task and showed a significant preference for the compound ( $t = 5.21$ , d.f. = 35,  $p = 8.59 \times 10^{-6}$ ). This result demonstrates that the ability to solve NP is not specific to the type of odour used in the protocol.

Despite their different complexity, the three protocols assayed opposed one or two  $CS^+$  to a  $CS^-$ . Under these conditions, an animal may learn to avoid the  $CS^+$ , to be attracted to the  $CS^-$ , or both. To determine the nature of the associations inculcated by DC, NF and NP, we studied if the  $CS^-$  (not reinforced) was learnt as a safe stimulus (inducing attraction), remained neutral (being indifferent to trained animals) or in the case of compounds sharing elements with the reinforced  $CS^+$ , also gained inhibitory strength, thus eliciting avoidance. To test these options, flies were first trained under the DC, NF or NP protocols (figure 2a) and then given a choice between the  $CS^-$  and a novel, neutral odour C. Here again, the preference of trained flies was compared to preference of flies after an unpaired protocol.

After being trained in the DC protocol (figure 2b, left panel), flies tested with the non-punished odour B versus the neutral odour C did not show any preference either after one training cycle ( $t = 0.48$ , d.f. = 26,  $p = 0.64$ ) or after five training cycles ( $t = 0.73$ , d.f. = 26,  $p = 0.47$ ). There was no significant difference between PIs corresponding to these different training amounts ( $N = 27$  and  $27$ ,  $t = -0.31$ , d.f. = 52,  $p = 0.75$ ). This result thus suggests that the non-punished odour was not perceived as attractive after DC training, and that the number of training cycles had little impact if at all.

After NF conditioning (figure 2b, middle panel), flies significantly preferred the novel odour C to the non-punished compound AB both after one ( $t = -3.78$ , d.f. = 26,  $p = 8.41 \times 10^{-4}$ ) and five training cycles ( $t = -4.15$ , d.f. = 26,  $p = 3.16 \times 10^{-4}$ ). This preference was maintained despite the increase in the amount of training ( $N = 27$  and  $27$ ,  $t = -0.03$ , d.f. = 52,  $p = 0.97$ ), thus showing that in learning this discrimination, flies assigned an aversive valence to the  $CS^-$  despite the fact that it was not paired with shocks. This inhibitory strength must have been acquired via the presence of the punished element A in the compound AB.

Finally, after NP training (figure 2b, left panel), flies also significantly preferred the new odour C to the non-punished compound AB both after one ( $t = -7.36$ , d.f. = 29,  $p = 4.12 \times 10^{-8}$ ) and five training cycles ( $t = -4.46$ , d.f. = 29,  $p = 1.12 \times 10^{-4}$ ). Yet, differently from NF training, the flies' aversion for the  $CS^-$  was reduced after five training cycles ( $N = 30$  and  $30$ ,  $t = -2.74$ , d.f. = 58,  $p = 8.10 \times 10^{-3}$ ), thus showing that increasing the amount of experience diminished the inhibitory strength of the compound. This variation is consistent with a nonlinear processing of the compound, which became less similar to the two single odour components, both being inhibitory after having been paired with shocks.

## 4. Discussion

Our results provide the first evidence of NP solving in an insect other than the honeybee [15,24]. Our results show that flies solved both an NF and an NP discrimination and preferred, in both cases, the odour compound AB that was unpunished during training. In both cases, the compound acquired some inhibitory strength as shown by its avoidance

when confronted with a novel stimulus C, a fact that could reflect an influence of the punished element (A in NF, A and B in NP) in it. Such an influence would be consistent with an elemental processing of the compound ( $AB = A + B$ ). Yet, in NP, increasing the amount of training diminished this influence, thus promoting a configural processing ( $AB = \text{new odour}$ ).

When interpreted in the light of these theories, our results cannot accommodate a strictly elemental or configural account. Even after five training cycles in NP, the flies still exhibited some avoidance of the  $CS^-$  mixture AB, implying that  $AB^-$  still carried some of the inhibitory strength associated with its constituents, a result that goes against a purely *configural* compound representation (figure 2b, right panel). In other words, the compound remained repulsive, but was preferred to the 'more repulsive' elements taken alone. This result differs from the response of naive flies, for which a compound is more repulsive than the single odorants, at the concentrations used (electronic supplementary material, figure S1). Our result could thus be interpreted as supporting the 'unique cue theory', which is consistent with observations both in honeybee and *Drosophila*. *In vivo* imaging of the antennal lobes, the primary olfactory centre in the insect brain, suggests that binary mixtures are not coded as the linear sum of their constituents but rather show both similarities and features that are unique to the compound considered [29–32]. However, the fact that the flies' initial choice, which was closer to an elemental representation, was reversed (although not entirely) after NP training shows that increasing training gradually changed the odour representation.

Our results show that the experience of an individual modulates the kind of processing adopted. This modulation is consistent with previous work on honeybees showing, in both the visual and the olfactory modality, that increasing the number of training trials promotes a configural stimulus representation [33,34]. Such a plastic stimulus representation has also been described in other learning paradigms (in crustaceans [14]; larvae [35] and adults *Drosophila* [21,36]; rabbits [37]; humans [38]).

How such a change of odour representation could be implemented in the fly's brain? Firstly, associative learning has been shown to modulate neural representation of odours in the honeybee antennal lobes, a structure involved in olfactory processing, also present in *Drosophila*. Over the course of  $CS/US$  associations, odours representation was modified, amplifying the  $CS^+$  representation and reducing  $CS^+/CS^-$  correlation. This could be a first explanatory mechanism for enhanced discrimination abilities necessary for NP resolution [39]. Moreover, pioneer work in the field identified the mushroom bodies as critical structures for the encoding of learnt behaviour in insect brain [40–42]. Interestingly, bees without functional mushroom bodies learn simple olfactory discriminations but are unable to solve NP tasks [43]. Such a failure in NP tasks (as well as in other tasks such as reversal learning) was related to the pharmacological blockade of a specific subset of feedback neurons providing GABAergic signalling to the mushroom bodies [43,44]. The functional equivalent of these neurons in *Drosophila*, the anterior paired lateral (APL) neurons, are equally necessary for reversal learning [45]. Together, this suggests that APL neurons play a crucial role for the modulation of odour representation, and thus for NP solving in flies.

The inhibitory feedback mediated by APL neurons enables a sparse encoding of sensory information by maintaining a low level of activity in the MB, which is needed to discriminate between similar olfactory stimuli [46]. Previous modelling work suggested that normalization at the antennal lobes level is crucial for non-elemental forms of learning as it leads to inhibition of element-specific features, thus enhancing compound-specific (unique cue) salience [26]. APL neurons activity also acts as a gain control mechanism and thus further enhances discrimination at the MB level, which would confirm a hypothesis already proposed in a previous work on NP in the honeybee [43]. Moreover, by modulating its inhibitory activity depending on the task, the APL neurons could mediate a shift along the generalization/discrimination balance as observed here. Indeed, after one cycle of NP conditioning, flies respond to AB as if it were A or B, consistent with odour generalization. Yet, after five cycles, flies change their behaviour and choose AB, even though the second experiment clearly shows that they still perceive the A and B constituents as aversive. This result, in contrast, can be attributed to better discrimination abilities. Interestingly, dopamine signals originating from PPL1 neurons (responsible for aversive US transmission) inhibit APL activity through D2-like receptors, thus modulating olfactory learning [47]. Moreover, APL neurons are activated differently by the CS<sup>+</sup> and CS<sup>-</sup> [48]. More specifically, the association between the CS and the US results in a diminished APL activity (leading to a reduction in inhibition towards the CS<sup>+</sup>, facilitating—in principle—its reinforcement). Thus, it is possible that during trials, a differential activation of APL in response to the CS<sup>+</sup> versus CS<sup>-</sup> produces a form of pruning, leading to the unequivocal reinforcement of the KC associated only with A or B while inhibiting the KC associated both to A/B (the CS<sup>+</sup>) and to AB (the CS<sup>-</sup>). If this process is slow compared to the acquisition of the original CS–US association, APL activity would gradually modulate stimulus

representation to facilitate the task resolution. In the case of NP, it would help to segregate the representation of AB from those of its elements. APL neurons could thus continually adjust and update representation along a generalization/discrimination continuum based on learning events, besides their role as regular gain control system. Understanding how APL activity might modulate non-elemental olfactory learning constitutes an interesting future research agenda.

So far, olfactory learning in fruit flies was limited to DC protocols, thus reducing the study of associative learning and its neural and molecular mechanisms to its most simple form. Our results thus open new perspectives to explore these mechanisms for higher-order forms of learning and determine if the same or different circuits intervene when different learning strategies are employed.

**Data accessibility.** Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.v9s4mw6t1> [49].

**Authors' contributions.** M.D. carried out all the experiments, analysed data, participated in the design of the study and drafted the manuscript; A.W. participated in the design of the study and critically revised the manuscript; P.A. conceived the semi-automated conditioning device; M.G. participated in the design and conceptualization of the study and critically revised the manuscript; G.I. conceived, designed and coordinated the study, and helped draft the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

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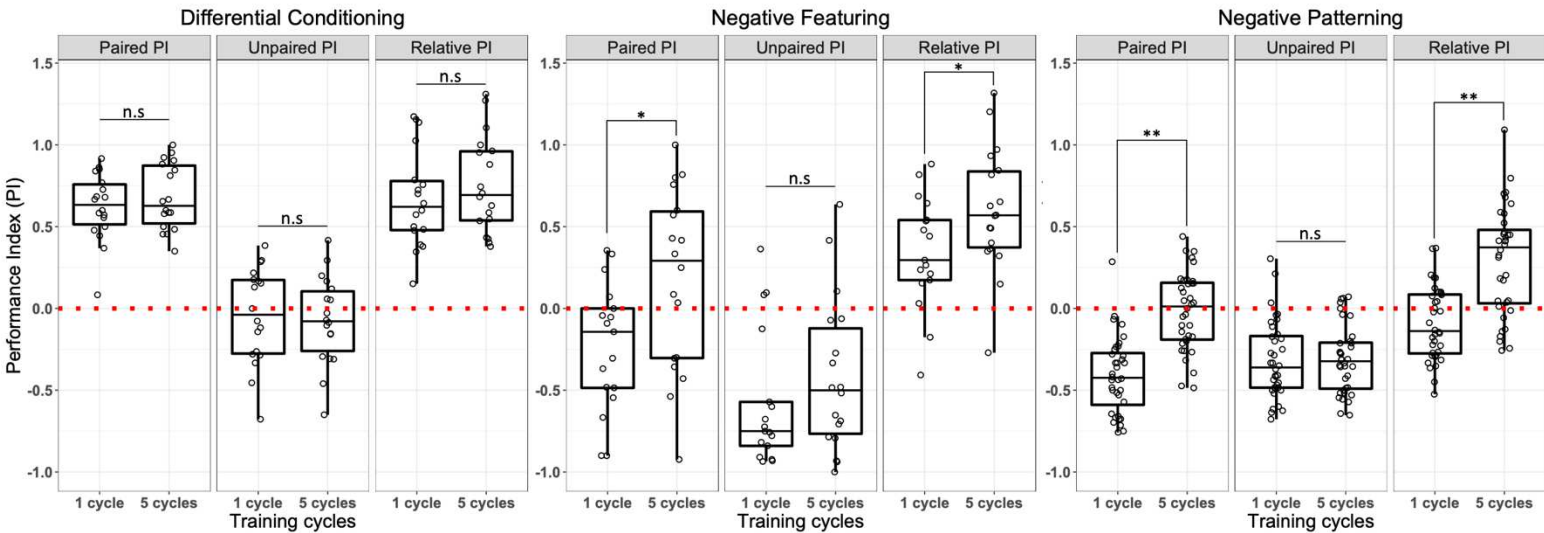
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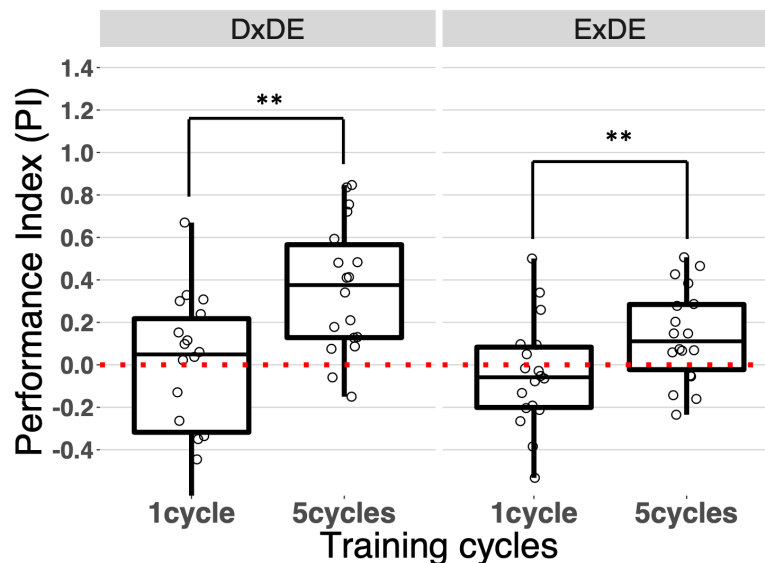
# Supplementary Information

Accessible at: <https://royalsocietypublishing.org/doi/suppl/10.1098/rspb.2020.1234>

**Figure S1** : Performance Indices from individual groups of flies trained with a Paired protocol, Unpaired protocol and the Relative PI computed as the difference between two subsequently tested paired and unpaired groups. 1 cycle and 5 cycles PI were compared to assess the impact of repetition on flies calculated performances. Data are plotted as boxplots. The middle line represents the median, the upper and lower limits of the box are the 25 and 75 percent quantiles. The whiskers are the maximum and minimum values of the data that are respectively within 1.5 times the interquartile range over the 75<sup>th</sup> percentile and under the 25<sup>th</sup> percentile. Raw data are superimposed as jittered dots. "n.s" stands for "non-significant", \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$  after a t-test.

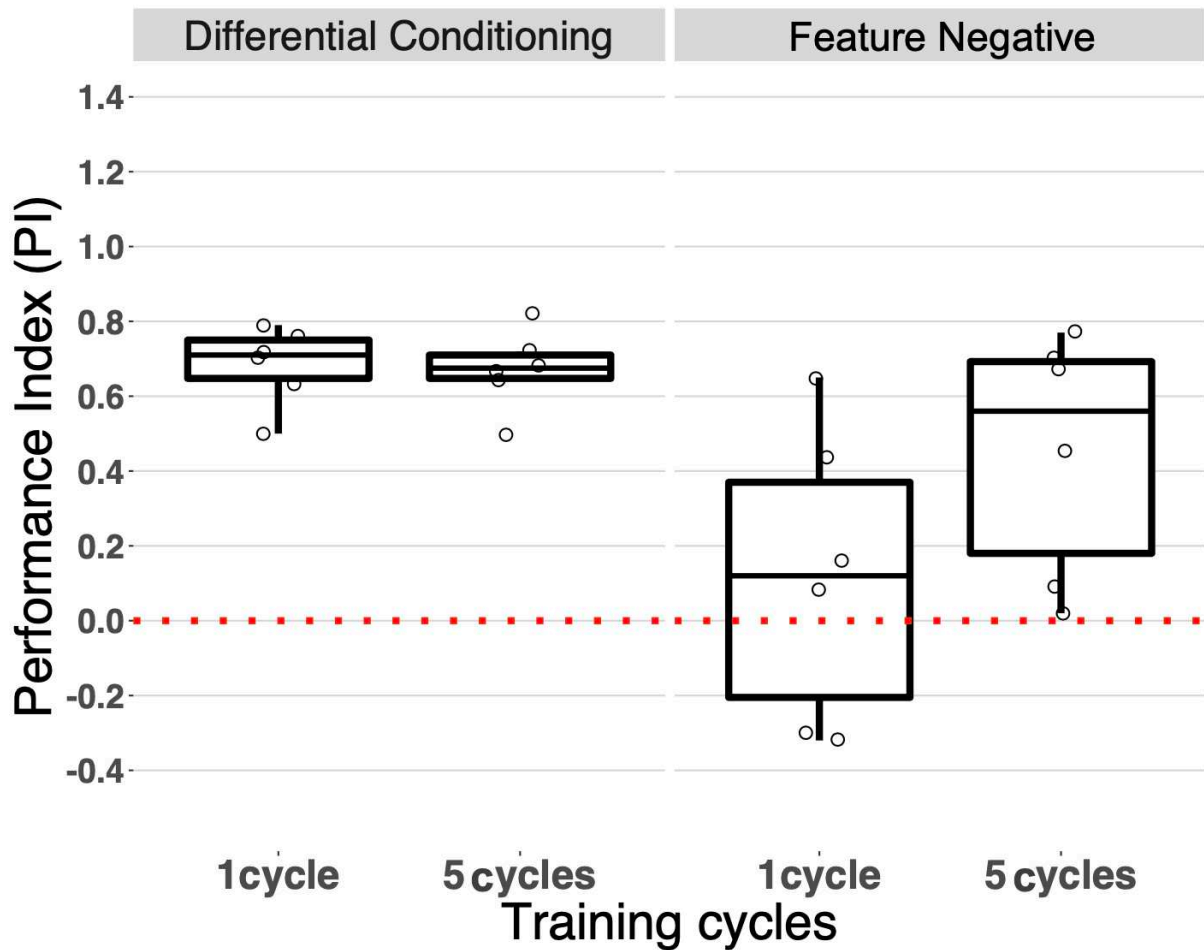


**Figure S2** : Relative performance indices (PI) of flies trained in a Negative Patterning discrimination with alternative odourants (D = Isoamyl Acetate, E = Ethyl Butyrate), computed as the difference between paired and unpaired scores. Data are plotted as boxplots. The middle line represents the median while the upper and lower limits of the box are the 25 and 75 percent quantiles. The whiskers are the maximum and minimum values of the data that are respectively within 1.5 times the interquartile range over the 75<sup>th</sup> percentile and under the 25<sup>th</sup> percentile. Raw data are superimposed as jittered dots. \*\* indicates  $p < 0.01$  after a 2-ways ANOVA





**Figure S3:** Relative Performance indices (PI) of flies trained either with a Differential Conditioning or Negative Feature discrimination, with 1 or 5 conditioning cycles, using B (4-Methylcyclohexanol) as CS+ and A (3-Octanol) as CS-. Data are plotted as boxplots. The middle line represents the median while the upper and lower limits of the box are the 25 and 75 percent quantiles. The whiskers are the maximum and minimum values of the data that are respectively within 1.5 times the interquartile range over the 75<sup>th</sup> percentile and under the 25<sup>th</sup> percentile. Raw data are superimposed as jittered dots.



## Chapter II: How targeted inhibition solves ambiguous learning situations in *Drosophila*

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## Introduction

Animals' survival depends on their ability to successfully find their way to beneficial resources and away from harmful situations. To do so, they can rely on associative learning, that is, link positive or negative experiences (called unconditional stimuli, US) with specific features extracted from their surroundings (called conditional stimuli, CS). That way, the CS is reinforced and becomes a predictor of the US usual outcome. However, a same CS may be associated with different outcomes depending on the context, and its value might change over time. For instance, a same odour molecule might be negatively reinforced when encountered alone but not when blended with other odours. Similarly, the food source associated with a specific scenery may be depleted. Such situations create discrepancies between previously learnt information and novel experiences. Animals must therefore be able to flexibly update their learnt associations with each learning event. One way to study how animal deals with such ambiguities is to use modified versions of Differential Conditioning (DC) experiments during which the animal has to discriminate between a previously reinforced CS (hence called CS+) and a non-reinforced CS (called CS-). In its simplest form, DC is performed using two easily distinguishable stimuli, A and B (the task may be summarized as A+ B-, meaning that only A has been reinforced while B has been presented without reinforcement). Various protocols are designed to introduce ambiguity in a DC paradigm, one of which, called Negative Feature discrimination (NF), consists in reinforcing A when presented alone but not when presented as a compound AB (which can be represented as A+ AB-). In an even more challenging alternative called Negative Patterning (NP), both A and B are reinforced when presented alone, but not when presented together (this task is represented as A+ B+ AB-). Interestingly, NP requires the involvement of specific brain regions, both in vertebrate and invertebrate, to be solved<sup>[36,196]</sup>. However, the actual neural mechanisms underlying animals' ability to learn to discriminate AB from its single components remain elusive. The fruit fly *Drosophila melanogaster* is well suited for such an endeavour as it offers powerful neurogenetic tools, and has been recently shown to be able to solve ambiguous learning tasks such as olfactory NF and NP<sup>[188]</sup>. Moreover, contrary to classical DC tasks, NP learning is not successfully acquired with one single training trial, but requires

several training trials to be solved<sup>[188]</sup>. This seems to be a general feature, as trials repetition is also required in other species to solve NP<sup>[34,197]</sup>.

Here, we investigate the biological processes explaining why training repetition may mediate ambiguous learning tasks acquisition. In the current accepted model of olfactory associative learning in *Drosophila*<sup>[198]</sup>, odours (here the CS) are represented as specific activity patterns at the level of glomeruli in the Antennal Lobes, the first olfactory processing centre<sup>[117]</sup>. Odour's activity patterns are then conveyed via projection neurons onto the Kenyon cells (KC) constituting the Mushroom Bodies (MBs), the main centre for olfactory associative learning in insects<sup>[198]</sup>. The US information is conveyed by specialized clusters of Dopaminergic Neurons (DANs), the larger protocerebral anterior medial cluster (PAM-DANs) being involved in reward learning<sup>[199,200]</sup>, and the protocerebral posterior lateral cluster (PPL1-DANs) being involved in aversive learning<sup>[84,201]</sup>, both projecting onto the KCs. Thus, olfactory associative learning occurs through the reinforcement of specific subsets of KCs representing the CS via DANs activity representing the US.

Overall KC activity is integrated in a small subset of Mushroom Body Output Neurons (MBONs) that drive avoidance or approach behaviour. In a naive state, both MBONs are equally activated by a given odour pattern, which is therefore neutral for the fly (apart from possible innate valence, which is mediated by another brain region, the Lateral Horn<sup>[202]</sup>). When an odour is associated with an aversive treatment (odour becomes a CS+), every associated KCs' activity is reinforced, which translates in a depression in synaptic weight between these KCs and the approach MBON. That way, the reinforced odour now elicits a greater avoidance MBON than approach MBON activity, which results in a behavioural bias toward avoidance<sup>[158]</sup>. In contrast, presentation of a non-reinforced odour (CS-) does not induce any plasticity at the MBONs level. Importantly, any of the CS+ KCs' might also be triggered, to some extent, by other odours, including the CS-. Thus, during testing phase where flies are simultaneously exposed to the CS+ and the CS-, the resulting behaviour may be computed as the relative difference of MBON activation elicited by the CS+ compared to the activation elicited by the CS- (termed " $\Delta$ MBON"). The more similar the CS+ and the CS- are, the bigger the overlap between CS+ and CS- associated KC is, and the lower the  $\Delta$ MBON activity (i.e., Learning-related behavioural bias) is. This approach is called the "Distance-Discrimination Model"<sup>[127]</sup>. From that perspective, if a stimulus elicits the activation of a large

number of KCs, the probability of a significant part of them being also activated by another stimulus is higher. On the contrary, a stimulus eliciting the activation of a small number of KCs should present less KCs in common also activated by other stimuli. In the *Drosophila* MBs, as also found in other insects<sup>[203]</sup>, odours are in fact sparsely encoded by KCs activity, which relies on the activity of a GABAergic neuron, the Anterior Paired Lateral neuron (APL)<sup>[171]</sup>. Sparse coding of olfactory stimuli has been shown to be especially crucial for similar stimuli discrimination, as APL selective inhibition prevent flies to discriminate similar but not dissimilar odours<sup>[171]</sup>. Interestingly, Honeybee APL homolog, the GABAergic A3v neurons have been shown to be required for NP, but not DC learning. Moreover, both APL and A3v neurons are necessary for Reversal Learning (RL)<sup>[178,179]</sup>. RL is a learning task divided in two phases. In the first phase a stimulus A is reinforced while another, B, is not. In a second phase, B is reinforced but not A (A+ B- => B+ A-). In other terms, RL is also an ambiguous learning task but the ambiguity is of temporal nature. Finally, imaging data from a previous work demonstrated that odour/shock pairing led to a modulation of APL activity, while at the same time APL also inhibit olfactory learning through GABAergic transmission<sup>[169]</sup>. Taken together, these results identify APL neurons as a prime candidate in the search of the neural mechanisms underlying NP learning through repetition.

In this study, we build a computational model based on the traditional *Drosophila* olfactory learning model presented above. We demonstrate that the modulation of KCs activity through training repetition qualitatively explains gradual acquisition of NP, whereas sparse coding alone cannot explain observed behavioural data. We characterize APL neurons activity as the likely *in vivo* implementation of KCs activity modulation through repetition as we show that APL disruption is detrimental for NF and NP but not DC learning, which we link to APL's GABAergic neurotransmission. Based on previous observations showing that APL activity is suppressed by the CS+ but not by the CS-<sup>[169]</sup>, we suggest that upon training repetition, the CS- presented without reinforcement leads to an APL inhibitory feedback that lastingly reduces the activity of CS- associated KCs, which facilitates NP by training flies to respond less to the AB compound than to the A or B components alone. We subsequently demonstrate the specific engagement of APL neurons during CS- presentation for NP acquisition and show that APL-mediated training repetition effect depends on a dopaminergic modulation of APL. Finally, we discuss how differential APL recruitment may generally mediate learning flexibility and ambiguity solving.

## Results and discussion

A model of targeted KCs activity modulation theoretically explains gradual acquisition of Negative Patterning learning

The MB is a key center for olfactory associative learning in insects, and training repetition is required for olfactory associative tasks such as Negative Patterning<sup>[188,197]</sup>. Therefore, the repetition of stimuli presentation during training might provoke neural changes in the MB. Indeed, neural recording in honeybees showed that repetition of odour presentation without coincident reinforcement leads to a decrease in KCs response, a form of “neural habituation” at the level of MBs<sup>[204]</sup>. Contrastingly, presentation of an odor together with reinforcement seems to increase the KC responses to this odor<sup>[204]</sup>. We therefore resonated that such a ‘KC habituation/potentialiation’ should happen across repeated presentation of the CS-/CS+, and that this effect may be key for flies to solve negative patterning tasks.

To confirm the viability of this hypothesis, we built a simple computational model of the flies’ MB based on the flies’ described anatomical features (figure 1, figure S1), and added this KC bidirectional modulation rule: the activity of KCs responding to the odor presented without reinforcement (CS-) would decrease across CS- repetition; and activity of KCs responding to the odor presented with reinforcement (CS+) would increase across CS+ repetition.

Remarkably, adding this rule to the model spontaneously enables NP to be solved through training repetition (Fig1d-right). No fine-tuned parametrization of the model is needed for the demonstration of the qualitative benefit of such a targeted ‘KC modulation’, as the chosen speed at which KCs are modulated across trials only impact the speed at which NP score improves across repetition. This ‘KC modulation’ seems key, as without it, the model appears unable to solve NP (Figure 1), even when we tried to manipulate actual learning rate (FigureS1).

a)

Paradigm	Conditioning Type	Training	Test
Differential Conditioning	Differential Conditioning	A+ B-	A vs B
Negative Featuring	Featuring	A+ AB-	A vs AB
Negative Patterning	Patterning	A+ B+ AB-	A/B vs AB

b)

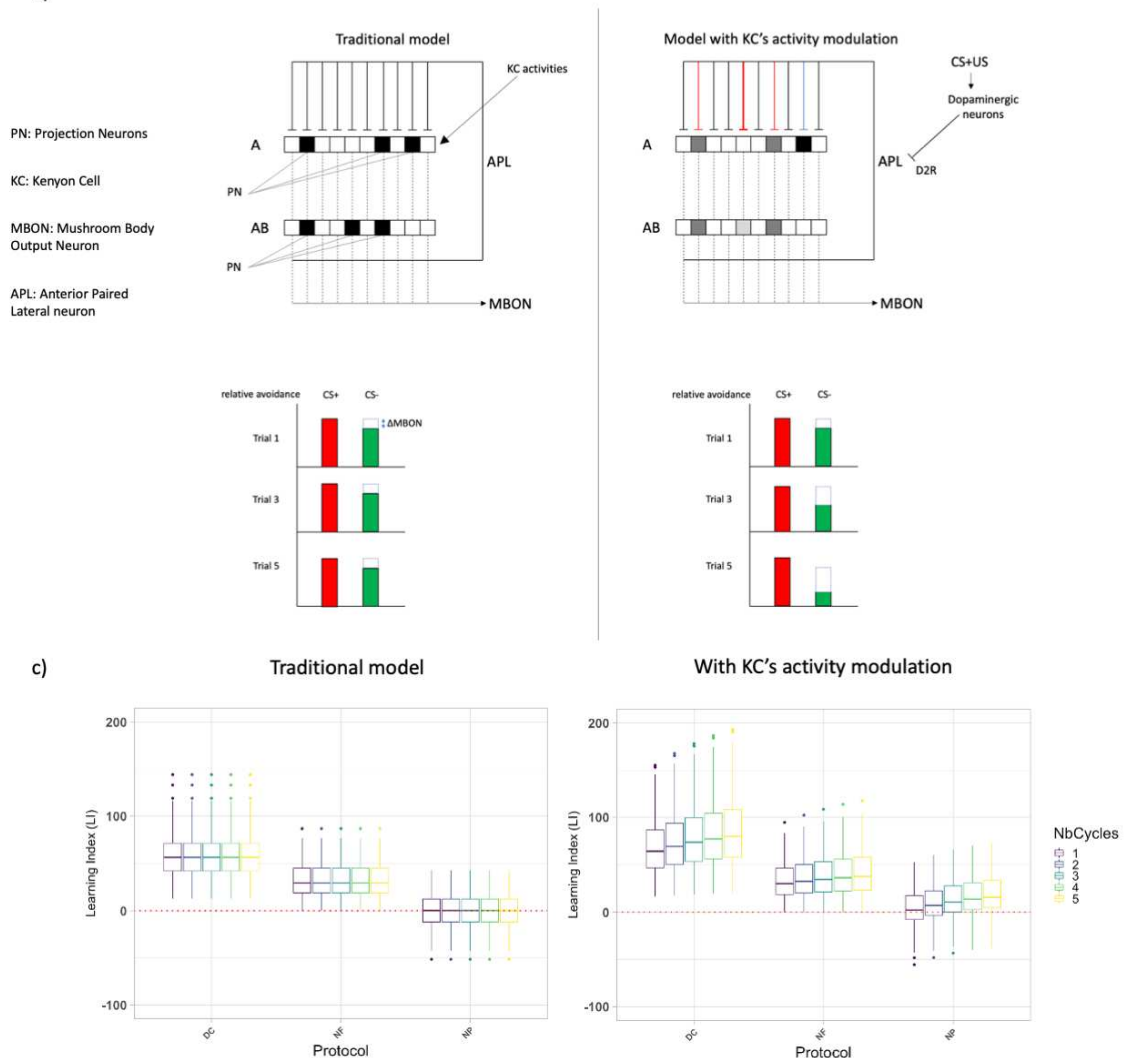


Figure 1 : a) Summary of all tested behavioural tasks b) Simplified example of simulated training involving a stimulus and a compound containing the same stimulus, without (left) or with (right) gradual modulation of KCs activity by APL neurons c) Effect of KC's activity modulation on learning performances : Theoretical performances expressed as relative  $\Delta$ MBON activity resulting from CS+ vs. CS- presentation. Performances were simulated for 5 experimental paradigms : Differential Conditioning (DC), Feature Negative (NF) and Negative Patterning(NP). Learning was assessed over the course of 5 conditioning cycles. N=100.

Interestingly, the model also spontaneously explains additional observations, such as the fact that avoidance to the CS- (AB) is strong after one training cycle but reduced by training repetition (FigureS2)<sup>[188]</sup>. Also, the model explains the observed positive but low scores (relative to Standard Conditioning DC) for Negative Featural (NF) and its improvement through cycle repetition<sup>[188]</sup>. DC performances in the model were also increased by repetition, which differed with our experimental observations but can easily be explained by considering a ceiling effect. Indeed, if maximum behavioural performances as measured in our T-maze are already reached at 1 training cycle, theoretical improvement may not be detected. Moreover, a previous study mentioned such ceiling effect when using the same experimental paradigm as we did<sup>[169]</sup>.

In any case, the importance of such a ‘KC modulation’ through repetition in these ambiguous learning paradigms seems to be a viable working hypothesis.

We next resonated that such KCs activity modulation may involve the APL neuron, as multiple evidence converges: First, APL is known to inhibit the KCs<sup>[169,171,205]</sup> and can do so differentially across individual KCs<sup>[206]</sup>. Second, APL activity is itself inhibited given the presence of a reinforcer<sup>[169,170]</sup> so it must act differently on CS+ and CS- during our learning paradigm. Third, functioning APL are necessary for the flies to solve complex learning paradigm such as Reversal Learning (RL)<sup>[179]</sup>, as is the ‘targeted KC modulation’ in our model (FigureS3). Finally, APL’s homolog in honeybee (the A3v neurons) is required for both RL and NP acquisition<sup>[36,178]</sup>, as again predicted by the model.

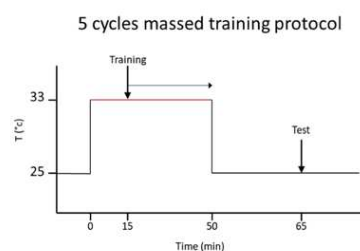
We further challenged the hypothesis that the APL neurons are involved in such a KC modulation across repetition by using neuro-genetic tool to test a series of key predictions.



## APL neurons are required for Negative Patterning learning

The first and foremost prediction of our model is that APL disruption should considerably impact NP but not DC performances. To test experimentally this prediction, we blocked APL synaptic transmission by expressing the dominant negative thermosensitive *Shibire<sup>ts</sup>* protein (using *UAS-Shi<sup>ts</sup>[194]*) in APL neurons (using the *VT43924-Gal4* driver<sup>[207]</sup>). In all our experiments, APL transmission was specifically blocked during training phase by submitting flies to 33°C 15min before conditioning. Right after training, flies were submitted to 25°C and animals were tested 15min afterwards. Flies were trained using different protocols (Fig1.a). Results are presented Fig2.b.

a)



b)

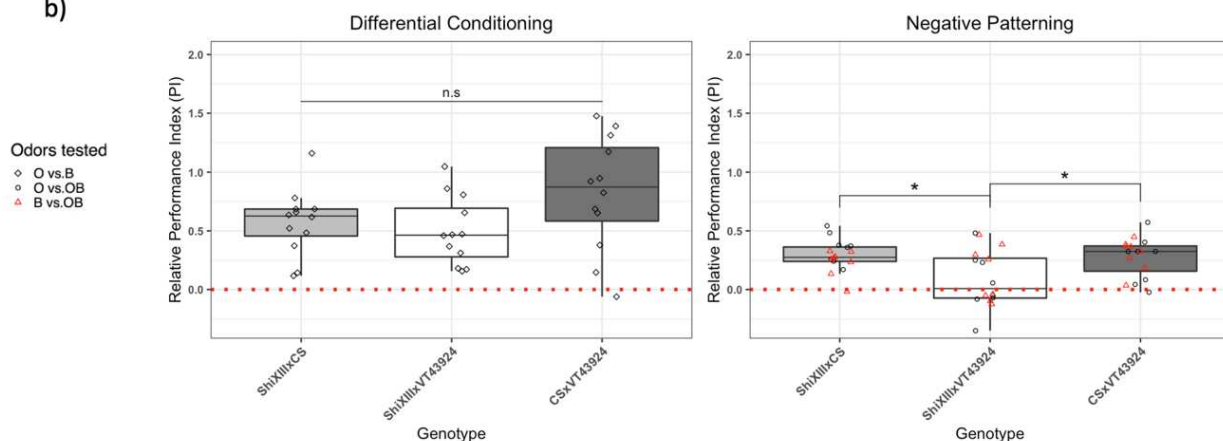


Figure 2: a) Time course of temperature variations during training/test procedures. b) Effect of APL neurons targeted inhibition on Differential Conditioning (left) and Negative Patterning (right). Relative performance indices (PI) computed as the difference between paired and unpaired indices. Performances are compared between genotypes within the same paradigms but not between paradigms (i.e. Differential Conditioning vs. Negative Patterning). "n.s" stands for "non-significant", \* indicates  $p < 0.05$

Flies trained with a Differential Conditioning were not significantly affected by APL disruption (ANOVA,  $F_{2,33} = 2.58$ ,  $p = 0.09$ ). Thus, this manipulation did not impact flies' ability to acquire associative learning in a classical context, which controlled for eventual perceptual or motor defects.

In contrast, when submitted to a Negative Patterning training, APL impaired flies showed significantly lowered performances (ANOVA:  $F_{2,45} = 5.25$ ,  $p = 0.0090$ , Tukey HSD post-hoc test: both controls are significantly different from the UAS-Shi<sup>ts</sup>/VT43924-Gal4 line ( $p=0.025$ ,  $p = 0.016$ ), but are not different from each other ( $p=0.98$ )). Without functional APL neurons during the training phase, flies' performances in NP were not distinguishable from chance (One sample T-test against 0,  $df = 15$ ,  $t=1.61$ ,  $p = 0.13$ ).

#### Improvement through repetition involves APL GABAergic transmission

Two neurotransmitters have been identified in APL neurotransmission, GABA and Octopamine (OA). OA from APL is required for aversive memory consolidation processes<sup>[207]</sup> and seems to facilitate olfactory learning<sup>[147]</sup>. In contrast, APL's GABAergic transmission has been demonstrated to inhibit KCs<sup>[169,171,205]</sup>. Moreover, APL's activity is specifically inhibited by the association between the CS+ and the US<sup>[169]</sup>, which suggests that KCs' inhibition through APL activity should rather occur during CS- presentation. Therefore, one way KCs activity modulation through repetition could be implemented is through a modulation of synaptic weights between APL and KCs, which would occur during CS- presentation. As our model predicts an inhibition of the CS- representation, APL's GABA transmission should be crucially involved in KCs activity modulation. To confirm that, we knocked down the expression of either glutamic acid decarboxylase (GAD) involved in GABA synthesis, or Tyramine- $\beta$ -hydroxylase (T $\beta$ h) mediating tyramine conversion to Octopamine, using RNA interference. RNAi knockdown was driven specifically within APL neurons using VT43924-Gal4 and restricted to adult stage using the GAL80<sup>ts</sup> repressor. Flies were placed at 30°C 5 days before experiments to trigger RNAi knockdown. Subsequently, flies were exposed to a Negative Patterning protocol. Training and test were performed at 25°C. As presented in Fig3, flies expressing GAD-Rnai under the control of VT43924-gal4 showed impaired NP performances (ANOVA :  $F_{2,61} = 20.67$ ,  $p = 1.4 \times 10^{-7}$ , Tukey HSD post hoc test: all genotypes

were found to show significantly different performances; Gal4 controls performances were significantly higher than UAS controls ( $p= 0.006$ ) and the UAS-Gal4 groups' ( $p= 0.006$ ), while UAS controls performances were significantly higher than the UAS-Gal4 groups' ( $p = 1.0 \times 10^{-7}$ ). In contrast, flies expressing Tbh-Rnai did not show significant performances variations compared to controls (ANOVA :  $F_{2,62} = 2.113$ ,  $p=0.13$ ). We conclude that KCs modulation through repetition crucially involves APL GABAergic transmission, which doesn't seem to be the case with Octopamine transmission.

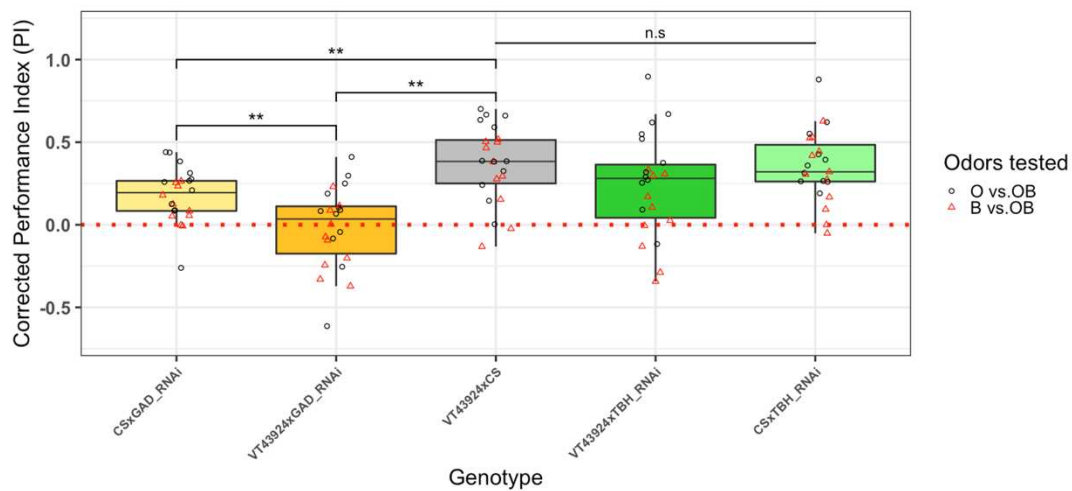


Figure 3 : Effect of Gad or Tbh knock-down in APL neurons on Negative Patterning. Relative performance indices (PI) computed as the difference between paired and unpaired indices. Data were compared across relevant genotypes (i.e. UAS-Gad control was compared to other Gad-related groups but not to Tbh conditions). "n.s" stands for "non-significant", \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$  )

APL neurons inhibition is specifically required during repeated CS- but not CS+ presentations

When comparing models without and with KCs activity modulation, simulated learning performances obtained after 1 cycle with KCs activity modulation are similar to performances obtained without KCs activity modulation. In other terms, learning enhancement induced by KCs activity modulation is predicted to only occur during conditioning repetition. In a previous study, we showed that 1 training cycle was not sufficient for flies to learn NP (Durrieu et al, 2020). In contrast, flies were able to learn a NF with 1 training cycle, although measured performances were significantly increased by training repetition. Therefore, NF is an interesting middle ground as KCs activity modulation is predicted to improve NF performances, but its absence shouldn't affect 1 cycle NF performances. In other terms, if KCs activity modulation is implemented by APL activity, APL inhibition during training should

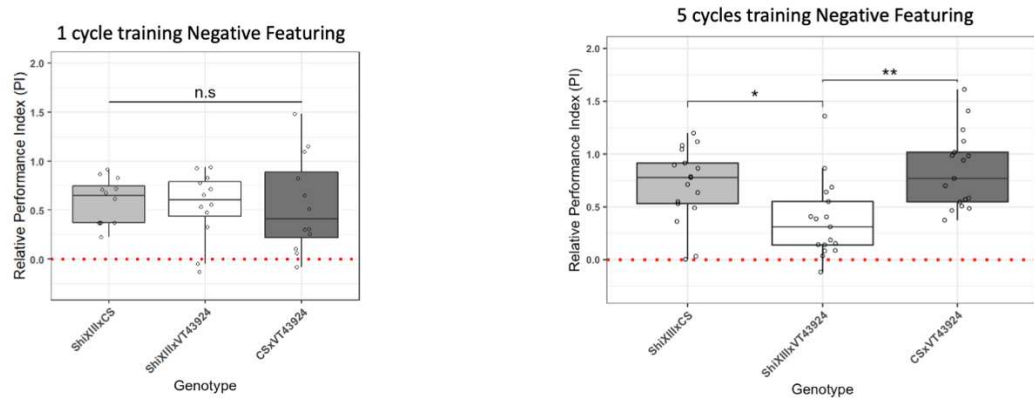
decrease 5 cycles but not 1 cycle NF performances. To test this assumption, we expressed UAS-Shits1 in APL neurons using VT43924-Gal4 and silenced APL neurons transmission during training by increasing temperature submitted to flies to 33°C 15 min before conditioning. Flies were trained with 1 or 5 cycles NF (Fig4a). After training, temperature submitted to flies was lowered back to 25°C and animals were tested 15min afterwards. Flies with silenced APL transmission showed no significant difference with the genetic controls after 1 cycle NF (ANOVA:  $F_{2,33} = 0.037$ ,  $p = 0.96$ ), as opposed to flies submitted to a 5 cycles NF (ANOVA:  $F_{2,48} = 7.71$ ,  $p = 0.0012$ , Tukey HSD post hoc test : both controls are significantly different from the UAS-Shits/VT43924-Gal4 line ( $p=0.0011$ ,  $p = 0.025$ ), but are not different from each other ( $p=0.51$ )). We formally validated the interaction of training repetition on APL disruption using a linear model ( $F_{1,77}=6.57$ ,  $p=0.012$ ). Thus, we conclude APL neurons are engaged specifically during training repetition.

Moreover, as APL role in NP has been demonstrated to be mediated by inhibitory GABA transmission, KCs activity modulation should be inversely related to APL activity. In other terms, APL activation should be necessary during CS- presentation to negatively modulate KCs activity. In contrast, APL should be inhibited during CS+ presentation to positively modulate KCs activity, as suggested in a previous study<sup>[169]</sup>. Therefore, APL inhibition specifically during CS+ presentation should have no effect on NP gradual acquisition, whereas APL inhibition during CS- presentation should prevent the negative modulation of KCs activity and impair NP acquisition. We tested this prediction by conceiving a protocol with extended breaks between CS+ and CS- presentation in order to enable Shibire mediated silencing specifically during either CS+ or CS- presentation (See Fig4b for detailed procedure).

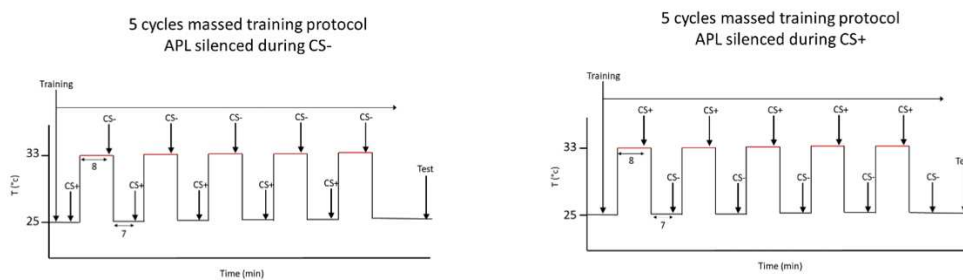
Using this procedure, we trained flies expressing UAS-Shi<sup>ts</sup> under the control of VT43924-Gal4 with a NP protocol. Flies were placed at 33°C either during the CS+ or the CS- phases, while being placed at 25°C for the rest of the training phase. Flies were subsequently tested at 25°C. Results are presented Fig4c. Animals with silenced APL specifically during CS- presentation showed impaired NP performances. (ANOVA:  $F_{2,33} = 6.52$ ,  $p = 0.004$  ; Tukey's HSD post hoc test : both controls are significantly different from the UAS-Shi<sup>ts</sup>/VT43924-Gal4 line ( $p=0.03$ ,  $p = 0.004$ ), but are not different from each other ( $p=0.69$ )). In contrast, flies with silenced APL during CS+ presentation showed homogeneous performances with that observed for controls groups (ANOVA:  $F_{2,33} = 0.21$ ,  $p = 0.81$ ). Furthermore, we compared

performances across conditions through a linear model and confirmed a significant contrast of APL silencing during CS- presentation compared to APL silencing during CS+ presentation ( $F_{5,67}= 5.7, p=0.020$ ).

a)



b)



c)

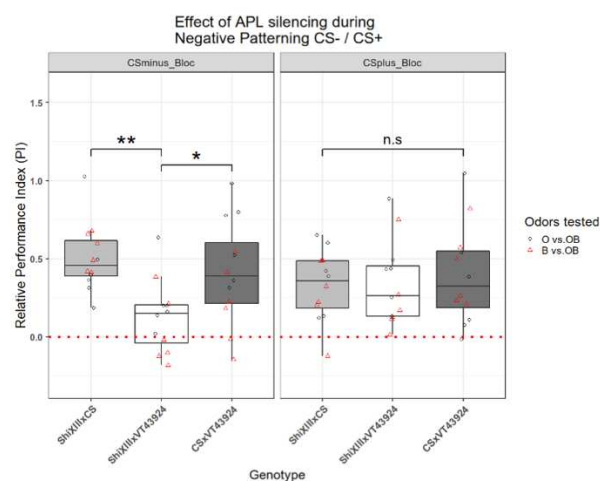


Figure 4 : a) Top: Time course of temperature variations during 1 cycle and 5 cycles training/test procedures. bottom: Effect of APL blocking on 1 cycle and 5 cycles Negative Featuring. Performances are compared between genotypes within displayed subsets (i.e. within the 1 cycle NF condition).

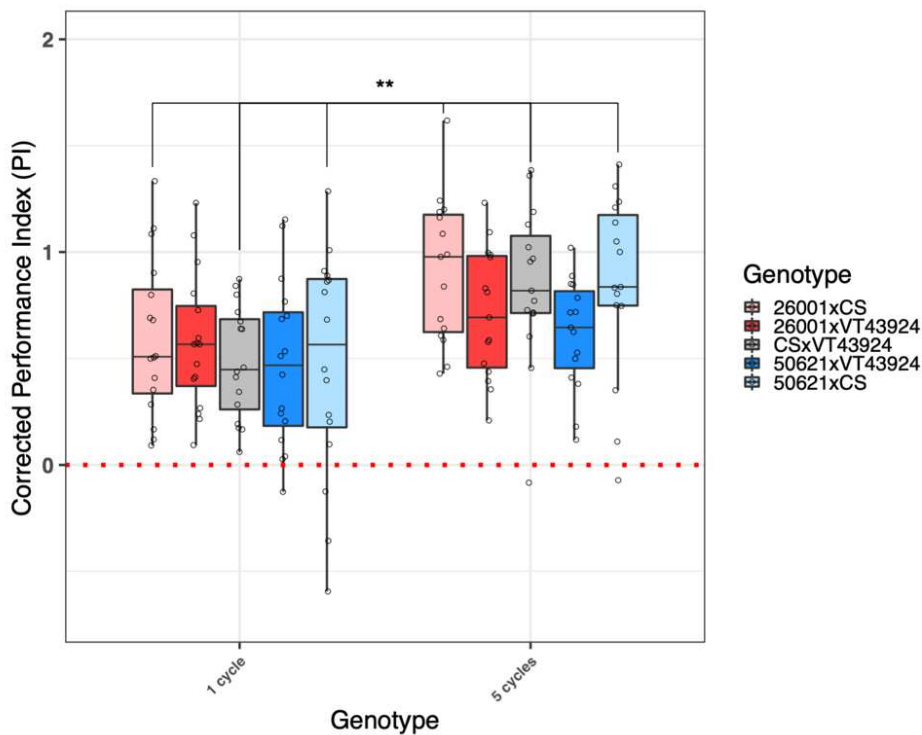
(b) Protocol used to block neurons only during CS+ or CS- presentation. c) Effect of APL silencing during CS+ or CS- presentation on Negative Patterning. Data were compared between genotypes within displayed subsets (e.g. Within the « Csmenus\_Bloc » condition). All data are Relative performance indices (PI) computed as the difference between paired and unpaired indices.

“n.s.” stands for “non-significant”, \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$  )

## APL neurons activity modulation through a D2 dependent pathway facilitates ambiguous learning

The core mechanism developed in this study relies on a differential APL activity depending on whether the presented stimulus is associated with a reinforcement (CS+) or not (CS-). Interestingly, previous studies showed that APL neurons express an inhibitory D2 dopamine receptor type<sup>[208]</sup>. APL D2 receptors have been shown to be responsible for the suppression of APL activity during odour/shock pairing via the activity of Dopaminergic subsets of neurons involved in reinforcement encoding<sup>[170]</sup>. Thus, D2-mediated APL modulation could mediate gradual learning enhancement through KCs activity modulation. To test this hypothesis, we expressed two distinct D2-RNAi lines (See method part for lines stocks detail) in APL neurons (VT43924-Gal4). Flies were trained using a Negative Feature discrimination protocol of either 1 or 5 training cycles. Data were analyzed through a linear model and are presented in Fig5. Within this model, the overall effect of training repetition was found to significantly enhance performances ( $F_{1,136} = 23.3$ ,  $p=3.6 \times 10^{-6}$ ). Moreover, no effect of genotype was found ( $F_{3,136} = 0.94$ ,  $p=0.42$ ) which allowed to compare pooled data including all control situations and both Rnai lines. Then, we used planned contrasts with a Tukey's adjustment to compare all relevant groups, that is, which shared at least one feature (for instance, controls were compared across 1 vs 5 cycles, and controls and mutants were compared within 1 cycle or 5 cycles but we didn't compare 5 cycle mutants with 1 cycle controls). Within these comparisons, training repetition enhanced control ( $p=7 \times 10^{-4}$ ) but not D2 knocked-down flies ( $p=0.42$ ). Moreover, we didn't find any difference between 1 cycle controls vs D2knocked-down flies ( $p=0.95$ ) whereas 5 cycle controls were found significantly different from D2 knocked-down flies ( $p=0.01$ ). Together, these results suggest that D2 receptors knockdown does not impair 1 cycle performances but prevents flies learning enhancement through repetition.

Taken together, our experimental results all verify the model's prediction which strongly support a D2-mediated differential APL neurons recruitment during CS+ vs CS- presentation as the likely mechanism to implement KCs activity modulation leading to ambiguous learning resolution.



*Figure 5: Effect of APL-D2 receptors inhibition on Negative Featuring. Relative performance indices (PI) computed as the difference between paired and unpaired indices. Data were compared between 1 cycle, 5 cycles and across genotypes.*

*“n.s.” stands for “non-significant”, \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$*

APL neurons are not required for Positive Patterning

In order to further test our model validity, we verified additional predictions arising from KCs activity modulation.

NP experiments are often complemented with Positive Patterning (PP) experiments, in which animals are exposed to the compound AB with reinforcement while both A and B are presented without reinforcement (AB+ A- B-). Interestingly, PP generally leads to better learning performances than NP, as shown in past studies both on rodents and humans<sup>[186,187]</sup>. By simulating PP with KCs activity modulation, we found that PP training led to robust simulated learning performances with only small improvement over training cycles (SuppFig4



a). We also tested Positive Feature discrimination (AB+ A-), as a mirror from the NF experiment. In that case, simulated performances were overall better than NF performances, although we did predict an improvement over training cycles. We verified APL neurons involvement in these tasks by inhibiting their activity using Shibire<sup>ts</sup> in the same procedures as used in Fig1 (SuppFig4b). As predicted, APL neurons inhibition did not affect PP performances after 5 cycles (ANOVA,  $F_{2,38} = 1.33$ ,  $p = 0.28$ ). We obtained mixed results regarding PF, where one control group was different from the APL inhibited flies while the other was not (ANOVA,  $F_{2,44} = 5.37$ ,  $p = 0.008$ , Tukey HSD post hoc test : Only one control is significantly different from the UAS-Shits/VT43924-Gal4 ( $p = 0.59$ ,  $p = 0.007$ ), while controls are not different from each other ( $p=0.08$ )). In any case, PP, and, to a lesser extent PF were shown to elicit robust performances and be less affected by APL inhibition than what was observed with their negative counterparts, consistently with the model predictions.

#### A sparse coding-independent mechanism

APL neurons, and their locust counterpart, the GGNs, also have a notorious sparse-coding function, ensuring that each stimulus is represented by few KCs, making their discriminability easier<sup>[171,203]</sup>. How to dissociate sparse-coding and KC's activity modulation repetition to account for the flies NP abilities? First, sparse-coding is especially prominent for discrimination. In the case of a classical associative learning paradigm, discrimination occurs during test phase as CS+ and CS- are presented simultaneously. In contrast, during training, CS+ and CS- are presented sequentially, which is why sparse-coding may not be as important during training than it is during test. Moreover, a recent study showed that APL activity is of two kind<sup>[209]</sup>. APL provide a global feedback inhibition of the whole population of KCs and a local lateral inhibition which might mediate a finely tuned inhibition. Global inhibition is likely to generate sparse coding whereas local inhibition could support KCs activity modulation through repetition. In any case, it is important to stress the fact that sparse coding alone is a gain control mechanism and cannot explain a gradual improvement of NP. We verified this by simulating learning with different combinations involving (or not) sparse coding, KC's activity modulation or both (SuppFig5). The results show that without APL activity, DC and NF are still learnt although without any improvement over trials. However, NP is not solved. Rather, the



compound elicits a significant level of repulsion, which is expected as the compound representation is almost entirely nested in the representation of its reinforced components. Interestingly, sparse coding alone improves discrimination and thus reduces the repulsion elicited by the compound during NP. However, APL inhibition during CS+ leads to overall decreased performances in all paradigms when only sparse coding is implemented, which is opposed to behavioural data and further confirms that sparse coding alone is insufficient to explain observed learning performances in vivo. Adding KC's activity modulation to the model introduces training repetition effects that can be separated in two categories: If APL are always fully activated, which leads to an inhibition of KC's activity during both CS+ and CS- presentation, all simulated performances tend to 0, which is expected as repeated inhibition of all odours' representations should lead to a decrease of behavioural responses altogether. Yet if APL are inhibited during CS+, performances are gradually improved over learning cycles and the condition where both KC's activity modulation and sparse coding are implemented in addition with APL inhibition during CS+ represents the best fit with overall behavioural data.

#### An alternative model involving parallel and opposing learning

How else could NP learning be explained? Recent publications present incremental evidence that the presentation of a non-reinforced stimulus might, in fact, elicit associative learning of opposing valence compared to the reinforced stimulus. Indeed, one study focused on extinction, a learning paradigm during which an initially reinforced stimulus is subsequently repeatedly presented without reinforcement, leading the animal's conditioned response to fade. The authors showed that the initial learning trace was not erased during extinction. Rather, a parallel learning trace of opposite valence was formed<sup>[153]</sup>. One recent study shows that aversive long-term memory is actually composed of two additive constituents, an aversive memory for the CS+ and an attractive memory for the CS-<sup>[165]</sup>. Could NP gradual acquisition be explained by the formation of an opposing memory for the compound over the course of cycles? We tested this hypothesis by simulating training cycles during which CS- presentation led to a gradual depression of the synapses between the CS- KCs and the aversive MBON (while CS+ presentation led to a rapid depression of the synapses between the CS+ KCs and the appetitive MBON, as always). Data are presented in SuppFig6a.

Interestingly, this model also explains gradual acquisition of the NP and fits with most of our experimental data with normal APL activity. However, simulated APL inhibition during CS+ decreases learning performances in all training tasks (SuppFig6c) while APL inhibition during CS- does not impair NP acquisition (SuppFig4d), which is in exact opposition with behavioural results. Thus, the formation of opposing learning traces during training repetition cannot explain APL role in it.

## General discussion

To summarize, APL neuron's role in associative learning is not limited to gain control through their sparse coding function. Here we demonstrate the key role of APL inhibitory action on the KCs over the course of repeated learning events. Through this function, flies' representation of the world is constantly updated and refined based both on associative (CS+ reinforcement) and non-associative (CS alone) events. A straightforward implementation of APL differential inhibition of the KCs is through the modulation of the APL-KC synaptic weights, which could happen simply by coincidence detection between the APL and the KC firing, following a form of Hebbian rule. Coincidence detection between the APL and an individual KC could yield a potentiation of their synaptic strengths; and conversely, the firing of a KC without the APL would decrease the strength of their connection. Because APL is inhibited during US presentation<sup>[169,170]</sup>, only the KC responding to the CS-, but not CS+, should see their connection to the APL reinforced across repetitions.

Alternatively, potentiation of KCs' responses for the CS+ could be achieved either by octopaminergic signal released in the calyx during US presentation (as shown in bees<sup>[210]</sup> and flies<sup>[147]</sup>), or through depression of APL-KC firing synapse. Interestingly, multiple evidence in honeybee shows that CS+ representation is also reinforced upstream at the level of Antennal Lobes, which could participate in optimizing the contrast between CS+ and CS-<sup>[211-214]</sup>. In any case, the inhibition of the APL by the US is key to prevent simultaneous opposite effect on synaptic strength.

Interestingly, we describe a kind of habituation which, in contrast with the classical definition (i.e., a non-associative process through which naïve response is lowered), enables flies to

solve both configural and temporal ambiguities (e.g., negative patterning and reversal learning tasks, respectively)) by inhibiting the expression of maladaptive learnt responses. We demonstrate that Dopamine pathways play a critical role in the modulation of APL activity, explaining why APL inhibition of KC activity occurs specifically during the CS- but not the CS+ presentation. Previous studies showed that PPL1 neurons, involved in aversive US encoding, likely project onto APL neurons and inhibit their activity via D2 receptors<sup>[170]</sup>. Incidentally, studies showed that APL's honeybee homolog, the A3v neuron is also involved in both appetitive Negative and Positive Patterning<sup>[36]</sup>. Thus, it is possible that Dopaminergic neurons encoding appetitive US (e.g., neurons in the PAM subset<sup>[148]</sup>) also project on and inhibit APL activity upon appetitive reinforcement.

Until now, the main source of learning and memory engrams was thought to lie in the synapses between KCSs and MBONs. In this study, we suggest that engrams may also be located at the synapses between APL and KCs, the molecular bases of which are yet to be discovered. Whether such plasticity arise from coincidence detection, as classically shown in the Mushroom Bodies<sup>[198]</sup> or through other, yet uncharacterized mechanisms hints at exciting future research prospects. A recent review highlighted the importance of inhibitory engrams (i.e., learning and memory traces in synaptic connectivity) in the formation of associative learning and memory<sup>[215]</sup>. Indeed, not responding to irrelevant stimuli is as important as correctly responding to appropriate stimuli. Because in ambiguous forms of training like NP, CS+ and CS- are intertwined, inhibitory pathways should be especially involved in learning the stimuli that the animal should or shouldn't respond to.

## Material & Methods

### Fly stocks

*Drosophila melanogaster* flies were reared in 25°C incubators under a 12: -12h light: -dark cycle. Flies were kept in 36x82 mm plastic tubes containing approximately 20mL of medium. Transgenic fly lines were outcrossed to a Canton-Special (CS) genetical background. Trained flies were 2-4 days old.

### Fly lines

UAS transgenes expression was driven specifically in APL neurons using the VT43924 gal4 line<sup>[207]</sup>. Conditional neuronal disruption was achieved using flies harbouring a double insertion of UAS-shibire<sup>ts1</sup> <sup>[194]</sup>. Dopamine receptor D2 knockdown was performed using Bloomington's 26001 and 50621 RNAi lines. To induce Gad and Tβh knockdown, we used the VT32344 and the VT51667 lines, respectively. In addition, in the case of Gad and Tβh, conditional knockdown was ensured by crossing these lines with tubGal80ts; VT43924 lines.

### Olfactory conditioning

Discriminatory olfactory aversive conditioning was performed according to a previously described protocol<sup>[188]</sup>. All procedures were performed using a semi-automated conditioning device<sup>[188]</sup> adapted from a previous study<sup>[184]</sup>. Odours used were 3-Octanol (termed 'A', 2.27 mM) and Benzaldehyde ('B', 1.89 mM) diluted in bottles of mineral oil. Odours were always delivered at the same concentration, but could either be presented as single components or diluted together in the same bottle, thus forming mixtures (e.g., 'AB'). Training trials consisted of 90s of acclimatisation, followed by one of the conditioning protocols described Fig1a. Odours were delivered as 2L/min air flows for 1 min. Reinforced olfactory stimuli (CS+) were paired with the US, which consisted of 12 pulses of 1.5s of 60 V electric shocks every 5s delivered through a metallic grid. The non-reinforced stimuli (CS-) were subsequently presented without US with an intertrial interval of 1 min. This sequence, considered as one conditioning cycle, was repeated either 1 or 5 times. The whole procedure was called "Paired" because of the explicit pairing of the CS+ with the US. Importantly, each paired replicate was complemented with an "Unpaired" procedure performed in parallel on another group of flies.

In that case, animals were exposed to the same stimuli used in the paired procedure, but the presentation of the US and CS+ were decorrelated. The unpaired procedure hence served as a control for experimental bias. For each experiment, flies whose performances were compared according to genotype were trained in parallel using the same experimental devices.

## Test

Following conditioning, flies were transferred to a T-maze where they could move freely between two compartments where previously learnt CS+ and CS- were delivered as 0.8L/min air flows for 1min without US. In the case of patterning protocols, as there are more than two trained odours, half of the tests were performed using one CS+/CS- combination (e.g., A vs AB), while the other half was performed using the alternative CS+/CS- combination (e.g., B vs AB). Flies exposed to paired and unpaired protocols were sequentially tested. After 1 min, flies in each arm of the T-Maze were isolated and counted. Performance Index (PI) was computed as (number of flies in the CS- arm – number of flies in the CS+ arm) / total number of flies). One Relative PI consisted of a 'paired group' PI from which the associated 'unpaired group' PI was subtracted<sup>[188]</sup>.

## Conditional neuronal manipulation using thermal treatments

In order to disrupt neurotransmission specifically during conditioning phase, flies were placed at 33°C 15 min before and during conditioning. Afterwards, flies were placed at 25°C for 15 min before and during test. To restrict Shibire disruption to the CS+ or CS- phase, we used a previously described time course<sup>[216]</sup>, also described in Fig4a. For conditional RNAi knockdown mediated by TubGal80ts, flies were placed at 30°C 5 days before conditioning. Training and test were performed at 25°C.

## Statistical analysis

Statistical analyses were performed using R software (4.0.2 v.). Data were plotted using the following packages: ggplot2, ggsignif, ggpubr, ggthemes, gridExtra, cowplot, magrittr. Data were plotted as boxplots. Raw data were superimposed as jittered dots and when different odour pairs were involved, tested odours were identified by dots shape and colour. Statistical

analyses were performed using the following packages: AICcmodavg, Emmeans. One-sample tests against chance level were performed using one sample two-tailed Student's tests. To compare relative performances of flies carrying both the UAS regulated transgene and the Gal4 regulator with controls carrying only UAS or Gal4 construct, we used analysis of variance (ANOVA) followed by Tukey's (HSD) post hoc tests. In each analysis, several statistical models were built taking into account all potential sources of variability such as experimental device, day of experiment, odours used and their interactions. Best matching model was selected using the Akaike Information Criterion (AIC) and tested for normality and homoscedasticity. Multi-factor analyses were performed using linear models. Here again, several possible models were built and best match with experimental data was selected using the AIC indicator and checked for normality and homoscedasticity. When appropriate, pairwise planned comparisons were performed with a Tukey's adjustment afterwards. A significance level of  $p < 0.05$  was set for every experiment.

#### Computational model description

Virtual olfactory stimuli are generated as random glomerular binary patterns with a fixed 0.69 proportion of glomeruli activated, based on electrophysiological recordings of antennal lobes<sup>[217]</sup>. Because olfactory stimuli are integrated in  $\sim 49$  glomeruli<sup>[103]</sup>, each odour is defined as a list of 49 zeros and ones, which is a fairly accurate approximation of odour combinatorial code in *Drosophila* brain<sup>[218]</sup>. For each simulated glomerulus, its attributed value is generated from a Bernoulli trial with a probability of 0.69 to be 1 and a probability of  $1 - 0.69$  to be 0. Compound stimuli are generated as the normalized sum of the glomerular patterns of both components, as already described in a previous model<sup>[182]</sup>. Each activated glomerulus then transmits a signal to the KCs through an average of 3 PNs<sup>[131]</sup>. Therefore, the initially generated list of 49 values becomes a list of 147 values. PN to KC connectivity is assumed to be random and each KC receives the input from an average of 6.5 PN<sup>[145]</sup>. In other terms, among the 294 000 ( $2000 \times 147$ ) potential PN/KC synapses, an average of 13 000 are actually formed. Thus, KCs activities are modeled as a list of 2000 values corresponding to the KCs number in a MB hemisphere<sup>[219]</sup>, and PN to KC connectivity is modeled as a matrix of 0 and 1 with a 0.044 ( $13\ 000 / 294\ 000$ ) probability for each PN/KC synapse to happen.

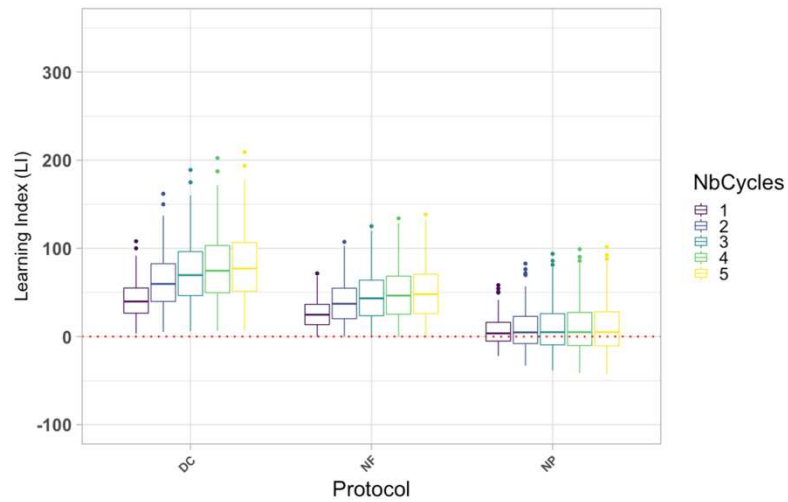
In a way, each connectivity matrix may be regarded as a unique individual. Finally, the resulting activated KC population is sparsened by the inhibitory activity of the APL neuron which provides global inhibition scaled on the overall KC activity, eventually applying a threshold that only leaves an average of 8,4 % of the KC activated<sup>[172]</sup>. Here, we modeled APL inhibition by dividing each single KC activity by an empirically estimated value so that between 5 and 10% KC remain active after inhibition.

Thus, each odour is identified by a pattern of approximately 168 KC ( $0.084 \times 2000$ ). Moreover, similarity between 2 odours may be computed as the Hamming distance between their activity pattern<sup>[125]</sup>, which is the ratio between the amount of commonly activated cells and either the amount of cells activated by the two odours combined (absolute similarity) or the amount of cells activated by one odour in average (relative similarity). Finally, all KC are matched with 2 MBONS, one driving avoidance while the other mediates approach. Each MBON is modelled as its synaptic weights with each KC. Thus, MBON are initialized with every synapse at 1. Upon an aversive associative learning, synaptic weight between the CS+ associated KCs and the approach MBON are depressed, switching their synaptic weights down, while leaving the avoidance MBON untouched. In contrast, when the CS- is presented without reinforcement, no change occurs in synaptic weights. Approach or avoidance behaviour toward each stimulus is computed as the difference of activity between avoidance and approach MBONS ( $\Delta$ MBON) elicited by the activation of the stimulus associated KCs. During test, the  $\Delta$ MBON associated with the CS+ is compared with the  $\Delta$ MBON associated with the CS- and a simulated learning index is computed as the relative  $\Delta$ MBON between CS+ and CS-. Model's architecture is displayed in Fig1b, along with an example of learning event Fig1c-left.

We simulated  $\Delta$ MBON activity for 100 random odour pairs and their compound in 3 different behavioural tasks with increasing ambiguity, including a typical Differential Conditioning (DC), Negative Feature discriminations (NF) and Negative Patterning (NP). Learning paradigms are summarized in Fig1a. Each conditioning protocol was repeated up to five times (i.e., 5 conditioning cycles). Generated Learning Indexes (LI), computed as the relative  $\Delta$ MBON between CS+ and CS- are displayed Figure1d-left.

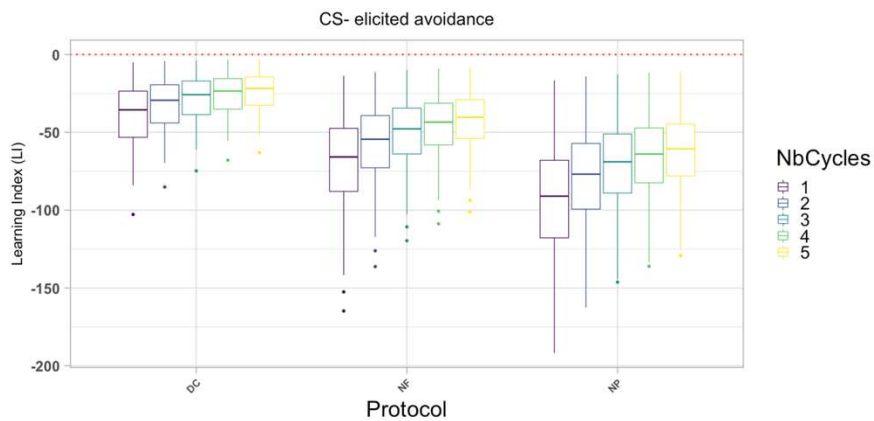
## Supplementary Material

### Supplemental figure1 : Classical model simulations with gradual learning rates



*Supplementary Figure 1 : Simulated learning performances in Differential Conditioning (DC), Negative Featring (NF), and Negative Patterning (NP) over the course of 5 trials with a gradual learning acquisition rate. Data are expressed as relative  $\Delta$ MBON activity resulting from CS+ vs. CS- presentation.*

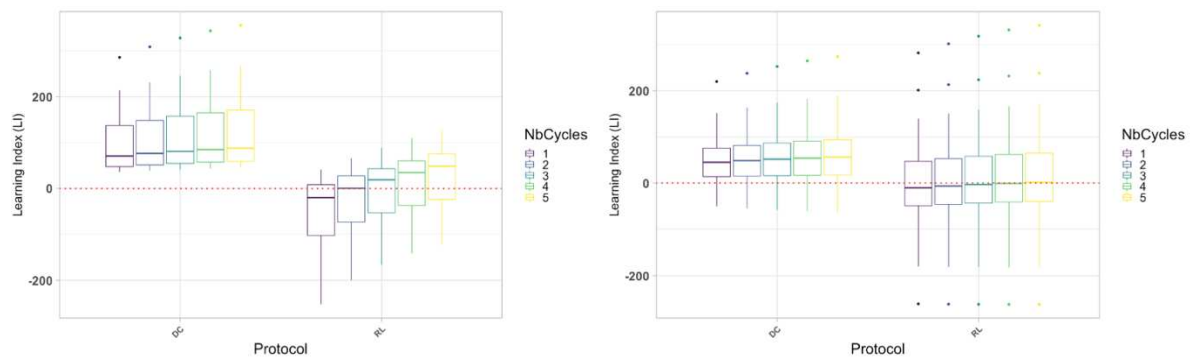
### Supplemental figure2 : CS- elicited avoidance after repeated KC's activity modulation



*Supplementary Figure 2 : Simulated avoidance toward the CS- elicited by Differential Conditioning (DC), Negative Featring (NF), and Negative Patterning (NP) over the course of 5 trials. Data are expressed as relative  $\Delta$ MBON activity resulting CS- presentation.*

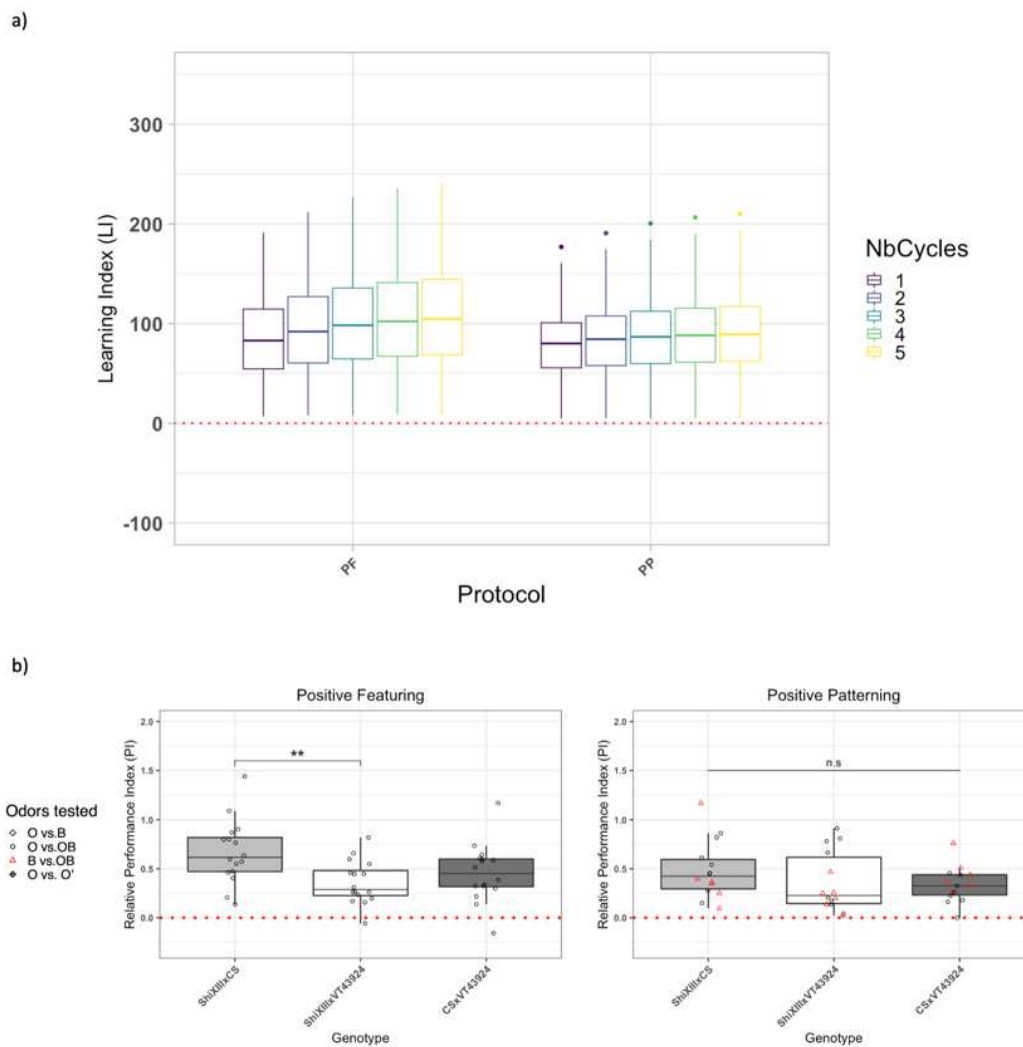


### Supplemental Figure3: Reversal learning simulation with and without KC's activity modulation



*Supplemental Figure3: Simulated performances in a Differential Conditioning (DC) followed by a Reversal Learning (RL) with (a) and without (b) KCs activity modulation through repetition. Theoretical performances are expressed as relative  $\Delta$ MBON activity resulting from CS+ vs. CS- presentation. Learning was assessed over the course of 5 conditioning cycles. N=100.*

Supplemental Figure4: Simulated and measured Positive Featuring and Positive Patterning

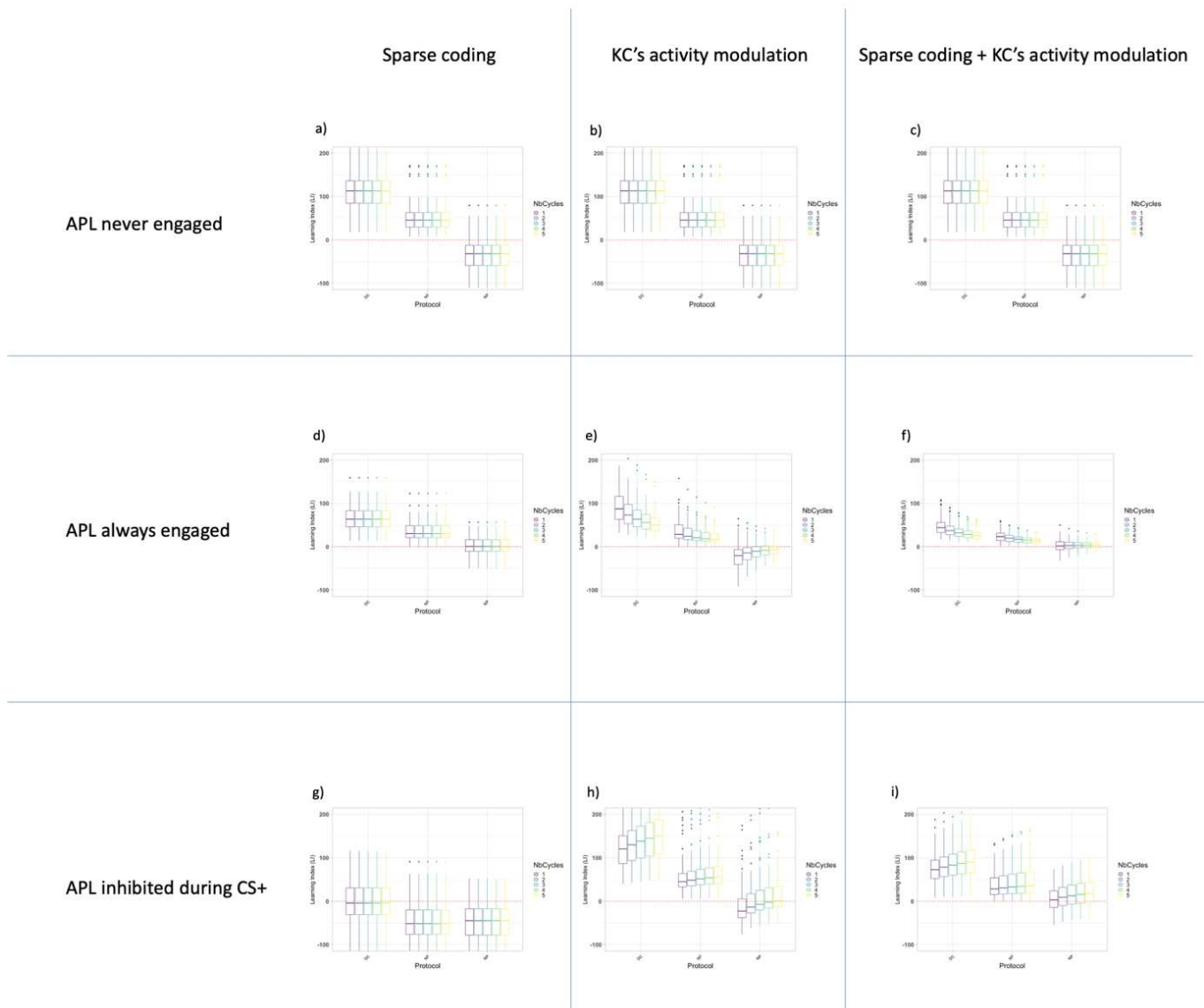


Supplementary Figure4: a) Effect of inhibition through repetition on Positive Patterning (PP) and Positive Featuring (PF) learning performances : Theoretical performances expressed as relative  $\Delta$ MBON activity resulting from CS+ vs. CS-presentation. Learning was assessed over the course of 5 conditioning cycles. N=100.

b) Effect of APL neurons targeted inhibition on PP and PF performances. Relative performance indices (PI) computed as the difference between paired and unpaired indices. Performances are compared between genotypes within the same paradigms but not between paradigms (i.e. PP vs. PF).

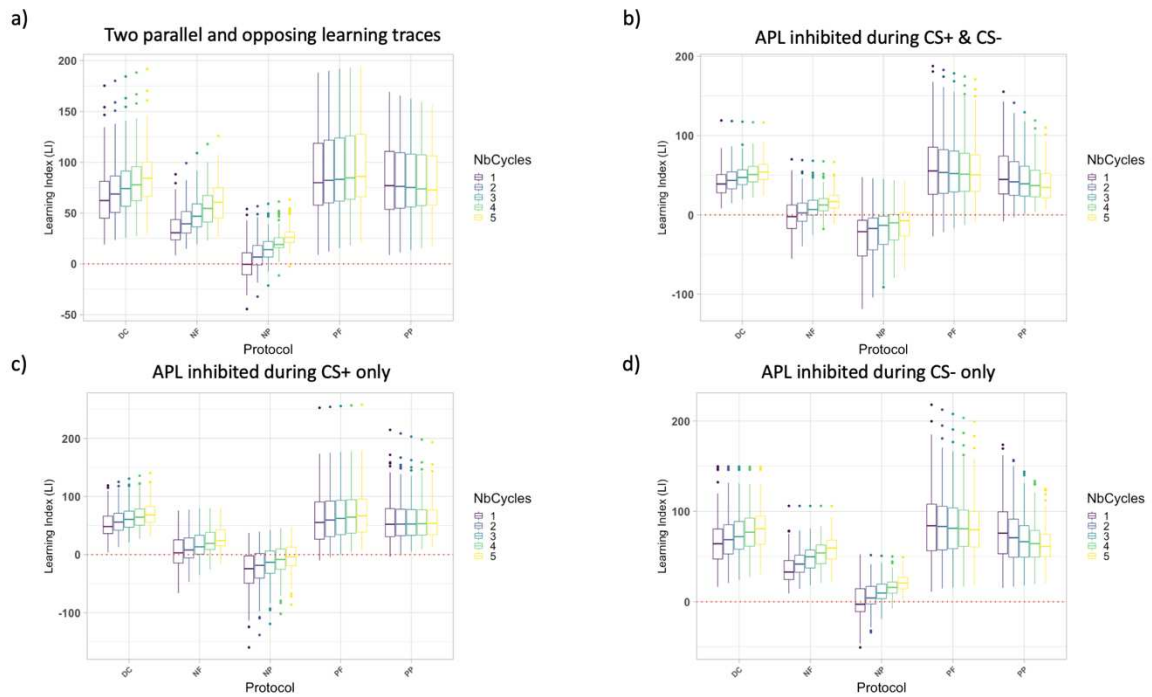
"n.s" stands for "non-significant", \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$

Supplemental Figure5: Relative involvement of Sparse coding and KC's activity modulation on associative learning acquisition



Supplementary Figure5 : Relative contribution of sparse coding and KC's activity modulation to Differential Conditioning (DC), Negative Feature discrimination (NF) and Negative Patterning (NP) acquisition. Data are expressed as relative  $\Delta$ MBO activity resulting from CS+ vs. CS- presentation. The simulations were run either without any APL activity (a, b, c), with APL activity both during CS+ and CS- (d, e, f) or only during CS+ (g, h, i). 3 conditions were tested where APL only mediated sparse coding (a, d, g), KC's activity modulation (b, e, h) or both (c, f, i).

Supplemental Figure6: Alternative model of parallel opposing learning traces



Supplemental Figure6 : Model of two parallel and opposing learning traces. Simulated learning performances in Differential Conditioning (DC), Negative Featuring (NF), Negative Patterning (NP), Positive Featuring (PF), Positive Patterning (PP) over the course of 5 trials. Data are expressed as relative  $\Delta$ MBON activity resulting from CS+ vs. CS- presentation. The simulations were run either with normal APL (a), APL inhibited during CS+ and CS- (b), only during CS+ (c) or only during CS- (d).



## Chapter III: Modulation of the discrimination- generalization trade-off through Differential Conditioning

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## Introduction

Associative learning's purpose is to establish robust predictive links between environmental cues. However, outside of the laboratory, perceived features previously involved in an associative learning event are almost never encountered again in the exact same conditions they were originally met with. Therefore, animals need to be able to apply previously learnt associations in similar but not identical contexts, an ability called Generalization. In other situations, perceived stimuli may be associated with distinct outcomes, which also requires the capacity to respond differently to similarly perceived cues, an ability called discrimination. Generalization and discrimination have an intricate relationship, which can be referred to as a trade-off, as an increase in one lead to a decrease of the other. Learning the association between a stimulus and a reinforcement spontaneously produces a 'response gradient' around the stimulus, which means that similar stimuli will also elicit a response, the magnitude of which is correlated to the degree of similarity with the originally presented stimulus<sup>[26,220]</sup>. Thus, part of the generalization / discrimination trade-off is learning-independent and directly related to perceptual aspects. Interestingly though, the generalization/discrimination trade-off may also be modulated by associative learning events. Multiple learning experiences have been shown to additively modulate the response gradients of previous associations<sup>[20]</sup>. Moreover, conspicuous stimuli are more likely to be associated with reinforcements and tend to conceal less noticeable ones, a phenomenon referred to as Overshadowing<sup>[221]</sup>. In the brain, stimuli are generally encoded as patterns of cerebral activity scattered across neural units. The degree of overlap between two stimuli-associated activity patterns is directly related to their perceived similarity<sup>[127,222]</sup>. Previous works showed that associative learning may modulate learnt stimuli-associated activity patterns enhancing discriminability<sup>[211,212,223,224]</sup>. However, the elucidation of the molecular and cellular mechanisms underlying the modulation of stimulus representation is still an open question. Here we explored how associative learning events may optimize the generalization/discrimination trade-off based on past experiences.

Fruit flies are gifted with a fine olfaction. As such, they constitute a robust model for olfactory discriminative learning, the neural correlates of which can be addressed using state-

of-the-art genetically encoded tools. Flies show remarkable discrimination abilities arising, in part from a sparse spatio-temporal encoding of olfactory stimuli within the Mushroom Bodies (MB), a major associative learning brain structure in insects<sup>[128]</sup>. Each olfactory stimulus is encoded by a small subset of Kenyon Cells (KCs), which are the building blocks of the MBs<sup>[172,219]</sup>. Upon association with a reinforcement, the activity of every KC associated with the stimulus is strengthened<sup>[151]</sup>. Importantly, the reinforced (CS+) and the non-reinforced (CS-) stimuli may share commonly associated KCs, which means that the CS+ reinforcement may lead flies to respond, to some extent, to the CS- as well. Therefore, the perceived similarity between CS+ and CS- and hence, flies' ability to specifically respond to the CS+ depends on how many KCs are activated by the CS+ only, compared to how many are activated by both the CS+ and the CS-.

Flies' discrimination abilities do not depend solely on the reinforcement of the CS+. In fact, the explicit presentation of a CS- during training generates conditioned inhibition which increases the contrast (and thus the discriminability) between CS+ and CS-<sup>[225]</sup>. A single pair of inhibitory neurons, the Anterior Paired Lateral (APL) neurons, was demonstrated to be key in the sparse encoding of odours within the MBs, minimizing CS+ and CS- representations overlap<sup>[171]</sup>. Moreover, APL's disruption impairs similar but not dissimilar stimuli discrimination<sup>[171]</sup>. APL were also demonstrated to be involved in conditioned modulation of odour representation during associative learning<sup>[169]</sup>, which was shown to be crucial for complex forms of learning such as Reversal Learning<sup>[179]</sup> and Negative Patterning (Durrieu et al., 2021, in prep). Interestingly, training repetition gradually reduces the degree of generalization displayed by trained flies<sup>[188]</sup>. Drawing on this set of evidence, we investigated how associative processes differentially elicited by the CS+ and CS- presentation and their representation's modulation by APL neurons may modulate the discrimination/generalization trade-off.



## Material & Methods

### Fly stocks

*Drosophila melanogaster* flies were reared at 25°C under a 12: -12h light: -dark cycle. Flies were kept in 36x82 mm plastic tubes containing approximately 20mL of medium. Wild-type flies were Canton-Special (CS) strains. In order to disrupt neuronal transmission in APL neurons, transgenic flies were generated by crossing flies carrying a double insertion of UAS-shibire<sup>ts1</sup> [194] with flies expressing VT-43924-gal4<sup>[207]</sup>. Both lines were outcrossed to a (CS) genetical background. All tests were performed with 2-4 days old flies.

### Olfactory conditioning

Discriminatory olfactory aversive conditioning was performed according to a previously described protocol<sup>[188]</sup>. All procedures were performed using a semi-automated conditioning device adapted from a previous study<sup>[184]</sup>. 3-Octanol (termed 'A', 2.27 mM) and 1-Octen-3-ol (termed 'A', 1.97 mM) were used as similar odorants along with Benzaldehyde ('B', 1.89 mM) as dissimilar control. 4-Methylcyclohexanol (termed 'C', 2.62 mM) was used as a novel odorant. All odorants were diluted in bottles of mineral oil. For experiments involving graded ratios, A and B at concentrations stated above were mixed together as performed in previous studies<sup>[171,211]</sup> with 3 conditions: 90:10 vs 10:90 A:B, 80:20 vs 20:80 A:B or 70:30 A:B vs 30:70 A:B.

The aversive reinforcement (US) consisted of 12 pulses of 1.5s 60V electric shocks every 5s delivered through a metallic grid. For each experiment, flies whose performances were compared according to genotype were trained the same days using the same experimental devices.

### Training

Training trials consisted of 90 s of acclimatisation, after which odours were sequentially delivered as 2L/min air flows for 1 min with a 1 min break between each odour presentation. Reinforced olfactory stimuli (CS+) were paired with the US, while the non-reinforced stimuli (CS-) were presented without US. This protocol is called "Paired procedure". In parallel, distinct groups were conditioned using an alternative Unpaired procedure in which flies were

exposed to the US alone for 1 min, followed by 1 min presentation of each odour alone, separated by 1 min breaks. In both cases, this sequence, considered as one conditioning cycle, was repeated 1 or 5 times. For absolute conditioning experiments, CS- was replaced with bottles only filled with solvent.

For experiments involving Shibire-induced synaptic transmission disruption, flies were placed at 33°C 15 min before training or test and the whole conditioning procedure was also performed at 33°C. If synaptic transmission disruption was performed during training, flies were placed at 25°C for 15 min before testing their learning performances.

### Test

Following conditioning, flies were transferred to a T-maze where they could move freely between two compartments presenting either the CS+ vs. the previously encountered CS- (differential conditioning); or presenting the CS+ vs. a novel odorant (absolute conditioning). Odours in the T-Maze were delivered as 0.8L/min air flows for 1min without US. Flies exposed to paired and unpaired protocols were sequentially tested. After 1 min, flies in each arm of the T-Maze were isolated and counted. Performance Indices (PI) were computed as (number of flies in the CS- arm – number of flies in the CS+ arm) / total number of flies). One Relative PI consisted of a 'paired group' PI from which an 'unpaired group' PI was subtracted to account for experimental bias.

### Glomerular activity patterns similarity computation

We extracted the glomerular activation patterns in the Antennal Lobes associated with 3-Octanol (A), 1-Octen-3-ol (A'), Benzaldehyde (B) and the 3-Octanol/Benzaldehyde compound (AB) used in Negative Patterning experiments (Durrieu et al., 2021, in prep) using the DoOR database<sup>[118]</sup>. AB glomerular pattern was calculated as the normalized sum of A and B, following known characteristics of mixture processing in Antennal Lobes<sup>[123]</sup>. The distance between A and B, A and A', A and AB, B and AB associated glomerular patterns was computed as the ratio between the number of commonly activated glomeruli ( $A \cap B$ ) and either the sum of the glomeruli activated by the two odours ( $(A \cap B) / (A \cup B)$ ; absolute similarity) or the average number of glomeruli activated by these odours ( $(A \cap B) / ((A+B)/2)$ ; relative

similarity), all of which weighed by the average glomerular activity measured across all glomeruli.

Odour pair	Absolute similarity	Relative similarity
<b>A vs B</b>	0.21	0.35
<b>A vs A'</b>	0.48	0.65
<b>A vs AB</b>	0.57	0.73
<b>B vs AB</b>	0.43	0.60

A and B have a low level of similarity (absolute and relative). In contrast, A vs A' present twice the absolute and almost twice the relative similarity observed with A and B, which confirms their proximity. A vs AB show an even higher level of similarity, while B vs AB displays a similarity consistent with A and A'.

#### Statistical analysis

All statistical analysis were performed using R software (4.0.2 v.). Data were plotted using the following packages: ggplot2, ggsignif, ggpubr, ggthemes, magrittr. Data were plotted as boxplots on which raw data were superimposed as jittered dots. Statistical analyses were performed using the following packages: AICcmodavg, emmeans. One-sample tests against chance level were performed using one sample two-tailed Student's tests. To compare relative performances of flies carrying both the UAS regulated transgene and the Gal4 regulator with controls carrying only UAS or Gal4 construct, we used analysis of variance (ANOVA) followed by Tukey's (HSD) post hoc tests. In each analysis, several statistical models were built taking into account all potential sources of variability such as experimental device, day of experiment, odours used and their interactions. Best matching model was selected using the Akaike Information Criterion (AIC) and tested for normality and homoscedasticity. Learning performances comparisons of flies trained using odours mix with varying ratios were performed using a linear model followed by multiple planned comparisons, for which a Tukey's correction was applied. A significance level of  $p < 0.05$  was set for every experiment.

## Results

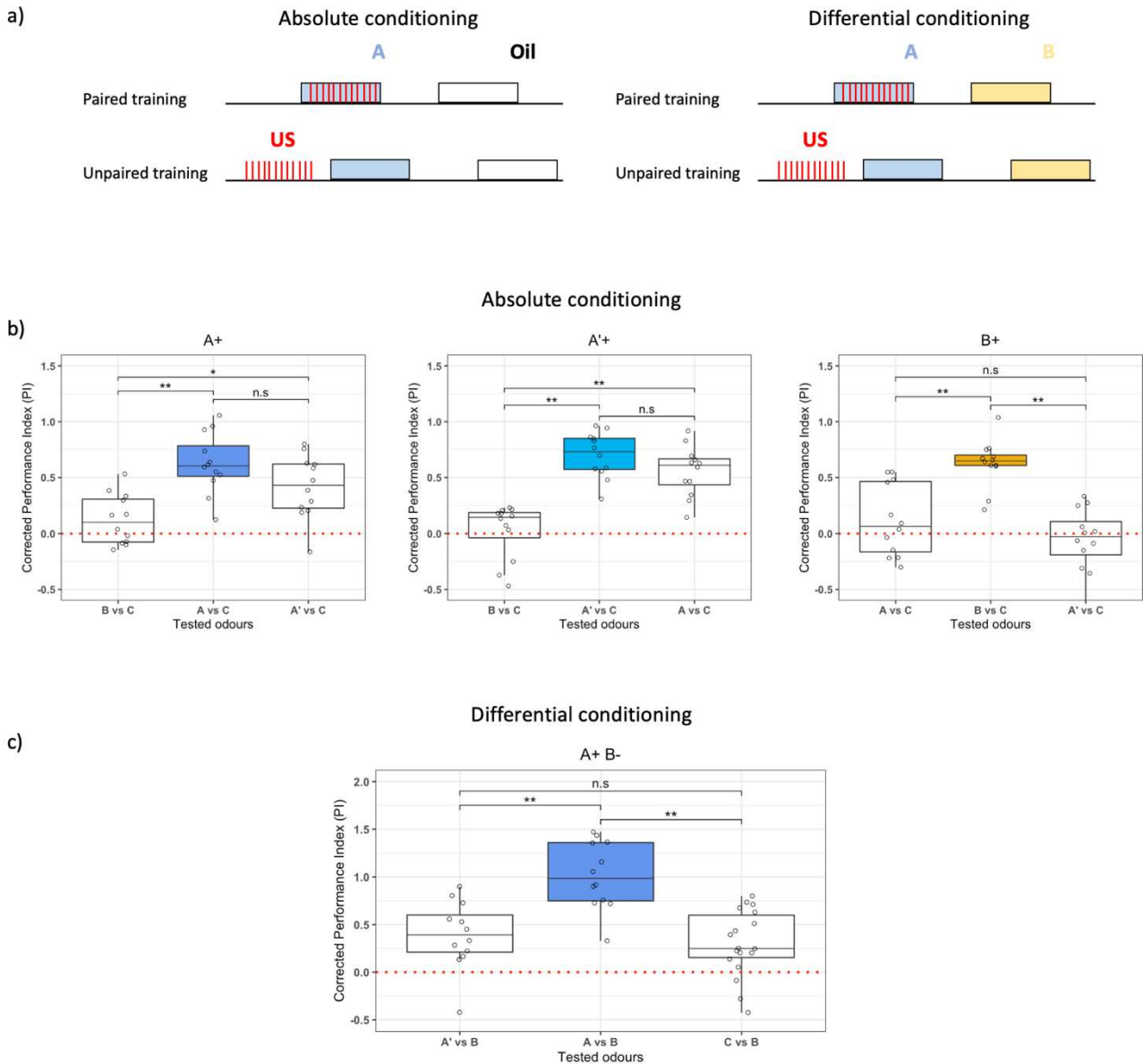
The following parts are three independent experiments where we investigate the role of the non-reinforced stimulus presentation in the modulation of the generalization/discrimination trade-off using complementary approaches.

### Differential conditioning suppresses acquired generalization

In a first approach, we selected two odorants previously shown to share a significant degree of similarity, 3-Octanol(A) and 1-Octen-3-ol (A')<sup>[225]</sup> and verified that these odorants were, indeed, similar for the flies. In other terms, we tested whether the association of one with an aversive reinforcement could elicit an avoidance response toward the other and vice versa through a generalization process. To do so, we trained flies with a single odour using an absolute conditioning procedure (details in Fig1 a) where flies were exposed to either one of the similar odours (A or A') or to a dissimilar odour (Benzaldehyde, B) associated with shocks. Subsequent flies' choices were tested in 3 conditions where flies were exposed either to the same odour they were trained with or to one of the two other odours they weren't exposed to. In all cases, the tested odour was presented along with a novel odorant (C) in order to balance the innate flies' behaviour. Data are presented Fig1 b). our results show that in both cases where flies were trained with one of the similar odours (A or A'), a robust generalization response toward the other (A' or A, respectively) was measured. Regardless of which one was reinforced, avoidance toward both odours was similar, but significantly higher than avoidance toward the dissimilar odour (For flies trained with A+ : ANOVA:  $F_{2,33} = 11.43$ ,  $p = 1.7 \times 10^{-4}$ ; a Tukey's HSD post-hoc test detected a significant difference between avoidance toward A vs B ( $p = 1.1 \times 10^{-4}$ ), A' vs B ( $p = 0.024$ ), but not A vs A' ( $p = 0.13$ ); For flies trained with A'+, ANOVA:  $F_{2,33} = 30.02$ ,  $p = 3.7 \times 10^{-8}$ ; a Tukey's HSD post-hoc test detected a significant difference between avoidance toward A vs B ( $p = 5.6 \times 10^{-6}$ ), A' vs B ( $p = 1 \times 10^{-7}$ ), but not A vs A' ( $p = 0.26$ )). In addition, flies trained with the dissimilar odour B showed a strong avoidance toward B but no generalization response toward A or A' (ANOVA:  $F_{2,33} = 19.98$ ,  $p = 2.1 \times 10^{-6}$ ; a Tukey's HSD post-hoc test detected a significant difference between avoidance toward A vs B ( $p = 2.1 \times 10^{-4}$ ), A' vs B ( $p = 2.3 \times 10^{-6}$ ), but not A vs A' ( $p = 0.29$ )). We concluded that A and A' could

be considered as presenting a perceptual similarity for the fly, thus confirming previously published data<sup>[225]</sup>. In a second approach, we exposed flies to a differential conditioning protocol where A was reinforced (A+) while B was presented without reinforcement (B-). Subsequently, flies were tested for their choice between A and B or A' and B to quantify any generalization response. In addition, flies' choice between B and a novel odourant C was tested as a negative control. Results are presented in Figure 1c). Strikingly, flies' avoidance toward A' was now analogous to their avoidance toward B, and both were significantly different from flies' avoidance toward A. (ANOVA:  $F_{2,39} = 16.14$ ,  $p = 7.8 \times 10^{-6}$ ; a Tukey's HSD post-hoc test detected a significant difference between avoidance toward A vs A' ( $p = 2.8 \times 10^{-4}$ ), A vs B ( $p = 9.0 \times 10^{-7}$ ) but not A' vs B ( $p = 0.77$ ). We also verified that flies exposed to a differential conditioning involving A' instead of A vs B- also elicited a suppressed generalization response, which was the case (SuppFig1).

Here again, our results are in line with previous data showing that Differential Conditioning enhances olfactory acuity<sup>[225]</sup>. Importantly, whereas the presentation of a CS+ followed by a CS- has been demonstrated to elicit a better discrimination between the same CS+ and CS-<sup>[225]</sup>, our experiment suggests that generally, presenting a non-reinforced odour after a reinforced one enhances the discrimination of the reinforced odour vs. any other odour.



**Figure 1 : Effect of differential conditioning on generalization response** a) Schematic representation of paired and unpaired absolute (left) and differential (right) conditioning procedures. b, c) Relative performance indices (PI) computed as the difference between paired and unpaired indices of flies exposed to either absolute (b) or differential (c) conditioning procedures and tested with different odour pairs. Performances are compared between tested odour pairs. "n.s" stands for "non-significant", \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$

APL neurons are required during a discrimination test but not during Differential Conditioning involving similar odours

CS- presentation has been shown to elicit an inhibitory memory trace<sup>[204,225]</sup>, which was suggested to be implemented by APL neurons and enable ambiguous forms of learning through odour representation modulation (Durrieu et al. 2021, in prep). Moreover, differential conditioning leads to a decorrelation of odour representations at the level of the

Mushroom Bodies' Kenyon Cells<sup>[225]</sup>. As the APL neurons are key in such decorrelation through their inhibitory activity, in a second experiment we tested APL involvement in a differential conditioning task involving A and A'.

We trained flies with a Differential Conditioning protocol using A as CS+ and A' as CS-. APL neurons activity was inhibited by driving the expression of UAS-Shibire<sup>ts</sup> with VT43924-Gal4. Inhibition of the APL was restricted either to the training or the test phase by placing flies at 33°C during the phase of interest (Fig1a). Flies with inhibited APL neurons during the training phase showed similar performances than controls (ANOVA:  $F_{2,33} = 1.407$ ,  $p=0.259$ ), which indicates that APL function is not required to learn this task. In contrast, inhibition of APL neurons during the test phase had a significant effect on flies' learnt performances (ANOVA:  $F_{2,33} = 14.85$ ,  $p=2.51 \times 10^{-5}$ ; Tukey' HSD test: Flies carrying both UAS and Gal4 constructs showed significantly decreased performances compared to both UAS and Gal4 controls ( $p=3.6 \times 10^{-4}$  &  $p=5.03 \times 10^{-5}$ , respectively), but no difference was found between controls ( $p=0.78$ )). Moreover, APL disruption during test prevented flies to discriminate A from A' (1 sample t.test did not show any difference with chance level (0):  $t=-1.08$ ,  $df = 11$ ,  $p=0.30$ ). When comparing flies' performances across the two conditions, we confirmed an interaction effect between the condition (APL inhibited during training or test) and flies' genotype (2 ways ANOVA:  $F_{1,68} = 7.261$ ,  $p=8.9 \times 10^{-3}$ ). As a control, we inhibited APL activity during test phase of a Differential Conditioning task involving dissimilar stimuli (A vs 4-Methylcyclohexanol, C, SuppFig2) and found no learning performances impairment in that case (ANOVA:  $F_{2,15} = 1.41$ ,  $p=0.28$ ).

This shows that APL inhibition during the discrimination test impairs flies ability to distinguish similar but not dissimilar odours (Fig2b, SuppFig2), which confirms previous results<sup>[171]</sup>. In contrast, APL are not required during the training phase even though the stimuli involved are perceptually similar.

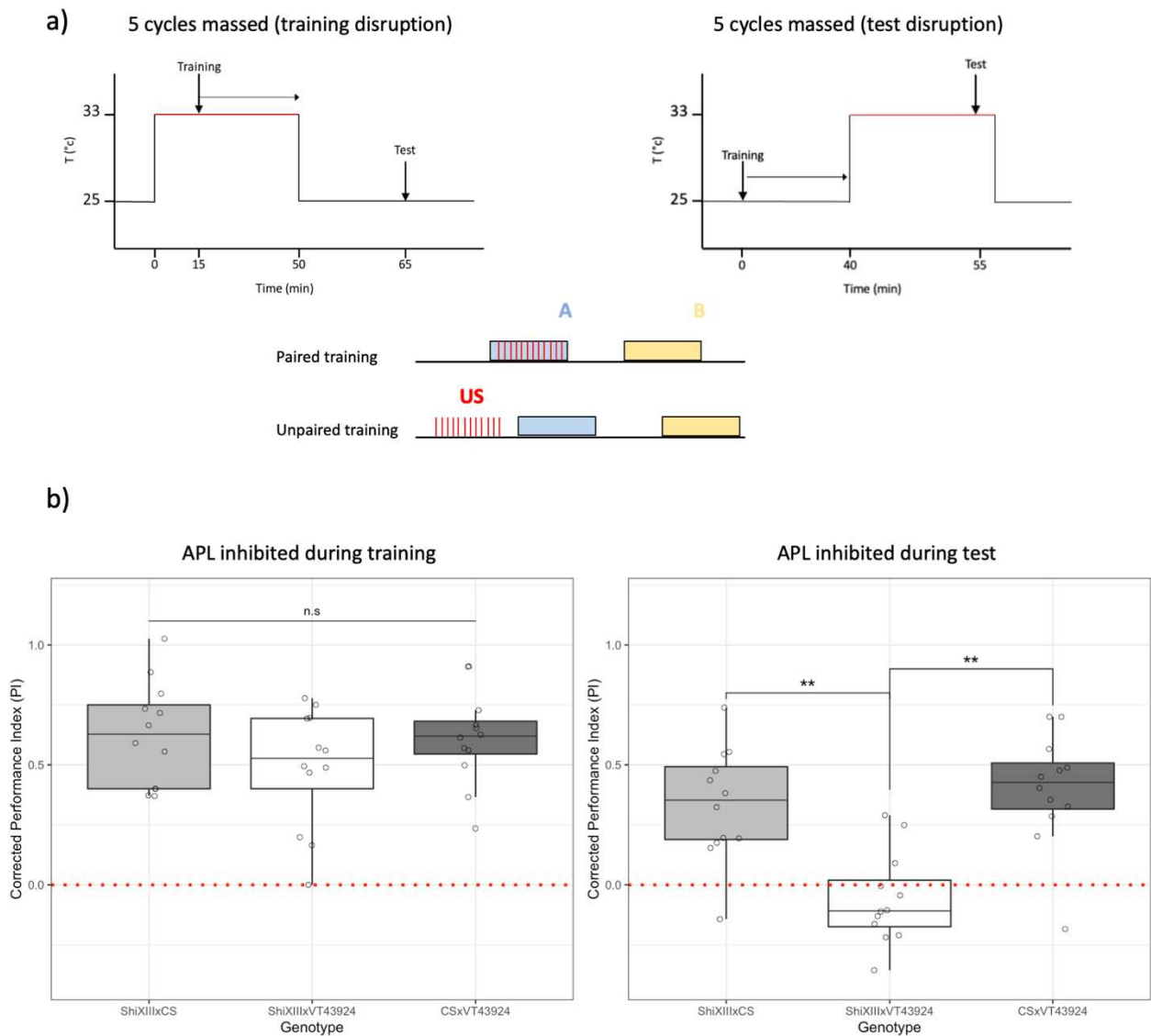


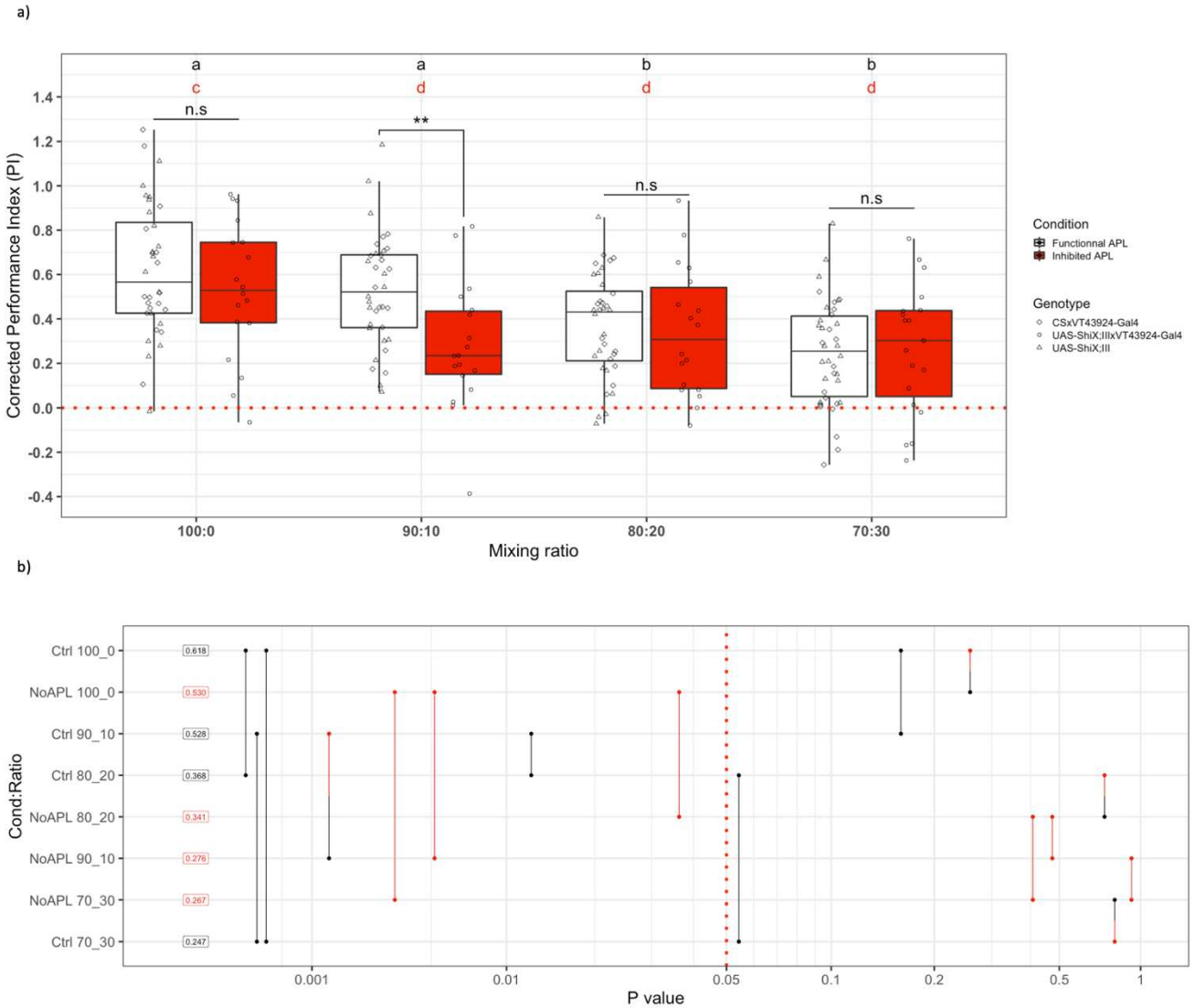
Figure2: Effect of APL disruption in differential conditioning involving similar stimuli. a ) Top: Time course of temperature variations during training/test procedures. Bottom : Schematic representation of paired and unpaired differential conditioning procedures. b) Relative performance indices (PI) computed as the difference between paired and unpaired indices. Performances are compared between genotypes within the same condition but not across conditions (i.e. APL inhibited during training v.s. test). "n.s" stands for "non-significant", \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$



APL neurons engagement is non-linearly correlated with stimuli similarity level.

Why would APL neurons be required for learning odour mixture discrimination such as A vs AB (Durrieu et al., 2021, in prep), but not for the discrimination of chemically close molecules (A vs A')? To answer this question, we first looked at the activity patterns triggered by these odours in the Antennal Lobes using experimental data uploaded on the DoOR database (see Material & Method for detailed procedure). Interestingly, odour mixtures show higher similarity than chemically close molecules. Thus, in a third and last experiment, we used odour mixtures to explore how APL involvement correlates with the degree of similarity shared by the CS+ and CS-. We argued that APL requirement during training might be determined by the degree of similarity between the CS+ and the CS-. In order to generate a controlled gradient of similarity, we mixed two dissimilar odorants with varying proportions. For instance, a A: B mixture with 90:10 respective proportions were used as CS+ and a A: B mixture with 10:90 proportions served as CS-. Similarly, 80:20 vs 20:80 and 70:30 vs 30:70 ratios were used. A 100:0 vs 0:100 condition was used as control. Here again, flies expressing UAS-Shi<sup>ts</sup> under the VT43924-Gal4 driver were exposed to 5 trials differential conditioning procedure, during which the training phase was performed at 33°C in order to reversibly disrupt APL activity. During test phase, APL neurons were always functional. Results are presented in Figure3. Learning performances were compared using a two-ways ANOVA taking into account the mix ratio and the condition (functional or inhibited APL neurons). Importantly, UAS-only and Gal4-only controls were pooled in order to avoid statistical power loss due to an excessive number of irrelevant comparisons. We checked the validity of this approach by testing the genotype effect in the ANOVA, which was shown to be non-significant ( $F_{1,210} = 0.563$ ,  $p = 0.454$ ). Following the ANOVA, data were compared following a planned contrasts procedure. We focused our analysis specifically on orthogonal contrasts, that is, comparisons between groups sharing at least one common level. For instance, all groups displaying functional APL were compared across the range of mix ratios, whereas in each mix ratio category, functional and inhibited APL groups were compared. However, groups that did not share a common level (e.g., Functional APL in the 100:0 category vs inhibited APL in the 90:10 category) were not compared. All comparisons and associated p-values are summarized in Figure2b. First, we found a significant effect of mix ratios on flies' performances with functional APL neurons. Indeed, 100:0 and 90:10 groups showed significantly higher

performances than 80:20 and 70:30 groups. Moreover, flies' performances generally seemed to decrease as the mix ratio converged towards 50:50, that is, became more similar across CS- and CS+. This result validated the mix ratios as a relevant model of incremental perceived similarity. Upon APL inhibition, flies exposed to dissimilar stimuli did not show any difference with the functional APL group. In contrast, when exposed to a differential conditioning involving 90:10 ratios, flies with inhibited APL neurons showed significantly reduced performances, that were lower than the dissimilar stimuli group but not different from the 80:20 and 70:30 groups. Surprisingly, we did not find any effect of APL inhibition on the 80:20 and 70:30 groups, for which we expected a greater engagement of APL neurons as they represented high-similarity groups. In sum, training flies with odour mixes of increasing similarity highlighted once more the impressive olfaction of flies as their behaviour was characterized by a smooth discrimination/generalization gradient nicely correlated with odours similarity. We expected APL neurons to be incrementally involved in differential learning as odours similarity increased. Instead, we found that APL inhibition specifically impacted a condition where odours displayed only a slight degree of perceived similarity for the fly. In fact, APL impaired flies displayed a stepped behaviour, showing robust discrimination when odours were not mixed, but, as soon as odours were even moderately mixed, flies' performances dropped and remained at the same level for every other degree of perceived similarity tested.



**Figure3: Effect of APL neurons inhibition on differential conditioning involving increasingly similar stimuli. a) Relative performance indices (PI) computed as the difference between paired and unpaired indices of flies trained with 5 trials differential conditioning procedures using either dissimilar (100:0) or increasingly similar mixes of odours (90:10, 80:20, 70:30). Performances are compared across ratios within the same condition (e.g. all groups in the “functional APL neurons” condition) in which case statistically different groups within the “Functional APL” (black) and “inhibited APL” (red) are symbolized by different letters. Performances are also compared between conditions within the same ratio (e.g. Functional APL vs inhibited APL in the 90:10 ratio). In that case, “n.s.” stands for “non significant”, “\*” indicates  $p < 0.05$ , “\*\*” indicated  $p < 0.01$ . b) Pairwise p-value plot summarizing all pairwise comparisons performed in terms of p-value. Colored labels are mean estimates of each group.**

## Conclusion

In this study, we demonstrate that the presentation of any non-reinforced odour during a differential conditioning seems to increase flies' discrimination abilities. What mechanisms could underlie this effect? Similarly to our results, a recent study on mouse fear conditioning showed that the presentation of a salient safety stimulus in addition to the punished one, enhanced mice discrimination abilities of the punished stimulus vs. any other one<sup>[226]</sup>. The authors suggest that such an effect could be linked to attentional processes. Indeed, absolute aversive conditioning of one stimulus could lead animals to exhibit exaggerated attention to the threatening stimulus, leading to an increased generalization. But if a second stimulus is also presented without reinforcement during the conditioning procedure, the attention bias associated with the CS+ could be counterbalanced, thus minimizing generalization. Such phenomenon has been extensively studied in vertebrates<sup>[227]</sup> and was also discussed in a honeybee study involving visual discrimination<sup>[228]</sup>. Thus, an interesting perspective would be to explore attention-like processes in *Drosophila* olfactory discrimination. Attention in *Drosophila* has been extensively studied in visual learning paradigms, as it is possible to measure objective features of attention-like processes like optomotor responsiveness<sup>[229]</sup>. In contrast, little is known about how attention might guide olfactory responses. Yet, classical memory mutants like *rutabaga* and *dunce* seem to affect attention-like processes which hints at shared circuitry between olfactory and visual sensory modalities<sup>[230]</sup>. What could be the neural correlates of attention-bias in *Drosophila* olfaction? Previous studies on rodent showed that auditory associative learning reshaped receptive fields in the auditory cortex through cholinergic modulation<sup>[231,232]</sup>. Similarly, a recent study on ALs modulation identified muscarinic cholinergic receptors as modulators of AL's local interneurons, as their manipulation shaped odour representation and flies' associated behaviour<sup>[233]</sup>. An interesting experiment would thus be to selectively inhibit AL's local interneurons' activity specifically during training phase but not test phase, by expressing *Shibire<sup>ts</sup>* under the control of *GH298-Gal4*. We expect to prevent differential conditioning involving similar but not dissimilar stimuli which would confirm the importance of odour representation shaping at the level of AL's during associative learning. Moreover, attention-based modulation of olfactory perception could also be imaged using fluorescent calcium probes.

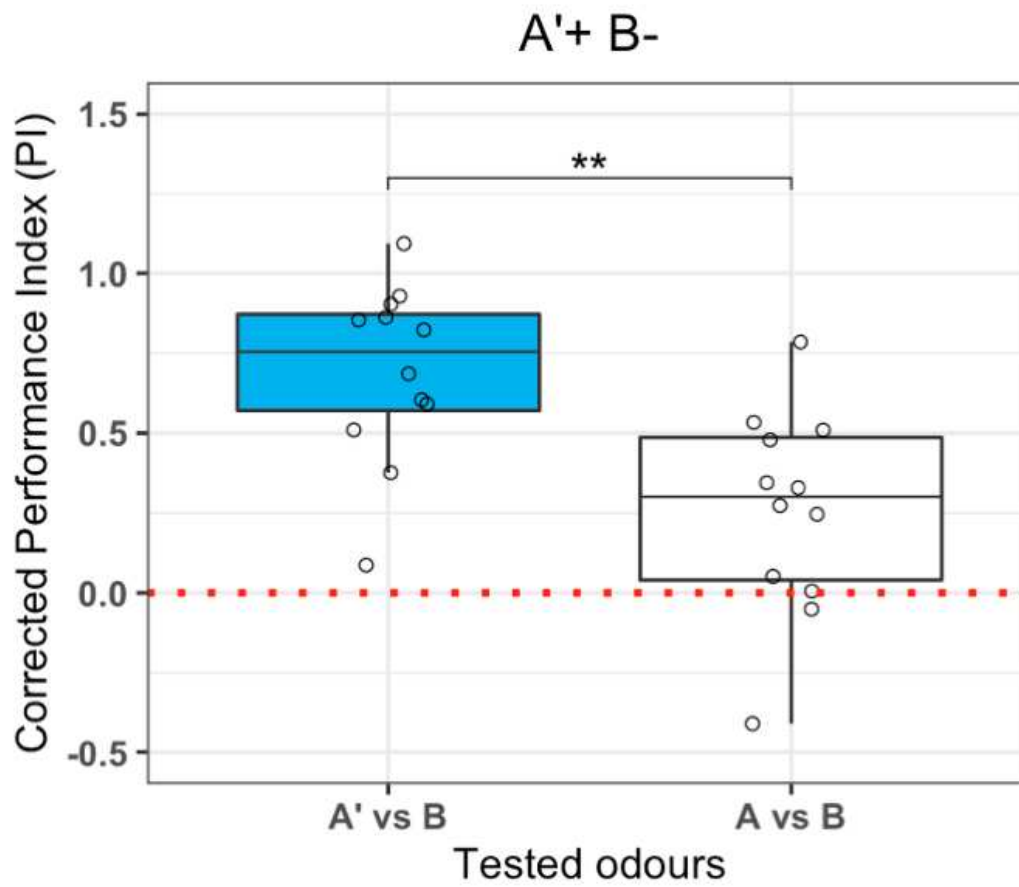
The fact that APL neurons' activity is dispensable during training involving similar stimuli was unforeseen, as they were shown to be involved during Negative Patterning and Negative Feature discriminations, two tasks involving similar stimuli (Durrieu et al., 2021, in prep). This observation may be explained by the existence of complementary mechanisms for odour representation modulation. Interestingly, Antennal Lobes (AL), which are the first integration centres of olfactory information, were classically thought to be mainly involved in stimulus pre-processing before learning. However, a growing set of evidence suggest that AL activity may also be modulated by associative learning<sup>[234]</sup>. In particular, several articles on honeybee demonstrate that olfactory conditioning increases odours discriminability at the level of AL by increasing the distance between CS+ and CS- representations<sup>[211,213]</sup>, especially in the case of similar stimuli such as odour mixtures<sup>[212,214]</sup>. Moreover, AL local interneurons inhibition impaired flies' ability to distinguish similar stimuli<sup>[225]</sup>. Together, these results hint again at a key role of ALs in the modulation of discrimination and generalization processes in insects.

Given more ambiguous situations, which seems to be consistently the case when mixtures are involved, AL's processing seems not to be sufficient and APL activity might be required to further enhance the contrast between CS+ and CS-. Indeed, A vs AB higher similarity supports our assumption that APL could be increasingly engaged with higher levels of similarity, as tasks involving A vs AB discrimination (Negative Patterning, Negative Feature discriminations) require functional APL neurons (Durrieu et al., 2021, in prep). Interestingly, B vs AB shows less similarity than A vs AB at the level of ALs, that is consistent with similarity observed between A and A'. Thus, an interesting experiment would be to test APL requirement for Negative Feature discrimination involving B and AB, in which case we would expect APL not to be necessary. Moreover, in this study we show that APL neurons are critically required to discriminate mixes of the same odours, even if these mixes are shown in very different concentration. This result suggests that even 90/10 A:B ratios are more similar for the fly than A and A'. In conditions involving mixtures, APL inhibitory activity could suppress overlapping representations and reduce generalization. The more similar odours become, the more relevant a generalization response is, which might explain why APL seem to have a lesser effect on ratios converging toward 50/50. In any case, an important next step would be to assess how similar odour mixtures with varying ratios used in this experiment really are, using

calcium imaging techniques. Then, a follow-up experiment would be to assess the ratio up to which APL are required, using for instance 95/5 and 97.5/2.5 ratios. As our previous study highlighted APL crucial role during CS- presentation (Durrieu et al., 2021, in prep), the same could be tested in the ratios protocol, where we would expect that inhibiting APL activity during CS- but not CS+ would produce the stepped behaviour observed in Figure3. Finally, flies were always able to solve the task as shown by relative performance indices above chance level (which was validated using 1 sample t.tests), which shows that APL neurons activity enhance CS+/CS- contrast but are not required to solve the task. These results are consistent with data on Negative Feature discriminations where APL were shown to be involved but not necessary. Moreover, following this logic, it is possible that ratios producing even greater levels of similarity could match results obtained with Negative Patterning, that is, control experiments would show a training repetition requirement for discrimination acquisition, and APL silencing would prevent flies from learning the task. Therefore, a future research agenda would include testing 65/45, 60/40 and 55/45 ratios as well.

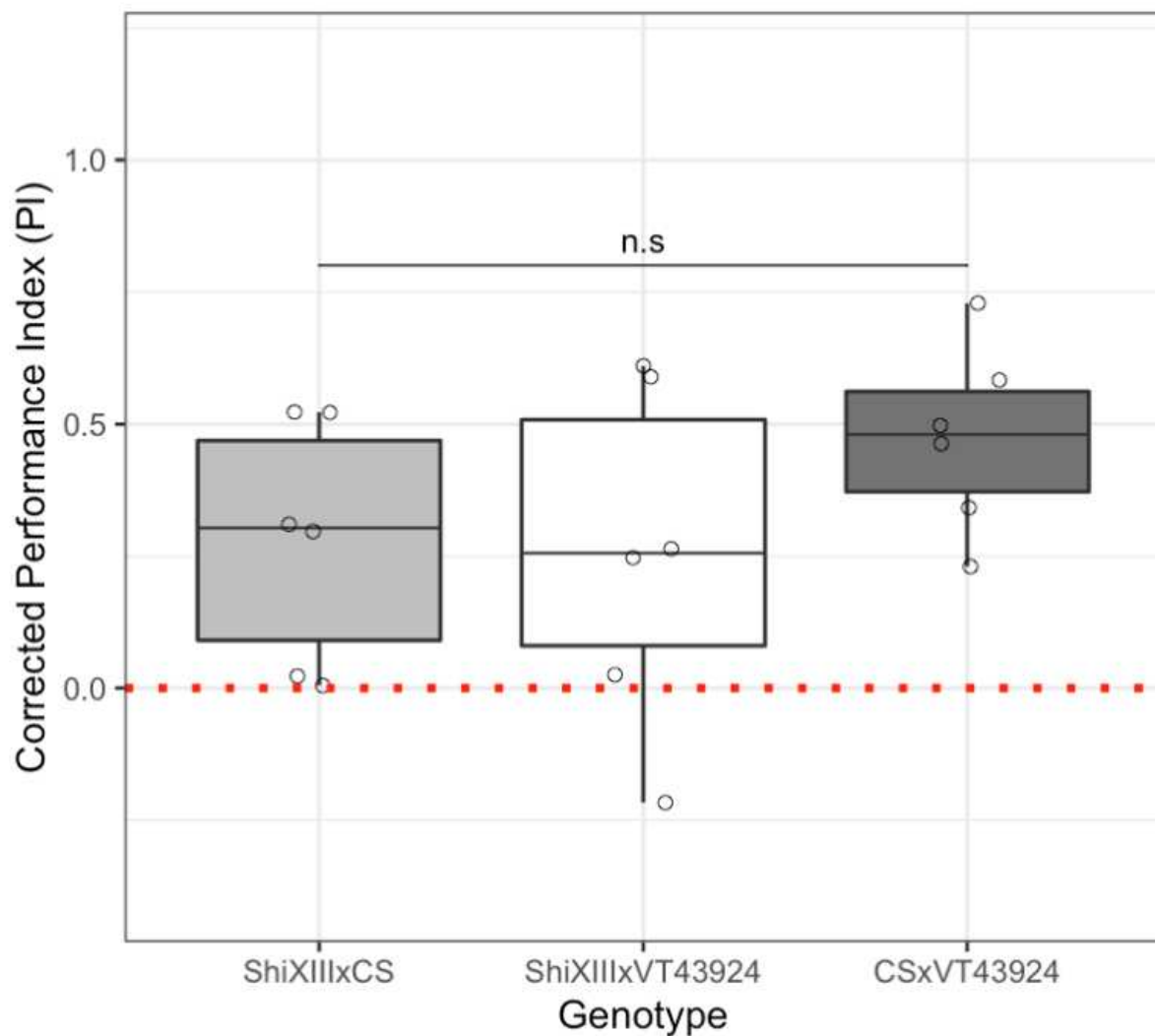
Another way to further test the relationship between perceived similarity and APL requirement would be to inhibit APL activity during differential conditioning involving more or less similar odour pairs, the similarity of which could be first assessed using the DoOR database, then directly inferred from flies generalization behaviour, as demonstrated in a previous work on *Drosophila* larvae<sup>[235]</sup>.

Importantly, while APL could be required for high similarity discrimination, processing at the level of ALs and MBs seem to be jointly contributing to the modulation of odours representation along the generalization/discrimination trade-off, as AL's local interneurons silencing impairs similar odours discriminations even with fully functional APL neurons, and substantial evidence disclosed the existence of a MB to AL feedback<sup>[236]</sup>. In conclusion, the interaction between ALs and Mushroom Bodies and how complementary modulation of odour representation in both regions participate to insect associative learning is a promising topic for future experiments.



SuppFig1 : Effect of differential conditioning on generalization across similar stimuli. *Relative performance indices (PI) computed as the difference between paired and unpaired indices of flies exposed to a differential conditioning procedure and tested with different odour pairs. Performances are compared between tested odour pairs.*  
**\*\* indicates  $p < 0.01$**

Supplementary figure 2



*SuppFig2: Effect of APL disruption in differential conditioning involving dissimilar stimuli. Relative performance indices (PI) computed as the difference between paired and unpaired indices. Performances are compared between genotypes. "n.s" stands for "non-significant"*





## General Discussion

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In this work we investigate ambiguous learning abilities and some of its neural correlates using a combination of approaches. First, we demonstrate that *Drosophila* can solve a non-elemental form of associative learning, the Negative Patterning, for which there wasn't former evidence in other insects than honeybee, let alone flies. Next, we characterize behavioural features associated with Negative Patterning resolution, two of which seem particularly prominent: Negative Patterning is acquired over multiple training trials, which is associated with a gradual shift in reinforced vs non-reinforced odours representation (chapter I). In the second part of our project, we focus on understanding how training repetition might modulate odour representation. To do so, we start from the traditional reinforcement model<sup>[198]</sup> and build a simple computational model based on already known neural mechanisms. The model successfully reproduces the training repetition effect on Negative Patterning by a gradual and differential modulation of reinforced vs non-reinforced stimuli representation at the level of Mushroom Bodies. We test this model validity back in flies using neurogenetically encoded tools and identify the inhibitory APL neurons activity as a plausible *in vivo* implementation of stimuli representation modulation.

#### On the benefits of modelling approaches

Importantly, flies' behavioural output is the result of a complex interaction of numerous cerebral functions, a significant part of which remains elusive and unaccounted for. One way to explore how each brain region contributes to associative learning is to selectively impair these regions and measure the resulting effect on the behaviour of interest. Once a particular region is demonstrated to be required for associative learning, a careful characterization of this region's activity allows to speculate on its function. This can be seen as a top-down approach. In a sense, modelling constitute a complementary approach, where these discovered functions can be assembled to understand how associative learning is implemented. In that case, all functions involved are known and the question is whether the already discovered mechanisms are sufficient to reproduce the behaviour of interest. This can be seen as a bottom-up approach. As neural mechanisms are intricated and often lead to non-linear computations, predicting the model's output can be challenging. This is where computational approaches become essential. In the scope of our work, computational simulations were used to test potential explanatory mechanisms in plausible conditions using a set of functions already described in insect brains. It allowed us to separate which mechanisms were likely to explain training repetition effect on negative patterning, and which mechanisms, although promising at first, were proven irrelevant. Moreover, the model exploration showed unforeseen effects that allowed us to make clear predictions which could be tested using neuro-genetically encoded tools. Lastly, almost anything is possible using flies' tools, but modelling approaches are also useful to select which experiments should be prioritized to test a given working hypothesis.

## Paired / Unpaired procedure as an alternative learning and memory procedure

In order to measure behavioural changes specifically related to an associative learning event, it is crucial to control for non-associative factors that could also drive flies' behaviour. During a Paired/Unpaired procedure, one group of flies is trained using a paired conditioning protocol where the CS+ is temporally paired with the US while the CS- isn't. In that case, flies' behavioural output reflects both associative learning-related behaviour and non-associative effects, such as naive preference and experimental bias. To disentangle associative and non-associative behavioural components, another group of flies is trained in parallel using an unpaired conditioning protocol where CS+, CS- and US are all presented in a decorrelated way. As the CS+ and the US are never explicitly paired, the resulting flies' behaviour only reflects non-associative effects. That way, it is possible to isolate the specific influence of associative learning on flies' behaviour by comparing Paired and Unpaired flies' response. Interestingly, the Paired/Unpaired procedure is almost non-existent in *Drosophila* learning and memory literature. Some articles presenting imaging data use the procedure to quantify learning related changes in neural activity<sup>[168,216]</sup>, yet a staggering majority of associative learning publications rely on the reciprocal method described in introduction<sup>[82,83]</sup>. Nonetheless, we argue that the Paired/Unpaired procedure could be an interesting alternative to the reciprocal method, as it presents key benefits. One of the main assumptions on which relies the reciprocal procedure is the symmetry between learning performances elicited by A+ B- and B+ A- which allows to compute the final performance index as the average between the performances elicited by each reciprocal procedure. However, depending on the odours used, symmetry between the reciprocal training performances is not manifest as odour-elicited activity patterns are not identical and sometimes nested in each other, which leads to differential performances depending on the odour reinforced<sup>[225]</sup>. Moreover, while in principle stimuli used as CS should be initially neutral, it is actually rarely the case, at least in *Drosophila* olfactory learning. Incidentally, the concentrations used vary across studies, which modulates both learning performances and innate preference. For instance, in our study 3-octanol, Benzaldehyde and 4-Methylcyclohexanol are slightly aversive for naïve flies whereas other studies showed innate attraction for the same odourants when

diluted with different concentrations<sup>[225]</sup>. The Paired/Unpaired procedure enables the quantification of learning performances elicited by one particular odourant and controls for any naïve bias through the unpaired performance indices. In addition, learning tasks involving a CS+ and CS- of differing nature, which is typically the case when compounds are involved, cannot be performed using a reciprocal procedure as described in the first chapter. In a previous study focused on elemental and non-elemental learning in *Drosophila*, authors used only a paired conditioning procedure to quantify associative learning and balanced odour concentrations using naïve flies behaviour as a control of experimental bias<sup>[181]</sup>. We already discussed why balancing odour concentrations might lead to challenging interpretations (chapter I) and in any case, it was not sufficient to detect any Negative Patterning ability in flies. In contrast, the Paired/Unpaired procedure led to robust and reliable performance indices, both in elemental and non-elemental paradigms. Also, our work shows that it is particularly insightful to explore flies' behavioural choice between a novel odourant and the CS+, the CS- or a stimulus that is similar to the CS+ (Chapters I and III). In addition, such procedure also ensured that in the case of a classical Differential Conditioning, the unpaired procedure did not elicit any behavioural bias. Indeed, odour presentation alone has been shown to elicit associative conditioning when associated with a reinforcement, even when the reinforcement is delayed up to 45 seconds after the odour presentation (which is called "Trace conditioning"). That is why we always applied >1 min breaks between the CS- presentation of a previous conditioning cycle and the US presentation of the following cycle. Comparing flies' behaviour toward the CS- and a novel odourant in a Differential Conditioning procedure confirmed that the unpaired procedure did not elicit any trace conditioning (Chapter I, Fig2).

Importantly, a critical criterion to assess the robustness of a *Drosophila* learning paradigm is the possibility to generate long-lasting memory with it. To further validate the Paired/Unpaired procedure as a general alternative to the reciprocal procedure, we explored memory retention of wild-type flies after 1h, 3h and 24h and a spaced conditioning which is known to elicit Long-Term Memory (LTM). The results presented in Figure32a show robust LTM performances at 24h. Thus, the Paired/unpaired procedure may be used in future experiments on memory in *Drosophila*.

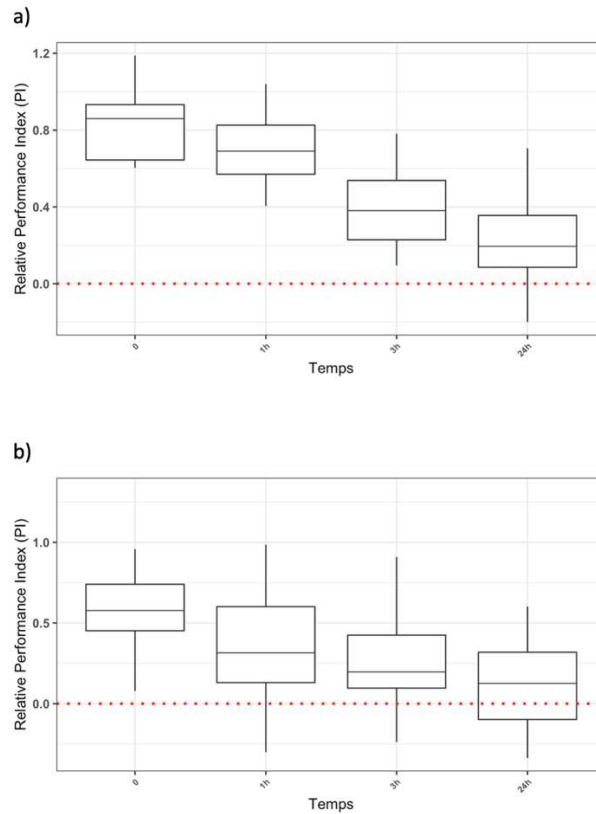


Figure 33: Differential Conditioning (a) and Negative Feature (b) learning performances of flies trained using either 5 trials massed (0, 1h, 3) or spaced (24h) conditioning and tested for short-term (1h), middle-term (3h) and long-term (24h) memory. Relative performance indices (PI) are computed as the difference between paired and unpaired indices.

Nevertheless, our results shed light on how non-associative processes such as the presentation of a non-reinforced odour (CS-) modulates stimulus representation. It is therefore important to stress that unpaired procedure is likely to induce non-associative learning which would be interesting to investigate. In chapter II we described how repeated CS- presentation may promote a lasting inhibition of its associated KCs responses through the activity of APL neurons. The unpaired procedure is basically the presentation of two CS-, therefore, this procedure may mediate such lasting KC inhibition through APL activity. However, as no reinforcement is applied and because all KCs should be modulated the same way, our model predicts no change in flies' choices after an unpaired training, regardless of APL state. We verified this by comparing flies unpaired performance indices in Negative Patterning with functional and inhibited APL (Figure33) and found no significant difference. Moreover, naïve bias for which the unpaired protocol controls is likely to be mediated by the

Lateral Horn, a region specialized in innate behaviour and, as far as we know, does not receive any APL input<sup>[202]</sup>. Taken together, we conclude that the Paired/Unpaired procedure is a robust alternative to perform olfactory conditioning with a T-Maze procedure.

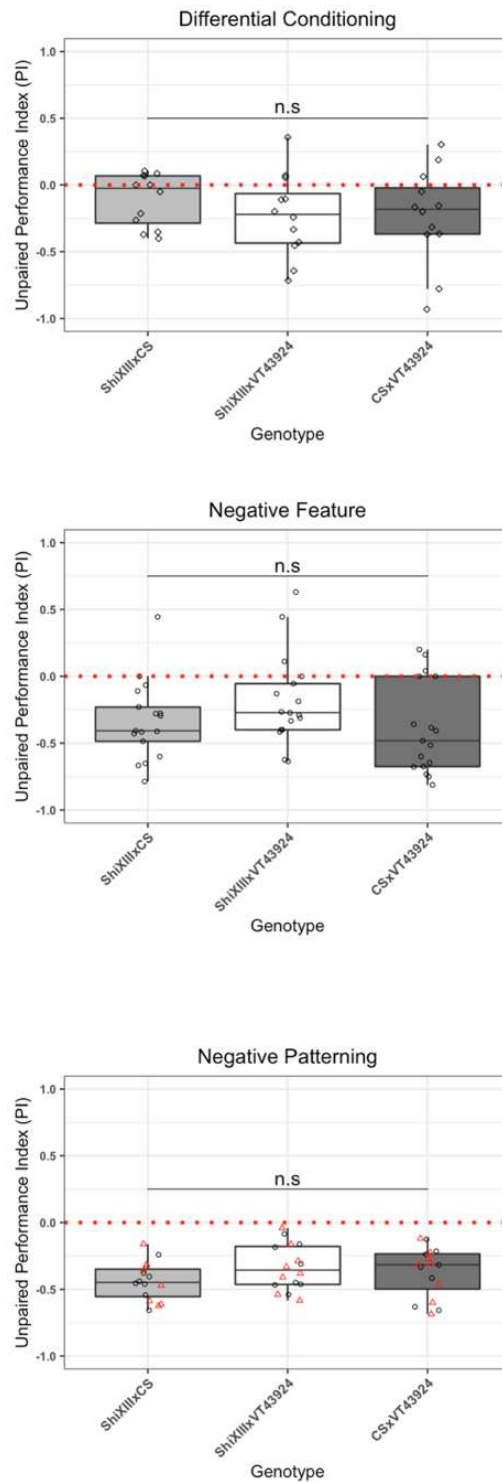


Figure 34 Effect of APL inhibition during training on Unpaired performances. Unpaired performance indices (PI) of flies trained either with a Differential Conditioning, Negative Feature discrimination or Negative Patterning. Data were compared across genotypes. "n.s" stands for "non-significant". Statistical tests are presented in Annex1.

## Alternative protocols for the study of Negative Patterning

In this study, we adapted the T-maze procedure to investigate Negative Patterning. As mentioned, one of the particularities of NP is that 3 distinct stimuli are involved, A, B and AB, whereas flies' performances are tested in a binary choice situation (either A vs AB or B vs AB). Because flies are the same age, same genetic background, trained and tested simultaneously and in large groups, it is possible to collect representative amounts of data for A vs AB and B vs AB, which can then be statistically compared. In every case, we showed that flies can both discriminate A from AB and B from AB after a NP protocol (chapter I). However, the traditional criterion for NP success is the ability of the same animal to perform both discriminations, which cannot be tested with our experimental setup. It would thus be interesting to conceive an alternative testing protocol where the same animals could be exposed to the two discrimination tests subsequently. One way to achieve it could be by testing flies for one discrimination, then keep flies from each T-Maze arm separately and test them again in the second discrimination. Of course, half of the flies would be exposed to A vs AB first and B vs AB next, while the other would be exposed to B vs AB then A vs AB in order to control for potential habituation effects. As a complement, flies could be tested alone and the time spent in each arm recorded as in previous experiments in order to access a more continuous measure of learning as opposed to a discrete position for each fly after 1 min<sup>[171]</sup>. Indeed, as flies exposed to ambiguous stimuli would in principle respond both to the CS+ and to the CS- (to some extent), we would expect them to behave accordingly. For instance, flies should spend more time in the CS+ arm when odours involved are similar and their position within the T-Maze should be closer to the middle instead of substantially biased toward the CS- compartment as would be expected for dissimilar odours. One problem with such protocol is that the fly may change its representation during the first test, where the CS+ is experienced without reinforcement, which would be challenging to measure as it would be dependent on the time spent by each fly in the CS+ and CS- T-maze compartments. Finally, a recent study highlighted 3D printed Y-mazes as a robust alternative to traditional T-maze<sup>[92]</sup>. It would be possible to adapt the Y-maze and test flies for their choice between three odours simultaneously. An example of three-ways Y-maze setup is represented in figure 34.



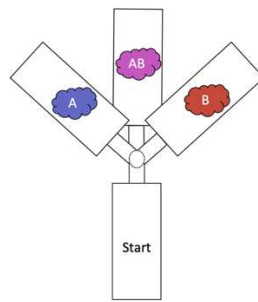


Figure 35 Double Y-Maze alternative for Negative Patterning testing

## How neural networks computations relate to elemental and non-elemental theories

In the first part of our work, we explore Negative Patterning acquisition in flies and show that reinforcement of A and B alone leads to an avoidance toward their compound, which is modulated by training repetition. Interestingly, each theory developed to account for non-elemental learning (elemental, configural, unique-cue, developed in introduction) contains complementary concepts to explain the observed behaviour. First, the Rescorla & Wagner (R&W) elemental model predicts that learning events are additive and each trial contributes to the final trained response. This is also what we observe with flies' behaviour. Indeed, training repetition effect is explained by the modulation of stimuli value at each trial. Moreover, in the second chapter, we develop how the CS- presentation modulates animals' response through an inhibitory learning trace, which is consistent with the R&W model.

The learning equation developed by R&W also relies on the crucial concept of prediction error. At each step of the learning process, learnt information is compared with the current experience and animals' behaviour is shaped accordingly. In flies' brain, the implementation of prediction error requires the comparison between the CS prediction (i.e., the MBON activity) and the US (i.e., the DAN activity). This could be achieved by a MBON/DAN interplay, where the presentation of a learnt CS+ would lead to the inhibition of the US DAN response through the MBON activity. That way, if both learnt CS+ and US are presented together, DANs activity would be neutralized. However, if a previously reinforced CS+ were to be presented

without US, associated MBON activity would lead to a 'negative' DAN activation, triggering learning in the opposite direction<sup>[153,156]</sup>. In chapter II, R&W prediction error is adapted in our computational model to drive synaptic plasticity in a biologically plausible way. Another important concept developed in the R&W model is the idea that every stimulus can be broken down in smaller elements that are individually associated with the US and contribute to the trained response. Remarkably, this concept is also verified in the neural architecture of flies' (and other animals, both insects and vertebrates) brains. A given olfactory stimulus can be broken down in a pattern of glomerular activities, which, in the case of insects, translates into a pattern of Kenyon Cells activity, which participate individually to the trained response by differentially driving the Mushroom Body Output Neurons activity.

To account for non-elemental learning, two theories are developed, the unique cue and the configural theory. In introduction we discussed how their most recent versions are very similar to one another and explain a significant portion of behavioural data. Indeed, our experimental data showing that A and B reinforcement leads to AB avoidance may be explained by a unique cue processing ( $AB = A + B + u$ ), where part of AB would be the sum of A and B. However, configural theory ( $AB = X$ ) also predict similar results if the configuration X generated by AB exhibits perceptual similarities with A and B (in a sense, this could be translated into  $AB = X = A' + B' + u$ ). Both accounts are consistent with how odours are processed In the flies' Antennal Lobes, as odour mixtures representation is largely predicted by their components representations, on which a normalization effects provided by inhibitory interneurons is applied, which leads to compound-specific features<sup>[123]</sup>. Moreover, as training repetition reduces flies' avoidance toward the compound in our Negative Patterning protocol (Chapter I), we propose that Elemental and Configural representations could be two virtual extremes of a continuum along which stimuli representation is shaped by learning experiences. This idea explains why depending on the task learned, the same stimuli representation may be modulated and is explain by different learning theories, which is highlighted in our work and in previous studies<sup>[188,237,238]</sup>.

## Multiple complementary mechanisms modulating olfactory discrimination

In the first chapter, we demonstrate that Negative Patterning training repetition leads to a representation shift of AB away from the representation of its constituents. In the second chapter, we describe the modulation of Kenyon Cells activity by APL neurons as a plausible mechanism for non-elemental learning acquisition. Interestingly, KCs activity modulation reproduce the representation shift of AB over the course of training cycles. Thus, representation modulation at the level of KCs is one way to increase discrimination abilities related to a particular ambiguous task. Importantly, in our model each KC is modulated independently which contributes to an overall representation shift. Therefore, in a supplementary experiment, we asked whether the modulation of only part of AB associated KCs through the presentation of B alone could also increase A vs AB discrimination. To do so, we trained flies with different conditioning paradigms, only presenting A paired with a reinforcement (A+) or presenting A+ followed by either B or AB as CS-. Results, presented in figure 35, suggest that presenting B alone without reinforcement could modulate AB representation and improve discrimination between A and AB, which is in line with our model's predictions. However, presenting AB without reinforcement seems to slightly better enhance A vs AB discrimination which once again suggests that AB carries unique but marginal features that are unrelated to either A or B and also contribute to the gradual acquisition of robust discrimination. However, in the chapter III, we also show that generally, the presentation of a non-reinforced stimulus during a classical conditioning experiment enhances the CS+ discriminability. Thus, the result we observe here should be compared with another experiment where A+ would be followed by C- before testing A vs AB discrimination. Moreover, AB representation shift through KCs activity modulation is APL dependent whereas differential conditioning effect on CS+ discriminability does not seem to be. Thus, it would be also interesting to inhibit APL activity specifically during the partial CS- presentation in order to determine the requirement of APL in that case. Nevertheless, our work hints at two potential sources of olfactory discrimination: one at the level of KCs that could modulate learnt odours representation, and another one at the level of Antennal Lobes which could modulate overall odours perception (although this remains to be tested).

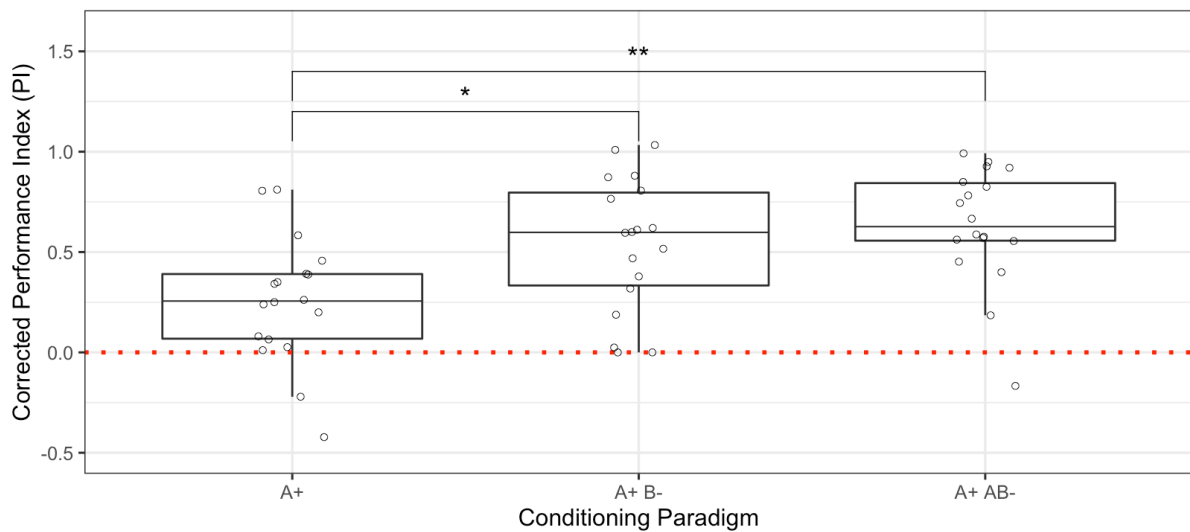


Figure 36 Partial CS- effect on compound discrimination. Relative performance indices (PI) of flies trained with three different conditioning paradigms, computed as the difference between paired and unpaired indices.

“\*” indicates  $p < 0.05$ , “\*\*” indicates  $p < 0.01$ . Statistical tests are presented in Annex2.

## Distributed memory traces encoding valence and discrimination/generalization

Our findings suggest the existence of plasticity at the level of the KC/APL synapses, the molecular mechanisms of which may be similar to those classically found for KC to MBON synapses<sup>[155,158]</sup>. That means that the KC's activity modulation, which we suggest is critical for ambiguous learning acquisition, could also be consolidated and stored over extended periods of time. Should that be the case, KC/APL and KC/MBON synapses would thus form two distinct memory traces that might be characterized by different dynamics. For instance, one memory could be extinguished while the other would endure, which would be measurable. Indeed, upon decay of KC/APL but not KC/MBON plasticity, animals would still show conditioned responses but their ability to discriminate ambiguous stimuli would decrease. Interestingly, the idea of distinct memory traces for valence on the one hand and discrimination/generalization on the other hand fits with various studies on vertebrates. Generalization modulation was observed in rodents, where time either increased<sup>[239]</sup> or decreased<sup>[240]</sup> generalization depending on the task tested. Indeed, generalization of learnt associations to new encountered situations has been shown to be crucial in knowledge

acquisition in humans<sup>[241]</sup>. Moreover, several disorders specifically impact discrimination and generalization abilities. For instance, children diagnosed with autism are notoriously known to be overselective and lack generalization skills<sup>[242]</sup>, whereas PTSD patients profile is often characterized by an overgeneralization of conditioned fear response<sup>[243]</sup>. The existence of a form of discrimination/generalization memory could also support the pattern separation/pattern completion concepts described in introduction. Indeed, heterosynaptic depression, which could be compared with the KCs' activity modulation we described, has been shown to be key in these processes<sup>[244]</sup>. Thus, it would be interesting to investigate memory consolidation and retention in ambiguous paradigms. It is important to stress the fact that although in our model only 2 MBONs were simulated, flies' actually exhibit 21 types of MBONs which are probably involved in different consolidated memories<sup>[245]</sup>. Thus, in combination with the APL/KC modulation, different MBON populations could encode different degrees of generalization. Preliminary experiments showed that NF discrimination can be measured after up to 3h, while 24h memory of NF was not ascertained (Figure 32b).

## Differential participation of Mushroom Body compartments to ambiguous learning

As mentioned in introduction, Mushroom Bodies are not entirely homogeneous structures, and while they are entirely composed of Kenyon Cells, they are subdivided in lobes which mediated different functions. Importantly, recent studies showed that APL inhibition on KC's is not homogeneous either, as some regions receive more APL's feedback than others<sup>[209]</sup>. In particular,  $\alpha'\beta'$  KCs receive more APL feedback and show higher sensitivity and discriminability. These results were complemented by another study showing that APL activity is localized and that activation of small subsets of KCs elicits local APL's feedback<sup>[206]</sup>. Therefore, a logical next step in the study of neural correlates of ambiguous learning will be to dissect the relative contribution of each Mushroom Body lobes in their acquisition, especially the  $\alpha'\beta'$  lobes. Interestingly, these lobes exhibit early memory traces, along with early learning traces both in Projection Neurons and APL's<sup>[142]</sup>. Yet, *ala* mutants, that lack either  $\alpha\alpha'$  or  $\beta\beta'$  lobes show normal short-term memory performances<sup>[184]</sup>, although  $\alpha'\beta'$  lobes inhibition during training was shown to disrupt 3h memory<sup>[246]</sup>. Taken together, these

data suggest that  $\alpha'\beta'$  lobes may be required for ambiguous learning and thus, *ala* mutants might show normal Differential conditioning but impaired Negative Patterning performances. Moreover, disrupting  $\alpha'\beta'$  lobes during training might differentially impact immediate learning performances of Differential Conditioning and Negative Patterning while impairing both after 3h.

## A possible interplay between APL neurons and DPM neurons through gap junctions

Overall, we showed that APL neurons play a key role in the modulation of odours representation over the course of learning events. Interestingly, APL are engaged in a reciprocal connectivity with another pair of neurons called DPM (Dorsal Paired Median) through gap-junctions. APL-DPM connectivity has been proven necessary for memory formation but not for associative learning<sup>[247]</sup>. Could DPM neurons be involved in ambiguous learning? We provide a first answer in Figure36 where we show that *Shibire<sup>ts</sup>* targeted inhibition of DPM activity during a NF conditioning had no visible effect on flies' performances. However, *Shibire<sup>ts</sup>* manipulation only impairs DPM synaptic transmission whereas DPM-APL connectivity relies on gap-junctions. Therefore, we cannot rule out an involvement of DPM neurons in the process of ambiguous learning. To formally test this, innexins that form the gap junctions between APL and DPM, *Inx7* and *Inx6*, should be knocked-down in each neuron, respectively. Moreover, DPM neurons have recently been highlighted in a study where we demonstrated their involvement in the social facilitation of a form of long-lasting memory<sup>[168]</sup>. Interestingly, imaging data showed that exposition to the social signal (CO<sub>2</sub>) led to an increasingly differentiated response of DPM to the CS+ compared to the CS- in previously trained flies, which was suggested to improve CS+/CS discriminability through a diminution of the CS- representation, not unlike APL's modulation of KC' activities. Incidentally, problem solving may be better achieved in groups<sup>[248,249]</sup>, which could be verified in the case of ambiguous olfactory learning if flies in groups perform better than alone. DPM's role in a potential social facilitation of ambiguous learning could then be investigated.

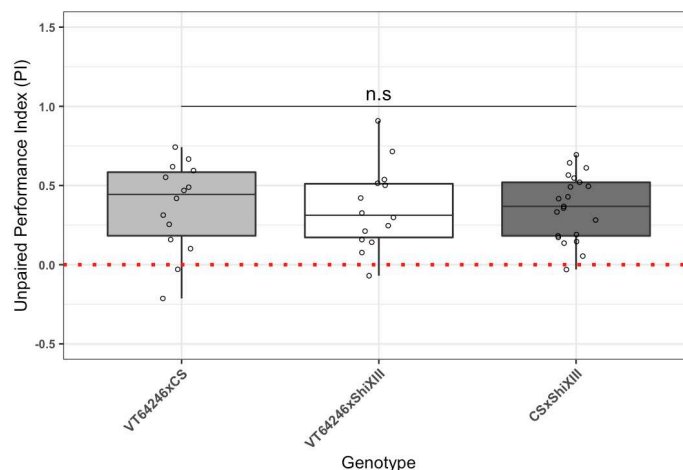


Figure 37 Effect of DPM inhibition on Negative Feature discrimination. Relative performance indices (PI) of flies trained with a 5 cycles NF are computed as the difference between paired and unpaired indices. Data were compared across genotypes. "n.s" stands for "non-significant". Statistical analyses are presented in annex3.

## Gradual KC's activity modulation through repeated associative learning events

At this point, we provide indirect evidence supporting the model of KC's activity modulation through training repetition. One way to further test the model's validity is to record KC's response to odours over the course of conditioning cycles repetition. The Gcamp fluorescent calcium reporter is a powerful way of quantifying neuronal activity in awake flies (Figure37a). Combining Gcamp with the UAS/Gal4 targeting system, it is possible to record fluorescence variations associated with KC's activity. Using 2-photon microscopy, KC's activity patterns could be recorded for each odour used in a given paradigm with high-resolution, as shown in previous publications<sup>[171,209]</sup>. Moreover, we recently developed a custom conditioning device designed to perform odour/shock associations under the microscope (Figure37b). Thus, the next step is to perform Differential Conditioning, Negative Feature and Negative Patterning protocols using flies expressing Gcamp in the KCs and record the gradual modulation of A, B and AB patterns of activation in the KCs over the course of training cycles., which would provide a direct proof of the KC's activity modulation mechanism. Alternately, Gcamp may also be expressed in the APL neurons, where we would expect a modulation of activity inversely correlated with KC's activity modulation.

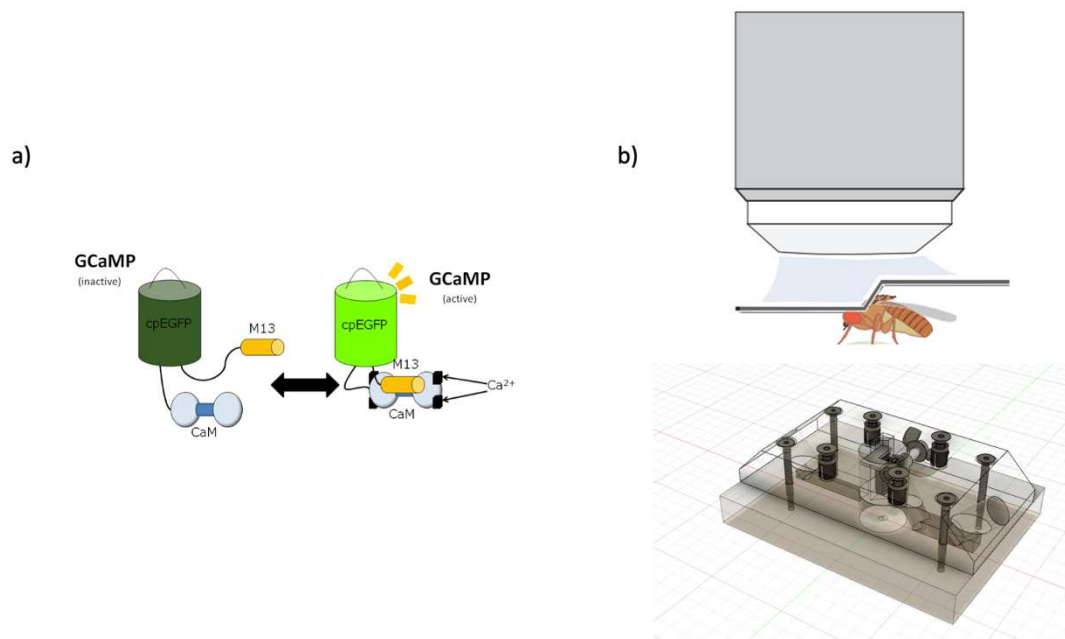


Figure 38 a) Schematic representation of the Gcamp fluorescent reporter. Upon binding with calcium, Gcamp conformation is shifted leading to a fluorescence increase<sup>[250]</sup>. B) Top: Illustration of a head-fixed fly under a microscope lens<sup>[251]</sup>. Bottom: Custom conditioning and imaging device. The device consists of a base structure designed to be secured under the microscope. The main structure is equipped with an air-channels enabling the presentation of odours and an actionable platform covered with a metallic grid which enables the presentation of electric shocks. 3D design was conceived using Autodesk Fusion 360 and is meant to be printed using SLA 3D printer systems.

## Ambiguous learning beyond aversive olfactory modalities

Although we focused on olfaction using aversive reinforcement, we expect that the mechanisms unveiled in our work could be, to some extent, applied to other sensory modalities. First, APL neurons have been shown to be involved in visual reversal learning<sup>[180]</sup>. Thus, it would be interesting to test APL role in visual Negative Patterning. Moreover, a significant part of the honeybee work on associative learning in general and ambiguous learning in particular has been performed using appetitive reinforcement. Importantly, the APL's honeybee homolog is required for appetitive Negative Patterning<sup>[36]</sup>. Yet in contrast with our experiments, in the honeybee case, APL's homolog is also involved in Positive



Patterning. An appetitive version of the semi-automated procedure we used in this work is available and could be used to test APL requirement in appetitive ambiguous learning. Importantly, while appetitive and aversive memory consolidation exhibits differing dynamics<sup>[185]</sup>, appetitive and aversive learning display a remarkable circuits symmetry<sup>[252]</sup>. Together, this suggests that appetitive ambiguous learning should also depend on KC's activity modulation.

It is important to consider the way odour blends are processed at the level of Antennal Lobes. Indeed, binary odour mixtures generally share significant similarities with their components<sup>[123]</sup>. Yet, local interneurons activity also leads to normalization effects which modulate compound representation. In contrast, a discrimination task involving two distinct sensory modalities would prevent that kind of interaction between compound components. An interesting perspective would be to replace one of the stimuli involved in the Negative Patterning with a visual stimulus (e.g., a colour dot). In that case, discrimination per se would be irrelevant as each stimulus would be easily distinguished from the other. Yet, it is still possible that some sort of pattern separation occurs for flies to learn to discriminate A and B alone from AB. Indeed, while Mushroom Bodies involvement in visual learning was debated for a long time due to mixed evidence<sup>[91,253]</sup>, recent studies suggested that olfactory and visual stimuli share common circuitry within the MB<sup>[254,255]</sup>, which was also observed in honeybee and ants<sup>[256,257]</sup>. If olfactory and visual learning traces are carried by the same cells, it is very likely that here again, representation modulation, possibly through APL activity, would be instrumental in ambiguous learning acquisition.

## Mushroom Bodies don't do it all: a potential role of Antennal Lobes in ambiguous learning

In the chapter III, we show that flies don't need APL neurons to learn a Differential Conditioning, even involving similar stimuli. APL rather seem to be involved in a fine modulation of the Discrimination / Generalization trade-off, when odours display a high-level of perceived similarity. One part overlooked in our work is the plausible implication of Antennal Lobes in the learning process. Increasing evidence in honeybee point toward a key role of ALs in early odour processing which shows learning-related plasticity<sup>[211,212,234]</sup>. Even

though the AL of flies is somewhat smaller than the bees<sup>[258]</sup>, the type of computation happening there seems conserved across insects, and thus we should not rule out the possibility that flies' ALs could equally show learning-related plasticity. Furthermore, MB to AL projections have also been reported<sup>[236]</sup>, which predicts a potential effect of learnt information on AL activity, although the underlying mechanisms are still unclear. In sum, beyond the role of Mushroom-Bodies related process, an important future research agenda consists of an extensive description of associative plasticity at the level of ALs, which could contribute to flies' discrimination abilities and co-operate with KC's activity modulation to further separate reinforced stimuli from non-reinforced ones.

## Concluding remarks

In this study, we provide yet another example of the remarkable behavioural flexibility of insects. Indeed, we demonstrate that the Fruit fly, *Drosophila Melanogaster* is able to solve a task of Negative Patterning, which requires flies to differentially respond to the same stimuli depending on whether they are presented alone or together. We show that fly's gradual acquisition of Negative Patterning relies on a shift in stimuli representations over the course learning trials. Based on extensive data resulting from decades of research on associative learning in insect, we build a model successfully explaining not only Negative Patterning, but also various ambiguous tasks resolution through a differential modulation of reinforced vs non-reinforced stimuli representation in the fly brain. Using genetically encoded tools, we validate the model's predictions and identify the APL neurons activity as a plausible in vivo implementation of stimuli representation modulation, which enables flies to separate highly similar stimuli and learn complex tasks. Our work provides an example of how implementing additional but likely naturally encountered challenges leads to the discovery of mechanisms that are not visible in simpler forms of learning tasks. Strikingly, our results are consistent with observations in Honeybee which suggests that despite their significant differences in ecological terms, flies and bees may rely on similar neural functions to overcome their respective challenges. Thus, in future research projects, ambiguous learning may be studied using a combination of approaches in both models. For instance, Bees may be studied in more ecological contexts in free flying experiments, while flies' benefit from powerful genetic tools

and extensive databases such as the recently published connectome of learning and memory centres. Finally, both flies and bees' brain functions may be explored using in vivo imaging and electrophysiology techniques in order to better understand how neural activity translates into behaviour. Taken together, the possibility of combined approaches such as the one developed in our work should lead to an increasing understanding of how apparently complex problems can be solved by remarkably simple mechanisms in the insect brain.

## Annexes

### Annex 1: APL inhibition effect on unpaired performance indices

Here we compared flies Unpaired performances indices when exposed to either a Differential Conditioning (DC), Negative Feature discrimination (NF) or Negative Patterning (NP), with functional or inhibited APL. No significant effect of APL inhibition was detected.

DC: ANOVA:  $F_{2,33} = 0.66$ ,  $p = 0.52$

NF: ANOVA:  $F_{2,48} = 1.82$ ,  $p = 0.17$

NP: ANOVA:  $F_{2,45} = 1.56$ ,  $p = 0.22$

### Annex 2 : Partial CS- effect on compound discrimination

Here data were compared across the three experimental conditions using an ANOVA:  $F_{2,51} = 6.97$ ,  $p = 0.002$ . Pairwise comparisons were subsequently computed using a Tukey's HSD post hoc test.

A+ vs A+B- :  $p = 0.03$

A+ vs A+AB- :  $p = 0.002$

A+B- vs A+AB- :  $p = 0.65$

### Annex 3: Effect of DPM neurons inhibition on ambiguous learning acquisition

Here we inhibited DPM neurons activity during NF conditioning and compared associated learning performances with genetic controls.

ANOVA:  $F_{2,46} = 0.007$ ,  $p = 0.99$

## Annex 4: Flies' feeding medium

Target quantity (L)	Water (L)	Agar (g)	Flour (g)	Yeast (g)	Tegosept (g/mL)	Propionic acid (g/mL)	Vitamin mix (g/mL)	Ethanol (mL)
1	1	9	70	70	0.5	0.15	0.0125	20

Note: blue shading indicates that these elements are diluted in water whereas red shading indicates a dilution in ethanol.

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## Résumé

Extraire les liens prédictifs au sein d'un environnement permet d'appréhender la structure logique du monde. Ceci constitue la base des phénomènes d'apprentissage qui permettent d'établir des liens associatifs entre des événements de notre entourage. Tout environnement naturel englobe une grande diversité de stimuli composés (i.e. intégrant plusieurs éléments). La façon dont ces stimuli composés sont appréhendés et associés à un renforcement éventuel (i.e. événement plaisant ou aversif) est un thème fondamental de l'apprentissage associatif. Théoriquement, un stimulus composé AB peut être appris comme la somme de ses composants (A+B), un traitement dit élémentaire, comme un stimulus à part entière (traitement configural,  $AB=X$ ) ou encore comme une entité comportant à la fois certaines caractéristiques de ses composants ainsi que des propriétés uniques (ou Indice Unique,  $AB = A+B+u$ ). Ces deux dernières théories permettent notamment d'expliquer la résolution de problèmes ambigus tels que le Negative Patterning (NP) au cours duquel les composants du stimulus AB sont renforcés lorsque présentés seuls mais pas lorsqu'ils sont présentés en tant que composé. Bien que les réseaux neuronaux impliqués dans l'apprentissage associatif élémentaire soient bien connus, les mécanismes permettant la résolution d'apprentissages non élémentaires sont encore peu compris.

Dans cette étude, nous démontrons pour la première fois que la Drosophile est capable d'apprentissage non-élémentaire de type NP. L'étude comportementale de la résolution du NP par les mouches montre qu'il passe par la répétition de cycles de conditionnement conduisant à un changement de représentation du mélange AB, s'éloignant peu à peu de la représentation de ses composants A et B. Nous développons ensuite un modèle computationnel à partir de données in vivo sur l'architecture et le fonctionnement des réseaux neuronaux de l'apprentissage olfactif chez la Drosophile, ce qui nous permet de proposer un mécanisme théorique permettant d'expliquer l'apprentissage du NP et dont la validité peut être testée grâce à des outils neurogénétiques. Lors d'un apprentissage de NP, les mouches acquièrent tout d'abord un premier lien associatif entre les composants A et B associés au renforcement, créant par la même occasion une ambiguïté avec leur mélange AB, présenté sans renforcement. Au cours des cycles de conditionnement, les représentations de A et B vis-à-vis de AB sont modulées de façon différentielle, inhibant progressivement la réponse neuronale au stimulus non renforcé tout en renforçant la réponse aux stimuli renforcés. Cette modulation augmente le contraste entre A, B et AB et permet aux drosophiles de résoudre la tâche de NP. Nous identifions les neurones APL (Anterior Paired Lateral) comme implémentation plausible de ce mécanisme, car l'engagement de leur activité inhibitrice spécifiquement durant la présentation de AB est nécessaire pour acquérir le NP sans altérer leurs capacités d'apprentissage dans des tâches non-ambiguës. Nous explorons ensuite l'implication des neurones APL dans un contexte plus général de résolution d'apprentissages ambigus.

Pour conclure, notre travail établit la Drosophile comme modèle d'étude d'apprentissage non élémentaire, en proposant une première exploration des réseaux neuronaux sous-jacents à l'aide d'outils uniques à ce modèle. Il ouvre la voie à de nombreux projets dédiés à la compréhension des mécanismes neuronaux permettant aux animaux d'extraire des liens associatifs robustes dans un environnement complexe.



## Abstract

Animals' survival heavily relies on their ability to establish causal relationships within their environment. That is made possible through learning experiences during which animals build associative links between the events they are exposed to. Most of the encountered stimuli are actually compounds, the constituents of which may have been reinforced (i.e., associated with a pleasant or unpleasant stimulus) in a different, sometimes opposed way. How compounds are perceived and processed is a central topic in the field of associative learning. In theory, a given compound AB may be learnt as the sum of its components (A+B), which is referred to as "Elemental learning", but it may also be learnt as a distinct stimulus (which is called "Configural learning"). Finally, AB may bear both constituent-related and compound-specific features called "Unique Cues" ( $AB = A+B+u$ ). Configural and unique cue processing enable the resolution of ambiguous tasks such as Negative Patterning (NP), during which A and B are reinforced when presented alone but not in a compound AB. Although neural correlates of simple associative learning are well described, those involved in non-elemental learning remain unclear.

In this project, we rework a typical olfactory conditioning protocol based on semi-automated olfactory/electric shocks association, allowing us to demonstrate for the first time that *Drosophila* is able to solve NP tasks. Behavioural study of NP solving shows that its resolution relies on training repetition leading to a gradual change in the compound AB representation, shifting away from its constituents and thus becoming easier to distinguish.

Next, we develop a computational model of olfactory associative learning in *Drosophila* based on structural and functional *in vivo* data. Exploratory simulations of the model allow us to identify a theoretical mechanism enabling NP acquisition, the validity of which can be tested *in vivo* using neurogenetical tools only available in *Drosophila*. We propose that during a NP training, flies first acquire associative links between A, B and their reinforcement, which induces an ambiguity as the compound AB is presented without reinforcement. However, over the course of training cycles, non-reinforced stimuli representation is inhibited while the reinforced stimuli representation is consolidated. This differential modulation eventually leads to a shift in odours representation allowing flies to better distinguish between the constituents and their compound thus facilitating NP resolution. We identify APL (Anterior Paired Lateral) neurons as a plausible implementation of this theoretical mechanism, as APL inhibitory activity is specifically engaged during the non-reinforced stimulus presentation, which is necessary for NP acquisition but dispensable for non-ambiguous forms of learning. Lastly, we explore APL role in a broader context of ambiguity resolution.

In conclusion, our work validates *Drosophila* as a robust model to investigate non-elementary learning, and present a promising model of the underlying neural mechanisms using a combination of behaviour, modelling and neurogenetical tools. We believe this opens the way to numerous interesting projects focused on understanding how animals extract robust associations in a complex world.